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ABSTRACT

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Multiple lines of evidence indicate that saturated fatty acids and cholesterol raise plasma cholesterol levels. Several methods for improving the nutritional properties of milkfat have been developed over the past few years. In this study, two processes were developed to reduce the hypercholesterolemic effect of milkfat. The first process was based on converting the hypercholesterolemic myristic acid to myristoleic acid using the Δ^9-desaturase system of hen hepatic microsomes. Desaturation of myristoyl-CoA to myristoleoyl-CoA was maximal at pH 7.4, linear with time up to 10min, and proportional to protein concentrations. The initial velocity was linear with substrate concentrations between 13 and to 200μM. The desaturation of myristoyl-CoA was stimulated by bovine serum albumin and reduced by cytoplasmic proteins. Direct desaturation of free myristic acid by hen liver microsomal Δ^9-desaturase without prior activation to myristoyl-CoA by the addition of adenosine triphosphate (ATP) and CoA was observed when the incubation medium was mixed at mixing speeds greater than 250 rpm in the presence of fatty acid-binding proteins (FABP). Direct desaturation was linear with time, proportional to the

microsomal protein concentrations, and maximal at pH 7.9. The greatest direct desaturation rate was observed at a mixing speed of 500 rpm in the presence of FABP. As the Δ^9-desaturase is inactive on triacylglycerol substrates, the application of the desaturation process on milkfat necessitates a lipase- catalyzed hydrolysis of milkfat triacylglycerols to liberate the free fatty acids (FFAs) prior to their desaturation, followed by re-esterification the FFAs. They would not all return to their original positions in the triacylglycerol moiety, hence the reaction of the final material to milkfat would be somewhat tenuous.

The second process is based on reducing cholesterol and FFAs in anhydrous milkfat through fat blending after the cholesterol was removed. The removal of cholesterol is based on the specific affinity of β-cyclodextrin for free cholesterol and cholesterol esters. The process, optimized by response surface methodology, consists of a sequence of steps: refining, dilution with distilled water, heating, mixing, and centrifugation. The process is efficient since the cholesterol was almost completely (>98%) removed. Free fatty acid reduction was greater than 92.40 %, which was more than 45 % greater than the prior-art processes. Gerbil feeding studies showed that modified milkfat (i.e., cholesterol-reduced milkfat) reduced serum total cholesterol by 28% and triacylglycerols by 47%. Also, modified milk fat could be used for whole milk reconstitution and consequently the whole spectrum of dairy products such as ice cream, yogurt, and cheese.

To my parents who

taught me the things that matter

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INTRODUCTION

Cardiovascular disease kills almost one million Americans annually. Another two million die from strokes. Forty million suffer from some forms of cardiovascular disease. Atherosclerosis affects 95 percent of all Americans over the age of 50. According to several studies during the last 20 years, scientists and doctors have pieced together much of the puzzle surrounding heart diseases. In 1994, an expert panel of clinicians and researchers convened by the National Heart, Lung and Blood Institute of the American National Institutes of Health sent out a clear message: high blood cholesterol is bad, and lowering it can reduce the risk of heart disease (Expert Panel, 1994).

Dietary management is the initial step in lowering blood cholesterol. Multiple lines of evidence indicate that dietary saturated fatty acids and cholesterol increase blood cholesterol and therefore increase the risk of heart disease. Better food habits can help reduce one of the major risk factors for heart attack – high blood cholesterol. The eating plan of the American Heart Association (AHA) and the National Cholesterol Education Program (NCEP, 1993) describes the latest advice of medical and nutrition experts. The best way to help lower blood cholesterol levels is to eat less saturated fat, cholesterol, total fat, and maintain a healthy body weight. The composition of this plan consists of Step I and Step II diets.

To achieve the recommendations of Step I diet, the saturated fat and cholesterol contents of a typical American diet must be reduced by approximately 25%. A Step II diet requires greater decreases in saturated fat and cholesterol: Both must be decreased by almost 50%. For both diets, total fat should provide ≤ 30% of calories, representing a reduction of approximately 15%.

Recognizing these concerns, the fat and oil industry is looking at new techniques to alter the composition of fats. The biggest challenge is to reconcile functional needs with nutritional concerns. With fats and oils, one cannot make a choice based on fatty acid composition alone. Taste is a very important factor.

The frying and the baking industry may have jumped from the "the frying pan into the fire" by switching to partially hydrogenated vegetable oils (PHVO) to maintain the textural and stability qualities of the saturated animal fats that they have replaced in industrial applications. *Trans* fatty acids generated during the processing of PHVO now appear as detrimental, if not worse, than saturated fatty acids (Ascherio et al., 1994).

The foremost element of this study is to produce a healthy natural animal fat that has a desirable flavor and functionality. This study addresses alternatives to the hypercholesterolemic animal fats and *trans*-rich PHVO by:

1- a) Developing an enzymatic process to convert the hypercholesterolemic myristic acid to myristoleic acid using the Δ^9-desaturase system of hen hepatic microsomes;

b) Elucidating at the biochemical level the underlying mechanism of the desaturation reaction vis-à-vis substrate specificity and the interaction between

substrate and fatty acid binding proteins and bovine serum albumin.

The primary aim of this part is to test the following hypothesis:

When a free fatty acid is bound to fatty acid-binding proteins (FABPs) in the form of protein-monomer complexes, its activation to the CoA derivative is not necessary in order to be desaturated by the Δ^9-desaturase system when using high mixing rates. FABP-fatty acid complexes are substrates for the Δ^9- desaturase in the presence of high mixing rates.

2- Reducing the hypercholesterolemic effect of animal fats by lowering their cholesterol contents by chemical means.

This study will provide basic knowledge for acceptable, cost- effective improvement of food lipids, particularly animal fats, to ensure the health of consumers and to find new uses of animal fats in food production.

CHAPTER 1

LITERATURE REVIEW

1.1 EFFECTS OF DIETARY FATTY ACIDS ON BLOOD CHOLESTEROL

Multiple lines of evidence indicate that saturated fatty acids raise plasma cholesterol levels. Both animal (Hegsted and Gallagher, 1967) and human studies (Keys et al., 1957) have demonstrated elevation in serum cholesterol levels following increased saturated fat intake. These observations, coupled with epidemiological evidence of increased risk of coronary heart disease (CHD) in populations consuming excessive amounts of saturated fats, explain the emphasis on the reduction of saturated fat by most health agencies.

Generally, saturated fatty acids increase plasma low density lipoproteins (LDLs) which are very atherogenic, partly by reducing receptor-mediated uptake (Grundy and Vega, 1988a). The high density lipoproteins (HDLs) provide protection against atherosclerosis by transporting cholesterol from tissue to liver for conversion to bile acids and excretion. Although saturated fatty acids maintain relatively high levels of HDL (Grundy and Vega, 1988b), it cannot be said that they have unique HDL-raising activity.

Dietary saturated fatty acids include caprylic, caproic, lauric, myristic, palmitic, and stearic acids. A key question is whether all saturated fatty acids raise the LDL-cholesterol level. If not, then it is inappropriate to consider all saturated fatty acids together when making dietary recommendations. Multiple evidence suggests that all

saturated fatty acids do not have the same effect on LDL-cholesterol concentrations. The focus has shifted to understanding the role that individual dietary fatty acids play in the health of the consumer. Thus, to examine the actions of saturated fatty acids on LDL-cholesterol concentrations, it is necessary to consider each fatty acid separately. The saturated fatty acids, lauric and palmitic acids increase plasma cholesterol and triacyglycerol levels (Denkee and Grundy, 1992), whereas polyunsaturated fatty acids (PUFAs) have the opposite effect (Mensink and Katan, 1992). Myristic acid is the most hypercholesterolemic long-chain saturated fatty acid, followed by lauric acid (Hayes, 1995).

1.1.1 Stearic Acid

It has been suggested that stearic acid does not raise the serum cholesterol level. Bonanom and Grundy (1988) produced a synthetic fat with approximately 45% stearic acid by chemical randomization between a completely saturated soybean oil and a high oleic-safflower oil. Oleic acid was the other predominant fatty acid (50%), while the palmitic acid content was approximately 5%. This synthetic fat was compared with palm oil which has about 45% palmitic acid and with high oleic safflower oil which is mainly oleic acid. The study showed that stearic acid did not raise total cholesterol (or LDL-cholesterol) compared to oleic acid, while palmitic acid markedly increased cholesterol levels. No other lipoproteins were changed by stearic acid.

The influence of butterfat, beef fat, cocoa butter, and olive oil on serum cholesterol and lipoproteins concentrations was studied by Denke and Grundy (1991). Olive oil served as a baseline against which the three highly saturated fats were compared. Of particular interest to this study was whether beef fat (tallow) and cocoa

butter which are rich in stearic acid, are as hypercholesterolemic as butterfat. The study was carried out in 10 middle-aged men under metabolic ward conditions. Liquid formula diets containing 40% of the calories from the test fat were fed in random order for three weeks each, with lipoprotein concentrations measured on the last five days. Butterfat raised LDL concentrations the most (4.23 ± 0.15 mmol/L; x ± SE). Beef tallow produced lower concentrations of LDL (4.03 ± 0.18 mmol/L), while cocoa butter resulted in even lower concentrations (3.82 ± 0.15 mmol/L). The lowest concentrations of LDL were observed with olive oil (3.62 ± 0.18 mmol/L). Because intakes of total cholesterol were balanced during this comparison, the different effects of these three fats (butterfat, cocoa butter, and beef tallow) on plasma cholesterol concentrations undoubtedly were related to differences in their fatty acid compositions. Cocoa butter was less hypercholesterolemic than butterfat and beef tallow because it contains a higher level of stearic acid and relatively low levels of cholesterol-raising saturated fatty acids such as myristic acid. This study provides additional support for the concept that stearic acid does not raise serum total cholesterol.

Why does stearic acid not raise the cholesterol level? There are at least two propositions that have been developed. First, the absorption of stearic acid might be incomplete as suggested by Apgar et al. (1987) or, second, stearic acid might be rapidly converted to oleic acid in the body (Elovson, 1965). The first proposition was not supported by Bonanome and Grundy (1989) who compared the amounts of fatty acids excreted in the human stool with the quantity ingested daily. Fecal excretion data indicated that oleic acid was 99% absorbed, palmitic acid was 96-97% absorbed, and

stearic acid was 90-94% absorbed. Therefore, the fact that stearic acid does not raise cholesterol levels cannot be attributed to a lack of absorption.

The second proposition is that stearic acid is rapidly converted to oleic acid in the body (Elovson, 1965). However, these investigators did not present any evidence for the accumulation of stearic acid in plasma lipids on feeding a high stearate diet, even though large quantities of stearic acid were absorbed. Instead, the oleic acid content of the plasma lipids was increased, thus suggesting the conversion of stearic acid to oleic acid.

1.1.2 Palmitic Acid

Palmitic acid is the predominant saturated acid in beef tallow, lard, butterfat, and even in certain vegetable fats and oils, e.g., palm oil and cottonseed oil. According to Keys et al. (1965), palmitic acid raises the serum total cholesterol by 2.7 mg/dl for every 1% in dietary calories. Studies with monkeys showed that palmitic acid is hypercholesterolemic compared to dietary oleic and linoleic acids (Pramod and Hayes, 1992). More recently, Khosla and Hayes (1993) and Pronczuck et al. (1994) reported that excessive intake of dietary cholesterol exerts a synergistic effect on the metabolism of palmitic acid-rich fats, causing them to be hypercholesterolemic. In the absence of dietary cholesterol and in individuals with normal lipoprotein profiles, palmitic acid does not ordinarily raise plasma total cholesterol concentrations or LDLs (Sundram et al., 1994,1995; Hayes, 1995).

1.1.3 Myristic Acid

Myristic acid is the most potent hypercholesterolemic long chain fatty acid,

followed by palmitic and lauric acid (Hayes, 1995). Zock et al. (1994) compared myristic acid with palmitic acid and oleic acid (all at 10% energy) and found that it significantly increased both total and LDL-cholesterol concentrations. Myristic acid also demonstrated a much greater cholesterol-raising effect than palmitic acid when substituted for oleic acid.

1.1.4 Lauric Acid

The cholesterolemic effects of lauric acid were evaluated in a study involving 14 subjects (Denke and Grundy, 1992). Compared with palmitic acid, lauric acid lowered total and LDL-cholesterol concentrations by 4 and 6 %, respectively. In contrast, when compared with oleic acid, lauric acid was hypercholesterolemic, raising both total and LDL-cholesterol concentrations by 11-12%.

1.1.5 Medium Chain Fatty Acids

Medium chain fatty acids such as caprylic acid and caproic acid are present in moderate amounts in butterfat. Currently, it is believed that they do not raise serum cholesterol levels, but apparently they can increase triacylglycerol concentrations when fed in large amounts (Larry et al. 1990). Nonetheless, the medium chain fatty acids which act like carbohydrates do have the potential as replacements of long chain, cholesterol-raising fatty acids in the diet (Kennedy, 1991)

1.1.6 Trans Unsaturated Fatty Acids

When vegetable oils undergo hydrogenation, a portion of the linoleic acid is completely saturated to produce stearic acid. Another portion is converted to mono or di-unsaturated fatty acids, *cis* and *trans*. Since double bonds can migrate during hydrogenation, several different *cis* and *trans* isomers are produced (*cis*-9, *trans*-13; *trans*-9, *trans*-12; *trans*-8, cis 12; *trans*-8, *cis*-13; or mono unsaturated *trans*-9 elaidic acid) (Ratnayke and Pelletier, 1992). *Trans* isomers have been implicated in increasing LDL-cholesterol levels. Zock and Katan (1992) studied the effects of elaidic (*trans* C18:1 (n-9)) on plasma total cholesterol. Compared to linoleic acid, they observed an increase of LDL-cholesterol of 9.3 mg/dl and a decrease of HDL-cholesterol by 2.3 mg/dl. No significant difference in serum triacylglycerol content was observed between the *trans* diet and the linoleate diet. They also observed a linear dose response relationship, but indicated that the metabolic effects of *trans* fatty acid as function of quantity ingested were not fully understood.

Margarine, the major source of *trans* fatty acids in the US diet, has increasingly replaced butter over the last 20 years (USDA, 1997). Ascherio et al. (1994) reported that 30,000 coronary deaths per year in the United States could be caused by consumption of hydrogenated fats and called for the elimination or a major reduction of these fats in foods.

1.1.7 Polyunsaturated Fatty Acids (W-3, W-6)

The major W-3 and W-6 fatty acids in foods are alpha-linolenic and linoleic acids, respectively. Humans can obtain W-3 and W-6 fatty acids only by ingesting them. They

are called essential fatty acids, because when a human cell builds a fatty acid, it cannot create double bonds in these sites.

1.1.7.1 Linoleic Acid (LA)

The plasma cholesterol-lowering effect of LA is one of the most consistent findings in nutrition. Studies carried out under strict metabolic ward conditions consistently show that the LDL-cholesterol concentrations are low when diets supplemented with LA are consumed. Iacono and Dougherty (1991) determined the effect of LA on plasma lipoproteins in a study carried out in 11 middle aged, healthy male subjects for two 40 day periods. The diets were designed to maintain saturated and monounsaturated fatty acids at 10 %, and LA at 3.8 and 10.8 %. LDL-cholesterol concentrations decreased 18% with the lower LA diet, and 22% with the higher LA diet. HDL-cholesterol concentrations were decreased by 2.3% on the lower LA diet and by 12.5% on the higher LA diet. The mechanism by which LA lowers the cholesterol contents of plasma LDL and HDL is not known. There has been concern expressed about the HDL- lowering effect of LA because HDL-cholesterol concentrations are inversely related to the risk of CHD (Mahley, 1985). Mensink and Katan (1989) showed that reasonable levels of intake of W-6 polyunsaturated fatty acids do not significantly lower HDL cholesterol concentrations.

1.1.7.2 W-3 Polyunsaturated Fatty Acids

The W-3 fatty acids are especially concentrated in some fish oils, canola, and walnut oil (USDA, 1991). It has been shown that fish oils may be equally or more

hypolipidemic than linoleic acid (Kromhout et. al. 1985). Recent epidemiological observations of low plasma lipids in populations consuming seafood reflect the effectiveness of dietary fish oils in reducing plasma lipids, especially triacylglycerols and their association with a decreased incidence of atherosclerosis, thrombosis, and coronary infarctions (Leaf and Webber, 1988).

1.1.8 Monounsaturated Fatty Acids

Oleic acid is the major monounsaturated fatty acid in foods. It is especially concentrated in canola oil and olive oil. Because of the concern about the HDL-lowering effect of linoleic acid, researchers re-examined the potential for monounsaturated fatty acid, especially oleic acid, as a replacement for saturated fatty acids. Mattson and Grundy (1985) compared oleic acid and linoleic acid as replacements for saturated fatty acids. Diets rich in the three types of fatty acids were fed in randomized order to 12 men. Oleic acid was as effective as linoleic acid in lowering LDL cholesterol, but without having the HDL- lowering effects of linoleic acid.

Furthermore, on the basis of epidemiological and metabolic ward studies, there is increasing evidence that olive oil may have a useful role in the prevention of CHD (Willet, 1994). This effect also has been observed with other monounsaturated fatty acids, so oils with high levels of monounsaturated fatty acids will be favored by consumers.

1.1.9 Cholesterol

Although the consumption of cholesterol does not seem to be a major factor in CHD, Khosla and Hayes (1993) and Pronczuck et al. (1994) reported that excessive

intake of dietary cholesterol exerts a synergistic effect on the metabolism of palmitic acid-rich fats, causing them to be hypercholesterolemic. In the absence of dietary cholesterol and in individuals with normal lipoprotein profiles, palmitic acid does not ordinarly raise total plasma cholesterol concentrations or LDL (Sundram et al., 1994, 1995; Hayes, 1995). Epidemiological studies further suggested that dietary cholesterol increases the risk of coronary heart disease beyond its serum cholesterol-raising effect (Stamler and Shekelle, 1988). Mechanisms for this latter effect are unknown.

Dietary cholesterol is prone to oxidation. Increased attention has focused on specific effects associated with certain cholesterol oxide products and the need to eliminate or prevent their formation in the human diet. Peng et al. (1979) reported that oxides of cholesterol are toxic and cause degeneration of aortic smooth muscle cells in tissue culture, and may lead to the development of atherosclerosis (Peng et al., 1991).

The overall conclusions from both experimental and epidemiological studies are consistent in showing that dietary saturated fatty acids elevate serum cholesterol concentrations. Three saturated fatty acids - palmitic acid, lauric acid, and myristic acid, undoubtedly increase LDL cholesterol concentrations. Thus, when dietary recommendations are made, the primary attention should be given to removing these three fatty acids from the diet.

1.2 ANIMAL FATS

Animal fats are stable to oxidation and possess uniquely pleasing flavors not found in other fats. Milkfat has received the most attention because of its commercial importance. It confers distinctive properties on dairy products that affect processing. Milkfat is a good source of essential fatty acids and it contains a high proportion of short

chain fatty acids which contributes to its ease of digestibility (Kennedy, 1991). Moreover, milkfat contains conjugated linoleic acids (CLAs) which are recognized for their potential ability to inhibit cancer (Clement et al., 1994). CLAs are unusual because they are abundant in products from ruminant animals. They are formed by biohydrogenation of PUFAs in the rumen of cows and subsequently find their way into milk (Gurr, 1994).

One epidemiological study compared dietary habits in rural Finland which has one-quarter the incidences of colon cancer with those in urban Copenhagen, Denmark (Maclennan et al., 1978). The community with the low incidence of colon cancer consumed more potatoes and whole milk than the higher incidence group which consumed less white bread and meat.

Of the natural fats, milkfat is the most varied in its chemical characteristics and functional properties. The melting point of milkfat increases with increasing saturation and chain-length of its constituent fatty acids (Walstra and Jenness, 1984). The melting point of milkfat is also affected by the positioning of the fatty acids on the glycerol molecule (Walstra et al., 1994). In its native form, milkfat does not suit various food formulations. For example, the wide melting range of milkfat, -40 to 40^{o}C (Walstra et al., 1994) makes it difficult to produce spreadable butter at refrigeration temperature. Consequently, new fields of application remain constrained due to the limited functionality (pourability and spreadability) of milkfat. An optimum fat cannot be always obtained from nature. Animal fats, when viewed in their native state, have limited use. But they can become an economic asset when viewed as raw materials to produce modified fats with desirable characteristics.

1.2.1 Methods to Modify Milkfat

The fat and oil industry is looking continuously at new techniques to alter the fat molecules. The biggest challenge is to reconcile the functional needs with the nutritional concerns. Several methods for improving the nutritional and physical properties of milkfat have been developed over the past few years. These approaches can be conveniently divided into biological (diet alteration), chemical (interesterification), or physical processes (distillation, crystallization, supercritical fluid extraction).

1.2.1.1 Biological Processes

1.2.1.1.1 Diet Alteration

Changing the fatty composition of milkfat by altering the diet of cows is an old concept which has received some recent attention (Banks et al., 1990). The inclusion of fats and oils in the diet of ruminants may exert the following effects, thereby influencing, either directly or indirectly, lipogenic pathways leading to milkfat secretion (Faulkner and Pollock, 1989; Banks et al., 1990).

1. The synthesis *de novo* of fatty acids such as palmitic acid and stearic acid by microorganims in the rumen may be reduced, leading to reductions in blood lipids, mammary gland uptake, and yield of long-chain fatty acids in the milk.

2. The amounts of long-chain fatty acids released in the rumen by lipolysis may be increased, leading to increases in the blood lipids, mammary gland uptake and yield of long-chain fatty acids in milk.

3. The intramammary synthesis of fatty acids may be reduced, thus decreasing the yields of short -and medium -chain fatty acids in milk.

These effects appear to be influenced by factors such as:(a) stage of lactation; (b) roughage-to-concentrate ratio and the fat content of the basal diet; and (c) amount, composition and physical form of fat added to the basal diet (Grummer, 1991).
A major technological breakthrough was the development of protected fat supplements obtained by crosslinking the fat with protein and treating with formaldehyde. The feeding of protected lipids was introduced as a means of producing fat with elevated linoleic acid levels. Feeding protected soybean oil to cows resulted in a 3.6 fold increase in linoleic acid (Banks et al., 1990) when compared to the basal diet. The degree of success in altering the fatty acid composition of milkfat through the feeding of protected lipid supplements has been inconsistent among different laboratories (Grummer, 1991).

1.2.1.2 Chemical Processes

1.2.1.2.1 Interesterification

Interesterification of milkfat alters the distribution of fatty acids in the triacylglycerol molecules and thus changes its physical properties such as melting behavior, crystallization, and plasticity. Interesterification can be accomplished by heating the fat or a mixture of fats and oils at a relatively high temperature (~ 200°C) for a long period of time. However, catalysts are commonly used that allow the reaction to be completed in a shorter time at lower temperatures. Alkali metals and alkali metal alkylates are effective low-temperature catalysts, sodium methoxide being the most

popular (Nawar, 1985).

Interesterification has not yet been applied to milkfat as its feasibility is restricted by the fact that it often requires two techniques that are deleterious to milkfat flavor, refining and deodorization, to remove the free fatty acids (0.2 to 0.4 %). These acids consume the catalyst or inactivate the active catalyst once it is formed (Sreenivasan, 1978).

Recently, the use of lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) to catalyze the interesterification reactions has received considerable attention. Triacylglycerol lipases can be used in several ways to modify triacylglycerols. In an aqueous medium with an emulsified triacylglycerol substrate, hydrolysis is the dominant reaction, but in organic media (heptane and hexane) esterification and interesterification become predominant (Bloomer, 1990). Enzymatic catalysts offer certain advantages over chemical catalysts including a controllable positional specificity and the ability to run the reaction at lower temperatures (Chang et al., 1990)

1.2.1.2.2 Complex Formation.

This technique is used to reduce cholesterol concentrations in milk and dairy products by complexing cholesterol and its esters with a complexing agent such as β–cyclodextrin. Cyclodextrins are cyclic oligosaccharides obtained by enzymatic degradation of starch. They consist of six, seven, or eight glucose monomers arranged in a donut shaped ring. These compounds are denoted alpha, beta or gamma cyclodextrin, respectively. β-Cyclodextrin is not hygroscopic and contains 13.6% moisture at 30°C and 86% relative humidity (Szejtli, 1982a). Cyclodextrins are water-soluble because of

the location of free hydroxyl groups on the external rim of the molecule (Szejti, 1982a). Solubility is a function of temperature, the higher the temperature the higher the solubility. The solubility of β-cyclodextrin increases from 0.8% at 0.5 °C to 39.7% at 90 °C. The internal cavity which is hydrophobic allows the cyclodextrins to complex molecules such as aromatic alcohols, fatty acids and their esters, and cholesterol.

Currently, cyclodextrins are used (1) to control volatility of agricultural compounds designed to control pathogens, insects, and weeds; (2) to stabilize pharmaceutical products (drugs, vitamins), fragrances, and skin care lotions through encapsulation; and (3) to enhance color, odor, and flavor stability in beverages and processed foods (Szejtli, 1982b). The most important parameters that determine whether a given molecule can form complexes with β–cyclodextrin are its hydrophobicity and its relative size and geometry in relation to the cyclodextrin cavity (Szejtli, 1982b). When dissolved in water, the cyclodextrin molecules are able to accommodate smaller guest molecules, or functional groups of molecules less hydrophilic than water in their internal cavities (Szejtli, 1982b). In aqueous solution, the slightly apolar cyclodextrin cavity is occupied by water molecules, an energetically unfavored process (polar-apolar interaction). These water molecules are therefore readily substituted by appropriate guest molecules such as cholesterol which are less polar than water (Szjetli, 1982b).

Several investigators have used β–cyclodextrin to reduce the cholesterol levels in animal products. Courregelongue et al. (1989) used 10 % β-cyclodextrin to remove 41 % of the sterols from anhydrous milkfat. Bayol et al. (1989) reported the removal of 80 % Δ^4-cholesten-3-one from anhydrous milkfat, while Roderbourg et al. (1993) demonstrated that at least 37% of the cholesterol could be removed from anhydrous milkfat in one

single step. Graille et al. (1991) reported the simultaneous removal of 50% cholesterol and 52 % FFAs from cream with β-cyclodextrin. In Australia, Okenfull et al. (1991) invented a new process called SIDOAK to reduce cholesterol in dairy products. The process consisted of adding β- cyclodextrin to milk and mixing below 10 °C. The insoluble complexes of cholesterol and β- cyclodextrin were removed by centrifugation. The maximum cholesterol reduction obtained was 80-90 %. Yen and Tsai (1995), using 10% β- cyclodextrin, indicated the removal of 95 % cholesterol simultaneously with 50 % FFAs from lard. Awad et al. (1996) used β- cyclodextrin to reduce the cholesterol level in egg yolk by 96%. The process consisted of mixing the diluted yolk with β-cyclodextrin at 50 °C for 10 min. The β– cyclodextrin -cholesterol complexes were removed from the medium by centrifugation.

1.2.1.3 Physical Processes

Milkfat fractionation using physical processes such as short-path distillation, melt crystallization, and supercritical fluid extraction has been applied in conjunction with cholesterol extraction.

1.2.1.3.1 Short-Path Distillation (SPD)

SPD is a relatively well known process and is based on the evaporation of molecules into substantially gas-free space, i.e., vacuum. The controlling factor is the rate at which the molecules escape from the heated surface of the distilling liquid and are received by the cooled condenser surface. Arul et al. (1988) fractionated milkfat by SPD into four fractions at temperatures of 245 and 265 °C and pressures of 220 and 100 μm

Hg. Two fractions were liquid, one was semi-solid, and one was solid at room temperature. The solid fraction contained cholesterol at a concentration of 0.2 mg/g fat compared to 2.6 mg/g fat in native milkfat or to 16.6 mg/g fat in the liquid fraction. SPD of milkfat offers an opportunity to obtain fractions with different chemical and physical properties. However, there are some major drawbacks to this technique: (1) the ratio of saturated fatty acids (C16:0, C14:0, C12:0) to the unsaturated fatty acids in the liquid and semi-solid fractions is 1.46 to 2.7 fold greater, respectively, than native milk fat; (2) the use of high temperatures might decompose or polymerize the triacylglycerols, particularly those with high unsaturation, even when distilled under vacuum; and (3) capital investment costs are high (Arul et al., 1988).

1.2.1.3.2 Melt Crystallization (MC)

This technique is based on a slow cooling of the melted milkfat, a short duration of stabilization at the fractionation temperature and the separation of the crystals from the liquid phase by vacuum filtration. Makhlouf et al. (1987) fractionated milkfat into seven fractions by MC at temperatures ranging from 26 to 9°C. Butter made from a blend of different fractions showed two to four fold improvements in spreadability at 7°C compared to regular butter. After crystallization, the concentration of cholesterol in the liquid fraction (3.8-mg/ g fat) was nearly twice that in the solid fraction (2.0-mg/ g fat). Despite its simplicity, MC is a tedious technique and requires many fractionation steps to obtain low melting point fractions. The major drawback of this technique is the significant overlapping of molecular weights of triacylglycerols (Makhlouf et al., 1987).

1.2.1.3.3 Supercritical Fluid Extraction (SFE)

In this process, a product is treated with a gas (usually carbon dioxide) of high density, low viscosity, and reduced surface tension under high pressure and temperature. The procedure has the advantage of absence of potentially toxic solvents and by-products (Friedrich and Pryde, 1984). Arul et al. (1987) fractionated milk fat into eight different fractions at 10 - 35 MPa and 50 to 70 °C. They found that cholesterol tended to concentrate in the low and intermediate melting fractions. Lim et al. (1991) acheived an overall cholesterol reduction of 92.6 % with a process yield of 88.5 %. The extraction was done at 40 °C and 24.1 - 27.5 MPa.

Recently, Bhaskar et al. (1993) fractionated milk fat into five different fractions (S1 – S5) with differing cholesterol levels at 3.5 - 24.1 MPa and 40 - 60 °C. The saturated to unsaturated fatty acid ratio along with cholesterol levels increased from fraction S2 – S5 compared to the native anhydrous milkfat. However, in fraction S1, the cholesterol level and the ratio of saturated-to-unsaturated fatty acids decreased by 51 and 24 %, respectively, with respect to the control. The decrease in the saturated-to-unsaturated fatty acid ratio in S1 was due to the decrease in short chain fatty acids C4:0 to C10:0 which may be viewed as undesirable due to their potential hypocholesterolemic effect (Grummer, 1991).

It can be concluded that all the approaches developed to modify the properties of milkfat do not improve the nutritional properties. None of these approaches alter the nature of the saturated fatty acids. Instead, the saturated fatty acids remain intact and are separated into different fractions. The fractionation of milkfat by physical processes has been effective to only a modest extent in improving its melting property. Moreover,

fractionation may have a negative impact on the aroma of milkfat.

The reported literature has recognized that modifying milkfat to reduce its hypercholesterolemic effects in order to fit under the current dietary recommendation guidelines will not be competitive in the manufacture of fat products, at least until a process is developed that will simultaneously remove cholesterol, free fatty acids, and reduce the myristic acid content.

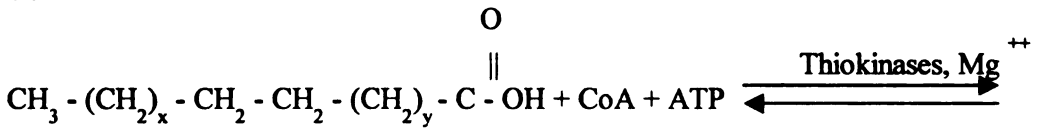
1.3 ENZYMOLOGY OF DESATURATION

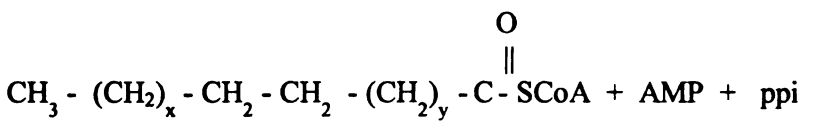
1.3.1 Biosynthesis of Unsaturated Fatty Acids

Saturated fatty acids are known to be the precursors of unsaturated fatty acids in higher organisms (Masoro, 1968). Many studies have been carried out with Δ^9-desaturase, which is responsible for the biosynthesis of monounsaturated fatty acids. What has been found to be true for the Δ^9-desaturase is, in general applicable, to the other fatty acyl CoA desaturases (Δ^6 and Δ^5) (Brenner , 1974). In contrast to the Δ^9 fatty acyl CoA desaturase which synthesizes monounsaturated fatty acids, the Δ^6 and Δ^5 fatty acyl CoA desaturases synthesize polyunsaturated fatty acids (Jeffcoat, 1979).

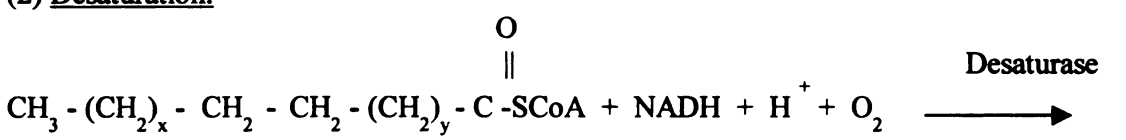
Early experiments using liver homogenates established that the desaturation of fatty acids involves two enzymatic reactions, *viz.*, the activation of the fatty acid to the acyl-CoA derivative by thiokinases in the presence of CoA, ATP and Mg^{++}, followed by the desaturation of the derivative by the acyl-CoA desaturase system (Masoro, 1968).

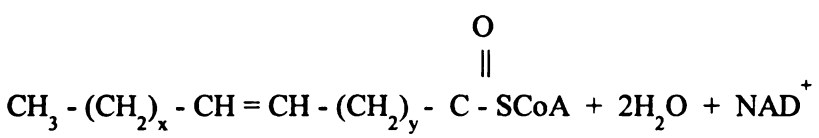
(1) Activation:





(2) Desaturation:





Acyl-Co A is the substrate and reduced nicotinamide adenine dinucleotide (NADH) is the reducing agent. Oxygen is a necessary requirement for the reaction.

Holloway (1971) reported that mammlian Δ^9 fatty acyl CoA desaturases are bound to the endoplasmic reticulum and have an obligatory requirement for NADH, molecular oxygen, cytochrome b_5, and NADH cytochrome b_5 reductase (EC 1.6.2.2). These data coupled to those of Raju and Reiser (1972) who demonstrated the lack of desaturation of lipid bound stearate, established fatty acyl CoA as the true substrate for stearoyl CoA desaturase. The same has since been demonstrated to be true for the Δ^6 and Δ^5 fatty acyl CoA desaturases (Brennner, 1971).

The rules for mammalian desaturation, as described by Jeffcoat (1979), are as follows:

(1) All desaturases require molecular oxygen and a reduced pyridine nucleotide to catalyse the direct oxidative desaturation of preformed fatty acids usually in the form of coenzyme A esters;

(2) Double bonds are always introduced into the methylene chain at a fixed position from the carboxyl group;

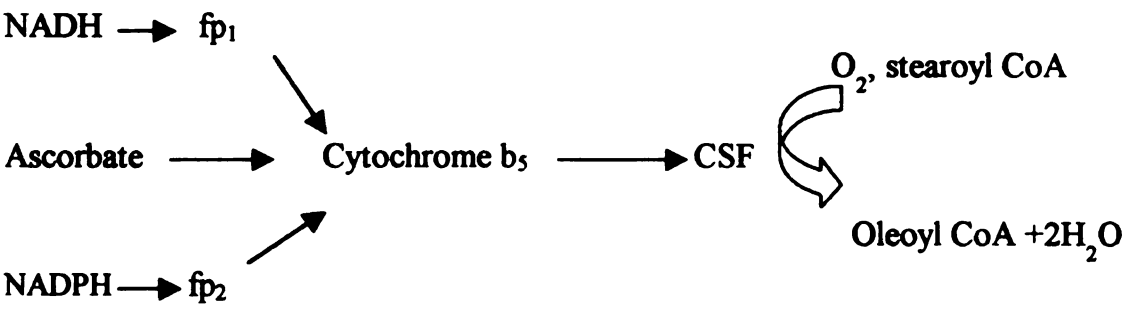
(3) When the substrate is a saturated fatty acid, the first double bond is inserted between carbon atoms 9 and 10. Unlike plants, animals cannot introduce double bonds farther away than nine carbons from the carboxyl group;

(4) When the substrate is already unsaturated, subsequent double bonds are inserted between the double bond nearest the carboxyl group and the carboxyl group itself, in such a way as to usually maintain the methylene interrupted distribution of double bonds;

(5) Unsaturated fatty acids with *trans* rather than *cis* double bonds are generally treated by the enzymes as saturated fatty acids.

1.3.2 Electron Transport Components

The requirment for three protein components in the stearoyl CoA desaturase has already been established.

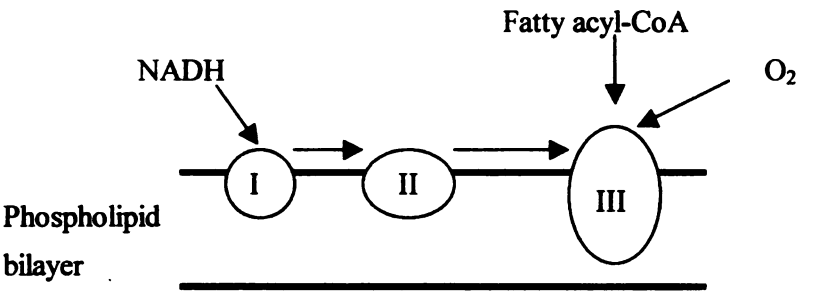


where fp_1: NADH - cytochrome b_5 reductase; fp_2: NADPH - cytochrome c reductase.

(adapted from Oshino et al., 1971)

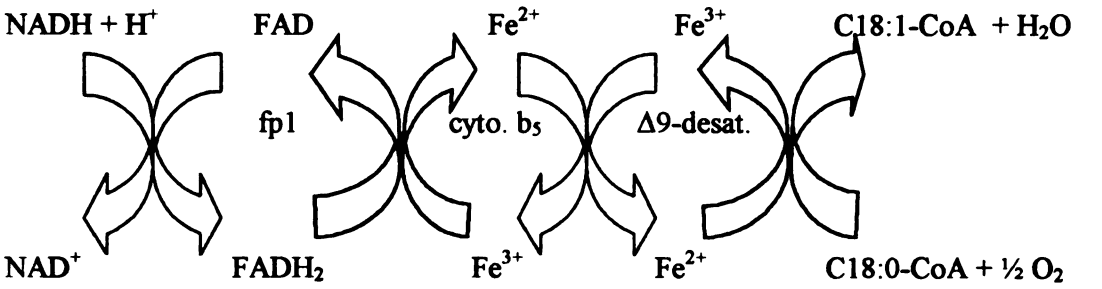
CSF: cyanide sensitive factor

These components have been isolated and characterized by Spartz and Strittmatter (1973) and Strittmatter et al. (1974). The purified cytochrome b_5 reductase (EC 1.6.2.2) and cytochrome b_5 consist of a hydrolytic catalytic segment exposed to the aqueous environment and a hydrophobic portion involved in binding to the microsomal membrane. The terminal stearoyl CoA desaturase is a more nonpolar polypeptide of 53000 daltons (62 % hydrophobic amino acid residues) (Strittmatter et al., 1974). It is completely submerged in the hydrocarbon region of the membrane with the catalytic domain exposed to the aqueous environment to permit reduction of its non- heme iron by cytochrome b_5 and interaction with the hydrophilic portions of substrate molecules (Enock et al., 1976).



Diagrammatic representation of the Δ_9 fatty acyl CoA desaturase complex. The location of the three proteins, NADH-cytochrome b_5 reductase (I), cytochrome b_5 (II), and the desaturase (III) are shown in the phosphilipid bilayer of the endoplasmic reticulum. (Diagram adapted from Jeffcoat, 1977).

The function of the electron transport proteins is to transfer reducing power from the reducing agent (NADH) to the desaturase protein. The heme iron of cytochrome b_5 accepts electrons from the reduced flavin nucleotide and passes them on the non-heme iron of the desaturase.



(Adapted from Jeffcoat, 1979).

1.3.3 Role of Membrane Lipid in the Desaturation Reaction

Studies by Rogers and Strittmatter (1974) and Strittmatter and Rogers (1975) have demonstrated a random distribution and translational movement of cytochrome b_5 and NADH-cytochrome b_5 reductase in the phospholipid bilayers during electron transfer from the flavoprotein to the heme protein. They also suggested that the most likely function of the lipid is to orient the proteins with respect to each other and therefore to act indirectly on the activity of the desaturase. Studies by Jones et al. (1969) showed that extraction of liver microsomes with 90 % (v/v) acetone resulted in 100% loss of stearoyl CoA activity, which could be restored to normal levels by the addition of the total extracted lipid fraction.

1.3.4 Role of Metal Ions in Δ^9-Desaturation

Strittmatter et al. (1974) showed that the purified rat liver stearoyl CoA desaturase is a non-heme iron-containing enzyme, as 80% of its activity was inhibited upon the addition of 10 mM bathophenathroline sulfonate (ion chelator). The stearoyl CoA desaturase iron participates in the catalytic process by mediating oxidation-reduction reactions through reversible changes in its oxidation state. Mg^{++} is not required for the

desaturation reaction per se. However, it is an obligatory component in the activation reaction of the fatty acid (Masaro, 1968).

1.3.5 Cytoplasmic Proteins and Desaturation

The biosynthesis of unsaturated fatty acids involves an interplay between soluble fatty acid synthetase and membrane-bound enzymes, the desaturases. Free fatty acids can be highly toxic within the cell, hence there are particular species of cytoplasmic proteins which bind to them to protect cell membranes and membrane-bound enzymes from their cytotoxic effect. This is analogous to the albumin in the circulation system (Bass, 1985). The functional role of these proteins is not clearly understood. In crude microsomal preparations, full activity of the Δ^9–, Δ^6–, Δ^5- fatty acyl-CoA desaturase system is expressed. Upon repeated washing, the activities of the three enzymes are lost, but can be restored by the addition of cytoplasmic proteins (Catala et al., 1975).
Fractionation studies of cytoplasmic proteins of the liver showed that Δ^9-desaturation is stimulated by a 46,000 molecular weight protein (Jeffcoat et al., 1976) in the way that bovine serum albumin affects enzyme activity. Brophy and Vance (1976) and Jeffcoat et al. (1977) suggested that this protein acts as a fatty acid binding protein which regulates the availability of fatty or fatty acyl-CoA for the lipid-metabolizing enzymes.
Jeffcoat (1977), working with purified Δ^9–desaturase, demonstrated that cytoplasmic proteins are not obligatory for the desaturation reaction per se and suggested that the role of bovine serum albumin is to protect the acyl-CoA substrate from the effect of acylthiolester hydrolases. Initially, it was believed that acyl-CoA (ACBP) and fatty acid binding proteins (FABP) were one and the same. However, more recent studies

(Rasmussen et al., 1990) have shown these functions reside in separate proteins having different molecular weights (FABP (M_r=12 - 15 $\times 10^3$) and ACBP (M_r = 9932) and no sequence similarities.

CHAPTER 2

DESATURATION OF MYRISTOYL-CoA TO MYRISTOLEOYL-CoA BY HEN LIVER MICROSOMAL Δ^9-DESATURASE.

2.1 ABSTRACT

Desaturation of myristoyl-CoA to myristoleoyl-CoA was measured in microsomal preparations of hen liver. Desaturation was maximal at pH 7.4. Enzymatic activity was linear with time up to 10 min and proportional to microsomal protein concentrations. The initial velocity was linear with substrate concentrations between 13 and to 200μM. Decrease in desaturation activity was observed at substrate concentrations greater than 266μM. There was an absolute requirement for reduced pyridine nucleotide (NADH), while maximum activity was observed at a myristoyl-CoA:NADH mole ratio of 1. Competitive inhibition studies of myristoyl-CoA desaturation suggest that the inhibitors stearyl and oleyl-CoA were more effective than palmitoyl-CoA. Free CoA did not inhibit the Δ^9-desaturase system. The desaturation of myristoyl-CoA was stimulated by bovine serum albumin and reduced by cytoplasmic proteins. The effect of cytoplasmic proteins in the enzymatic reaction was completely abolished by trypsin digestion and boiling for 30 min. On the basis of the present data, it was concluded that 9,10-desaturation of acyl-CoA derivatives containing 12 to 18 carbon fatty acyl chains are catalyzed by the same enzyme.

2.2 INTRODUCTION

Enzymatic desaturation of saturated fatty acids to form monounsaturated fatty acids is known to occur in various aerobic organisms including yeast and several animal species (Brett et al., 1971). Studies with liver homogenates have established that the desaturation of fatty acids involves two enzymatic reactions, *viz.*, the activation of the fatty acid to the acyl-CoA derivative by thiokinases in the presence of CoA, ATP and Mg^{++}, followed by the desaturation of the derivative by the acyl-CoA desaturase system (Masoro, 1968).

(1) Activation:

$$CH_3 - (CH_2)_x - CH_2 - CH_2 - (CH_2)_y - \underset{\underset{O}{\|}}{C} - OH + CoA + ATP \xrightleftharpoons{\text{Thiokinases, } Mg^{++}} CH_3 - (CH_2)_x - CH_2 - CH_2 - (CH_2)_y - \underset{\underset{O}{\|}}{C} - SCoA + AMP + ppi$$

(2) Desaturation:

$$CH_3 - (CH_2)_x - CH_2 - CH_2 - (CH_2)_y - \underset{\underset{O}{\|}}{C} - SCoA + NADH + H^{+} + O_2 \xrightarrow{\text{Desaturase}} CH_3 - (CH_2)_x - CH = CH - (CH_2)_y - \underset{\underset{O}{\|}}{C} - SCoA + 2H_2O + NAD^{+}.$$

Acyl-Co A is the substrate and reduced nicotinamide adenine dinucleotide (NADH) is the reducing agent. Oxygen is a necessary requirement for the reaction.

Holloway (1971) reported that mammalian Δ^9 fatty acyl-CoA desaturases are bound to the endoplasmic reticulum and have an obligatory requirement for NADH,

molecular oxygen, cytochrome b_5, and NADH cytochrome b_5 reductase (EC 1.6.2.2). These components have been characterized by Spartz and Strittmatter (1973) and Strittmatter et al. (1974). The purified cytochrome b_5 reductase (EC 1.6.2.2) and cytochrome b_5 consist of a hydrolytic catalytic segment exposed to the aqueous environment and a hydrophobic portion that is involved in binding to the microsomal membrane. The terminal stearoyl CoA desaturase is a more nonpolar polypeptide of 53000 daltons and contains 62 % hydrophobic amino acid residues (Strittmatter et al., 1974). It is completely submerged in the hydrocarbon region of the membrane with the catalytic domain exposed to the aqueous environment to permit reduction of its non- heme iron by cytochrome b_5 and interaction with the hydrophilic portion of substrate molecules (Enock et al., 1976).

Using purified stearoyl-CoA desaturase, Enoch et al. (1976) reported that 9,10-desaturation of fatty acyl-CoA derivatives with chain lengths of 12 to 19 carbon atoms is catalyzed by the same enzyme, with stearoyl-CoA being the best substrate. These studies, coupled with those of Raju and Reiser (1972) who demonstrated the lack of desaturation of lipid-bound stearate, established fatty acyl-CoA as the true substrate for stearoyl CoA desaturase. Similar conclusions have been established for the Δ^6 and Δ^5 fatty acyl CoA desaturases (Brennner, 1971).

As free fatty acyl-CoA derivatives can be highly toxic within the cell, there are particular species of cytoplasmic proteins which bind to them to protect cell membranes and membrane bound enzymes from their cytotoxic effect. This is analogous to the presence of albumin in the blood circulatory system (Bass, 1985). The functional role of these proteins is not clearly understood. Brophy and Vance

(1976) and Jeffcoat et al. (1976, 1977, 1978) suggested that these proteins act as fatty acid-binding proteins which regulate the availability of fatty acids or fatty acyl-CoA for the lipid-metabolizing enzymes. The latter investigators, working with purified Δ^9-desaturase, demonstrated that bovine serum albumin (BSA) is not obligatory for the desaturation reaction *per se*, suggesting its role to be the protection of the fatty acyl-CoA substrate from the effect of acylthiolester hydrolases.

In this study, the initial objective was to develop an assay system to monitor the desaturation of myristoyl-CoA to myristoleoyl-CoA by Δ^9-desaturase system in hen liver microsomes. This assay procedure was subsequently used to study several properties of the enzyme system including specific activity, substrate specificity and the interaction of the substrate with bovine serum albumin and cytosolic proteins.

2.3 MATERIALS AND METHODS

2.3.1 Materials

Myristoleoyl-, myristoyl-, palmitoyl-, stearoyl-, oleoyl-CoA (lithium salt), tetradecanoic, *cis*-9-tetradecenoic and pentadecanoic acids. BSA, trypsin, trypsin inhibitor, and reduced nicotenamide adenine dinucleotide (NADH) were obtained from Sigma Chemical Co. (St Louis, MO). The standards were of greater than 98 % chemical purity according to the manufacturers. Magnesium chloride and potassium hydroxide were obtained from Aldrich Chemical Co. (Milwaukee, WI). Laying hens (White Leghorn) were obtained from the MSU Poultry Farm.

2.3.2 Preparation of hen liver microsomes

For each set of experiments, three laying white Leghorn hens were sacrificed. The whole livers were excised and cooled immediately over ice. The livers were minced and mixed together to decrease possible variations in desaturation yield from one single liver preparation to another. A 20g sample of the ground livers was immediately homogenized in a Waring blender for 30 sec with 60 ml of chilled 0.1 M potassium phosphate buffer (pH 7.4) containing 0.3 M sucrose. The temperature of the buffer solution was 0 to 2°C. The resulting homogenate was centrifuged at 15,000 x g for 30 min at 2 °C. The sedimented cellular debris and mitochondria were discarded. Microsomes containing the Δ^9-desaturase system were sedimented from the postmitochondrial supernatant fraction by centrifuging at 105,000 x g for 1 hr at 2 °C. The resulting microsomal pellet was either suspended in the homogenizing buffer or in the microsomal supernatant (containing the cytoplasmic proteins) to provide a final concentration of 2.66 mg of microsomal protein/ml. The protein concentration was determined by the method of Lowry et al. (1951). The remaining ground livers were stored at -20 °C for no longer than 10 hours. Independent studies indicated that there was no loss in enzymatic activity over the duration of the holding period.

2.3.3 Enzyme assays

The process to convert myristoyl-CoA to myristoleoyl-CoA using the Δ^9-desaturase system of hen liver microsomes is outlined in Figure 1. The rationale for each subsequent step in the process is described below.

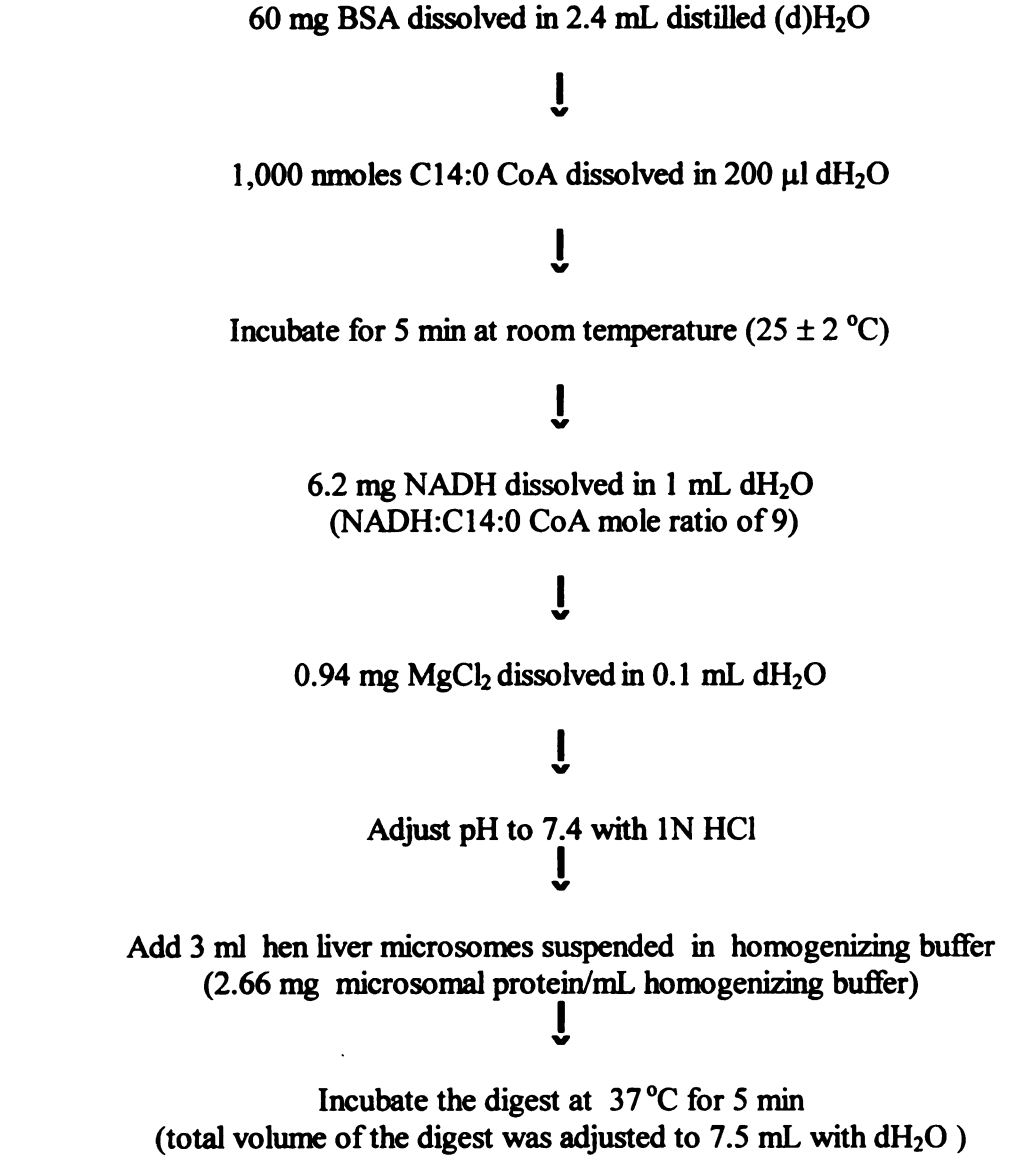


Figure 1- Process for converting myristoyl-CoA to myristoleoyl-CoA using the Δ^9-desaturase system of hen liver microsomes. The total volume of the digest was adjusted to 7.5 mL by the addition of 0.8 mL distilled water. The microsomes were suspended in the homogenizing buffer with a vortex mixer.

2.3.4 Lipid extraction and fatty acid methyl esterification

The enzymatic reaction was stopped by adding 4 ml of 10 % methanolic KOH to saponify the lipids. The methanolic KOH solutions were heated at 65° C for 30 min and then acidified with 7 N HCL. Samples were extracted three times with a mixture of hexane: isopropanol (3:2, v/v). The combined solvent extracts were evaporated under nitrogen. The internal fatty acid standard C15:0 (0.02 mg) was added to each of the samples which were then derivatized to their fatty acid methyl esters by the boron trifluoride/methanol esterification procedure of Morrison and Smith (1964).

2.3.5 Gas chromatographic (GC) and GC-MS analyses

Gas chromatographic analyses were carried out on a Hewlett-Packard 5890 A gas chromatograph (Avondale, PA) equipped with a flame ionization detector (FID), split-mode(1/12), and a fused silica capillary column (30 m x 0.25 mm) coated with a 0.25 μm film of DB-225 (J & W Scientific, Folsom, CA). Operating conditions of the gas chromatograph were as follows: temperatures – injector 275 °C, detector 300 °C, column 165 °C; hold 5 min; increase over temperature 0.5 °C/min to 180 °C, hold 20 min, increase 10 °C to 215 °C, hold 10 min. Fatty acid methyl esters were identified by comparing their relative gas chromatographic retention times with those of known standards. Standard curves were generated from known concentrations of pure methyl ester standards of C14:0 and *cis*-9-C14:1 ranging from 7.8 to 62.5 pg and containing 0.01 pg of C15:0. The areas of the C14:0, C14:1, and C:15:0 peaks were determined using a digital integrator. The C14:0 and C14:1 peak areas were divided by the internal standard area to obtain the response ratios for both standards and samples.

The standard response ratios for C14:0 and *cis*-9- C14:1 were plotted against their corresponding concentrations. Standard response ratio plots should bracket the sample response ratio. The C14:0 and *cis*-9-C14:1 concentrations in the sample extracts were quantified from the standard response ratio plots and corrected for the dilution factor (df = 2). Initial analyses indicated that there was no endogenous *cis*-9-C14:1 in the medium. Thus, the *cis*-9-C14:1 observed was derived as a result of the desaturation reaction *per se*.

The identity of *cis*-9-C14:1 was confirmed by comparing the mass spectra of authentic *cis*-9-C14:1 standard and that derived from the desaturation of C14:0 (Appendix 1). The GC-MS analyses were carried out on a JEOL A × 505H double focusing mass spectrometer equipped with a Hewlett-Packerd 5890 J GC. The fatty acid methyl esters, dissolved in hexane, were injected using a splitless injection technique onto a 30m x 0.25 mm i.d., 25μm coated DB 225 capillary column interfaced directly into the ion source. The GC oven temperature was programmed from 100°C to 165 °C at 5 °C per min and held for 5 min then to 172 °C at 0.5 °C per min and finally to 230 °C at 10°C per min. The injector and transfer line temperatures were maintained at 230 °C and 230 °C, respectively. Fatty acids were analyzed in the m/z range of 45 – 600 for the methyl ester derivatives in positive electron ionization mode (EI). The analyses indicated that the fatty acid resulting from the desaturation of C14:0 by the hen liver desaturase system was indeed *cis*-9-C14:1.

2.3.6 Trypsin digestion

To a 6 mL aliquot of the microsomal supernatant containing 31.5mg/mL of cytosolic proteins, 340 mg of trypsin were added, and the mixture was incubated for 1

hour at 37 °C. Proteolysis was stopped with the addition of 680 mg trypsin inhibitor.

2.3.7 Statistical analysis

All treatments were performed in duplicate (n=2). Statistical analyses were based on the polynomial regression and analysis of variance (ANOVA) procedures of SYSTAT 7.0 (SPSS Inc., 1997). ANOVA was also used to analyze the data and included NADH, competitive inhibitors, heat treatment, trypsin digestion, BSA and microsomal supernatant (MS) as main effects and BSA x MS as interaction. The Tukey multiple comparison procedure was used for mean separation (Berk, 1998). If levels within a factor were significantly different, they were examined further using the student t-test for differences among individual means. Results were judged to be statistically significant based on type I error rate of 5%.

2.4 RESULTS AND DISCUSSIONS

Mammalian desaturases have been known for many years to be bound to the endoplasmic reticulum and require oxygen and NADH (Oshino et al., 1966; 1971). In mammalian tissues, the formation of monoenes from free fatty acids is a two-stage reaction: activation of the saturated fatty acid to fatty acyl-CoA by acyl-CoA synthetase, followed by desatuartion of the acyl-CoA derivative (Masoro, 1968). In the activation reaction, the energy is generally supplied by adenosine triphosphate (ATP) conversion to adenosine monophosphate (AMP) and pyrophosphate (Pande and Mead, 1968). Previous studies of desaturation generally used free fatty acids as the substrate in the presence of CoA, Mg^{++}, and ATP, and thus the rates of both

reactions were measured. In the present study with Δ^9-desaturase of hen liver microsomes, the desaturation was studied as a single reaction using the activated form of the substrate (myristoyl-CoA) in order to ensure that the results were not due to inhibition of the initial activation of free myristic acid to myristoyl-CoA. When the fatty acyl-CoA is used as the substrate, the activation reaction of the substrate does not take place and therefore it is not a rate limiting step in the desaturation reaction.

2.4.1 Factors that modify the yield of desaturation of myristoyl-CoA

Hen liver microsomes, like microsomes from other sources (Brett et al. 1971), catalyzed the oxygen-and reduced pyridine nucleotide-dependent desaturation of myristoyl-CoA to myristoleoyl-CoA. In order to test the reliability of the assay conditions, the effects of time, microsomal protein concentration, substrate concentrations, reducing agent, pH, competitive inhibitors, BSA and microsomal supernatant on enzyme activity were investigated.

2.4.1.1 Effect of incubation time

Under the assay conditions, the amount of myristoleate formed was directly related to the incubation times. When the desaturation reaction was stopped after 5 min, a linear relationship between desaturation and time was observed (Figure 2). A similar relationship was observed when the reaction was terminated at 10 min. The linear relationship between desaturation rate and incubation times (up to 10 min) is indicated by the equation $Y = 3.7643X$ ($R^2 = 0.93$). Monoene production declined sharply after 30 min of incubation which may be due to the decrease in substrate

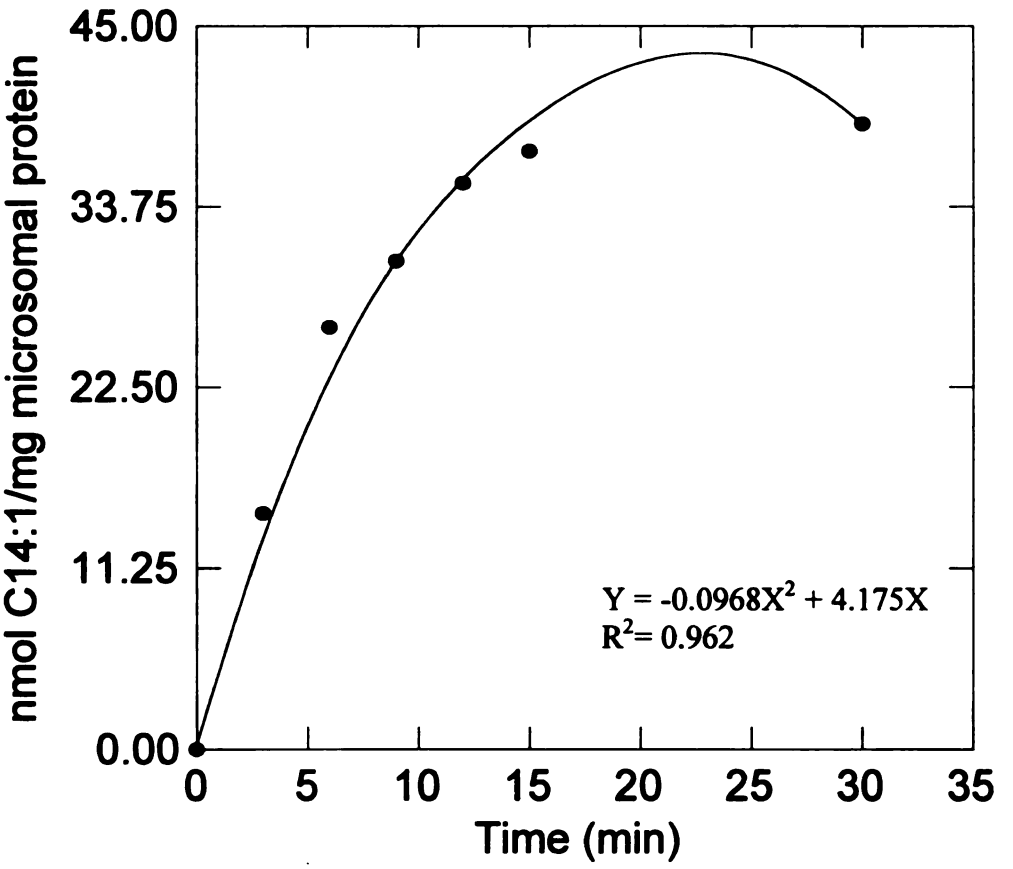


Figure 2- The desaturation of myristoyl-CoA as a function of time by the Δ^9-desaturase of hen liver microsomes. The desaturation activity was measured at 37°C under conditions described in Figure 1 with different incubation times. The complete system contained 8 mg hen liver microsomal protein, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 133 μM C14:0CoA, 60mg BSA, 1.16 mM NADH, 1.31 mM $MgCl_2$, pH 7.4, in a total volume of 7.5 mL.

concentration as a result of its utilization in other enzymatic reactions. In the liver cell, any saturated acyl-CoA can suffer a variety of fates, such as being hydrolyzed to the unesterified fatty acid, converted into lipid, or undergoing β–oxidation, chain elongation or desaturation (Voet, 1990).

2.4.1.2 Effect of microsomal protein concentration

The rate of desaturation of myristoyl-CoA was shown to be directly proportional to the amount of the microsomal protein over the range of 2 to 8 mg per 7.5 ml of final incubation volume (Figure 3). The linear relationship between the desaturation rate and the microsomal protein concentration (up to 8 mg per 7.5 ml of final incubation volume) is indicated by the equation $Y = 5.1419X$ ($R^2 = 0.99$). Enzyme activity was slowed at protein concentrations greater than 10 mg per 7.5 ml of incubation medium. A relatively high microsomal protein concentration of 8 mg per 7.5 ml incubation volume was purposely employed in all the assays, unless otherwise indicated, to assure that there was sufficient production of myristoleate over the incubation time of the assay in order to be quantifiable by the gas chromatographic procedure. At constant levels of substrate, monoene formation was slowed at high protein concentrations and slowed by prolonged incubation. These latter two findings were also observed in rat brain desaturation systems (Cook and Spence, 1973) and could be due, in part, to the addition or accumulation of desaturation product.

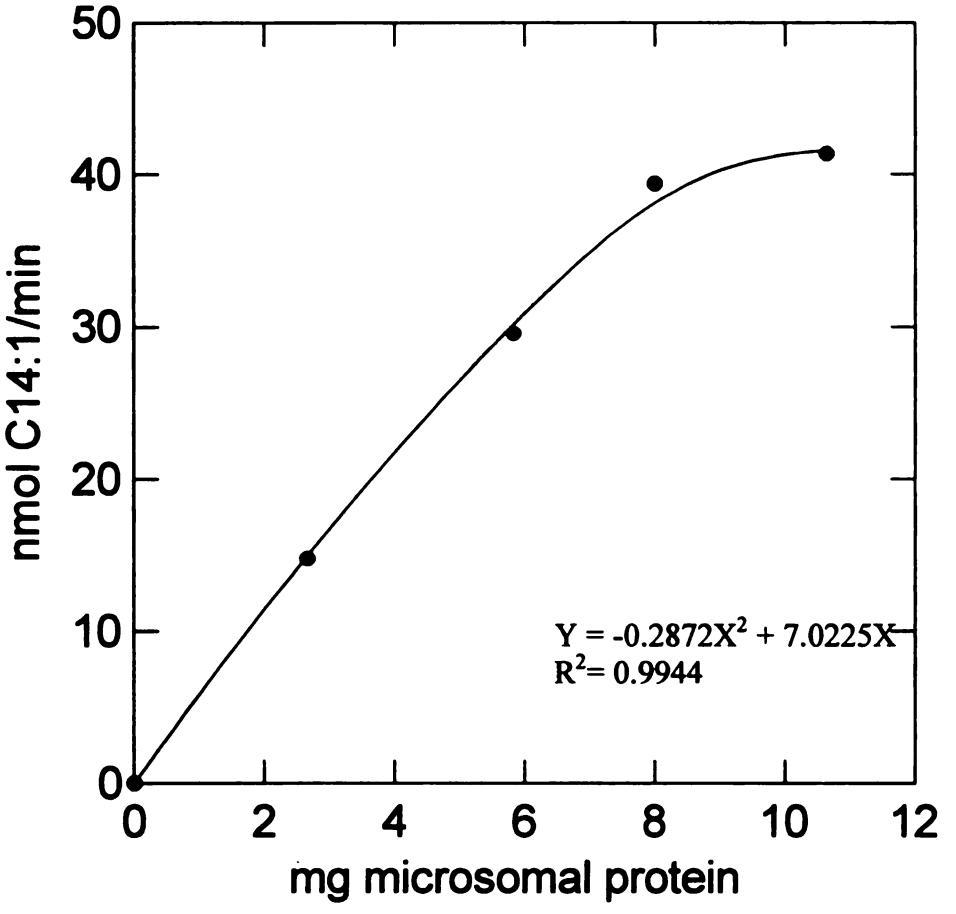


Figure 3- The desaturation of myristoyl CoA as a function of microsomal protein concentration by the Δ^9 desaturase of hen liver microsomes. The desaturation activity was measured under the conditions described in Figure 1 with different microsomal protein concentrations. The complete system contained 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 133 μM C14:0CoA, 60mg BSA, 1.16 mM NADH, 1.31 mM $MgCl_2$, pH 7.4, in a total volume of 7.5 mL. Incubations were at 37°C for 5min.

2.4.1.3 Effect of substrate concentration

Figure 4 reveals the relationship of initial velocity to substrate concentration for the hen microsomal Δ^9-desaturase catalyzes reaction. The plot was linear between 13 and 133 μM. Inhibitory effects of substrate concentration occurred at concentrations in excess of 300 μM. The assays of initial rates could not be measured accurately at substrate concentrations lower than 13 μM, because it was difficult to have sufficient product to be accurately determined by gas chromatography. Because of the inhibition at high substrate concentrations and variation at the very low concentrations (i.e., less than 13 μM), it was difficult to determine K_m for the desaturation reactions. Unless otherwise indicated, most assays were carried out at a substrate concentration of 133 μM, allowing recovery of product significantly above background values (if any) and avoiding the possibility of substrate inhibition. Further, the use of this constant substrate concentration decreased the likelihood of non-first order reaction conditions of reduced substrate availability, by incorporation into lipids by acyltransferase. These data, together with those in Figures 2 and 3, clearly show that the assay described here gives a true measure of the initial velocity under pseudo first order reaction conditions.

2.4.1.4 Effect of reducing agent

The reducing equivalent in the desaturation reaction is used to reduce the Δ^9-desaturase system through an electron transport chain (Oshino et al., 1971).

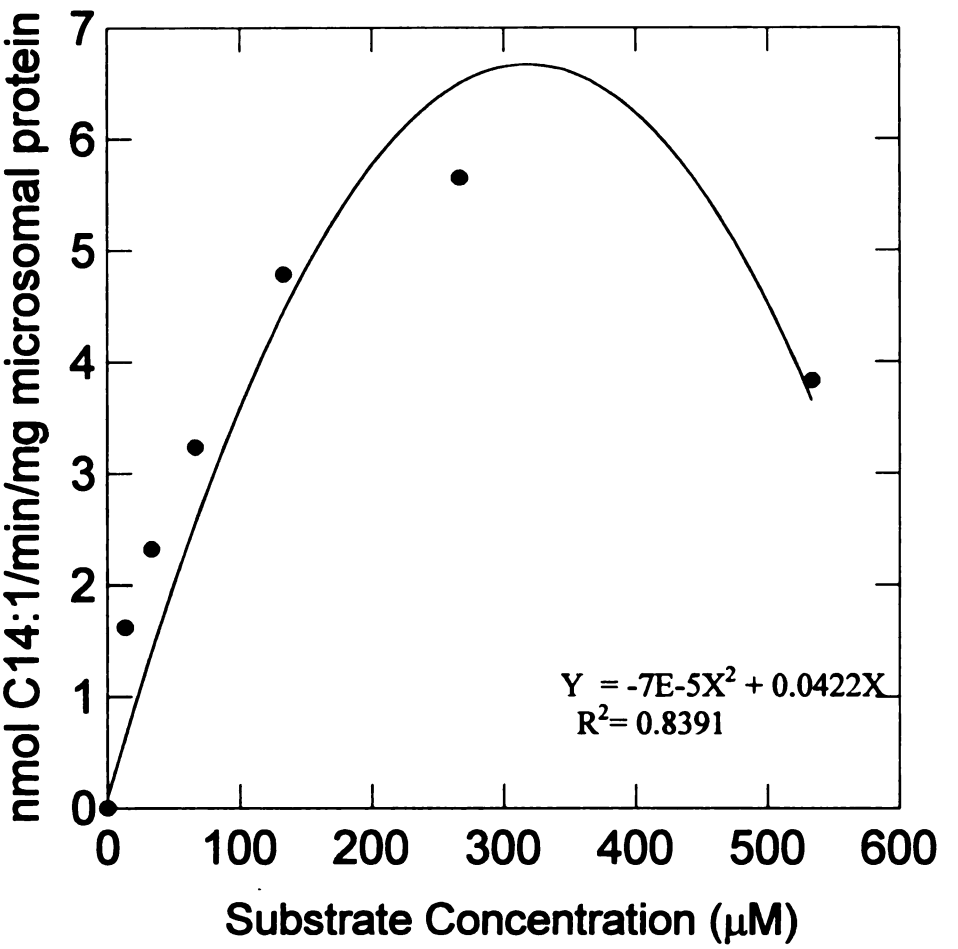


Figure 4- The relationship of initial velocity to substrate concentration for the hen microsomal Δ^9-desaturase catalyzed reaction. The desaturation activity was measured under conditions described in Figure 1 with different substrate concentrations. The complete system contained 8 mg hen liver microsomal protein, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 60mg BSA, 1.16 mM NADH, 1.31 mM $MgCl_2$, pH 7.4, in a total volume of 7.5 mL. Incubations were at 37°C for 5min.

The microsomal nonphosphorylating electron transport chain involved in the fatty desaturation includes a flavoprotein, the desaturase enzyme attributable to an hemo-protein and cytochrome b_5 (Oshino et al., 1971). The reducing equivalent required for the desaturation site is supplied by the NADH *via* cytochrome b_5. The rate of cytochrome b_5 reduction by NADH in rat liver microsomes is in the order of 1,000 nmoles per min per mg protein. In contrast, the activity to desaturate stearyl CoA is, at most, less than 10 nmoles of oleate formed per min per mg protein in even highly "induced microsomes" (Oshino et al., 1971). Thus, the capacity of the microsomal system to supply reducing equivalents to cytochrome b_5 is greater than that required for the desaturation of fatty acyl CoAs.

The effect of NADH concentrations on the desaturation of myristoyl-CoA is shown in Figure 5. A wide range of NADH:myristoyl-CoA mole ratios was assessed under the experimental conditions described in Figure 1. In the absence of NADH, no product formation was observed suggesting the absence of endogenous reducing equivalents. At NADH: myristoyl-CoA mole ratios ranging from 1 to 9, the desaturation was maximal. Decrease in desaturation was observed at NADH: myristoyl-CoA mole ratio of 12. A NADH: myristoyl-CoA mole ratio of 9 was employed for all the assays, unless otherwise indicated, to ensure that the electron transport system was not rate-limiting in the desaturation reaction.

2.4.1.5 Effect of pH

The velocity of enzyme-catalyzed reactions depends on pH. The importance of pH in the desaturation reaction is due to its ability to: (1) change the ionization state

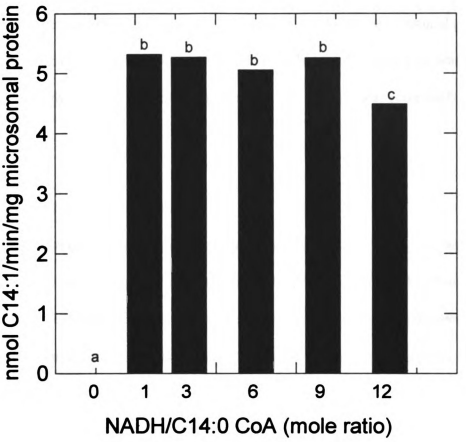


Figure 5- Effect of increasing concentrations of reduced pyridine nucleotide (NADH) on the desaturation of myristoyl-CoA by the Δ^9–desaturase system in hen liver microsomes. The assay was performed as described in Figure 1 with different NADH:myristoyl-CoA mole ratios. The complete system contained 8 mg hen liver microsomal protein, 133 μM C14:0 CoA, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 60mg BSA, 1.31 mM $MgCl_2$, pH 7.4, in a total volume of 7.5 mL. Incubations were at 37°C for 5min.

[a-c] Means with no common superscript are significantly different ($p<0.05$).

of the Δ^9-desaturase system, (2) change the ionization state of the substrate which in turn affects the reactivity of the reaction, and (3) change the solubility of the substrate solubilizing protein (Voet, 1990). The Δ^9-desaturase system gave a bell-shape curve of velocity versus pH (Figure 6) which is typical of most enzymes. Optimal pH for the formation of monoenes from myristoyl-CoA in hen liver microsomes was 7.4. At physiologically extreme pH values, the desaturation reaction was completely inhibited.

2.4.1.6 Effect of competitive inhibitors

Paulsrud et al. (1970) reported that desaturase activity in rat liver microsomes increases with increasing fatty acid carbon number from 10 through 18. Brett et al. (1971), who examined four other desaturation systems, *viz.*, microsomal preparations of goat mammary gland, hen liver, and whole cells of *Torulopsis bombicola* and *Chlorella vulgaris*, concluded that a widely distributed single enzyme whose activity is maximal with stearic acid and high with palmitic acid also, inserts the double bond between C_9 and C_{10}. Working with purified stearyl-CoA desaturase, Enoch et al. (1976) reported that 9,10-desaturation of fatty acyl-CoA derivatives of varying chain lengths (12 to 19 carbon atoms) by rat liver microsomes are catalyzed by this enzyme, with stearyl-CoA being the best substrate. This is consistent with the observation that oleate is the major monounsaturated fatty acid of rat liver lipids. Similar observations are presented in Figure 8 when several acyl-CoA derivatives were used as inhibitors. Stearoyl-, oleoyl-, and palmitoyl-CoA derivatives were effective competitive inhibitors for the Δ^9-desaturase of hen liver microsomes.

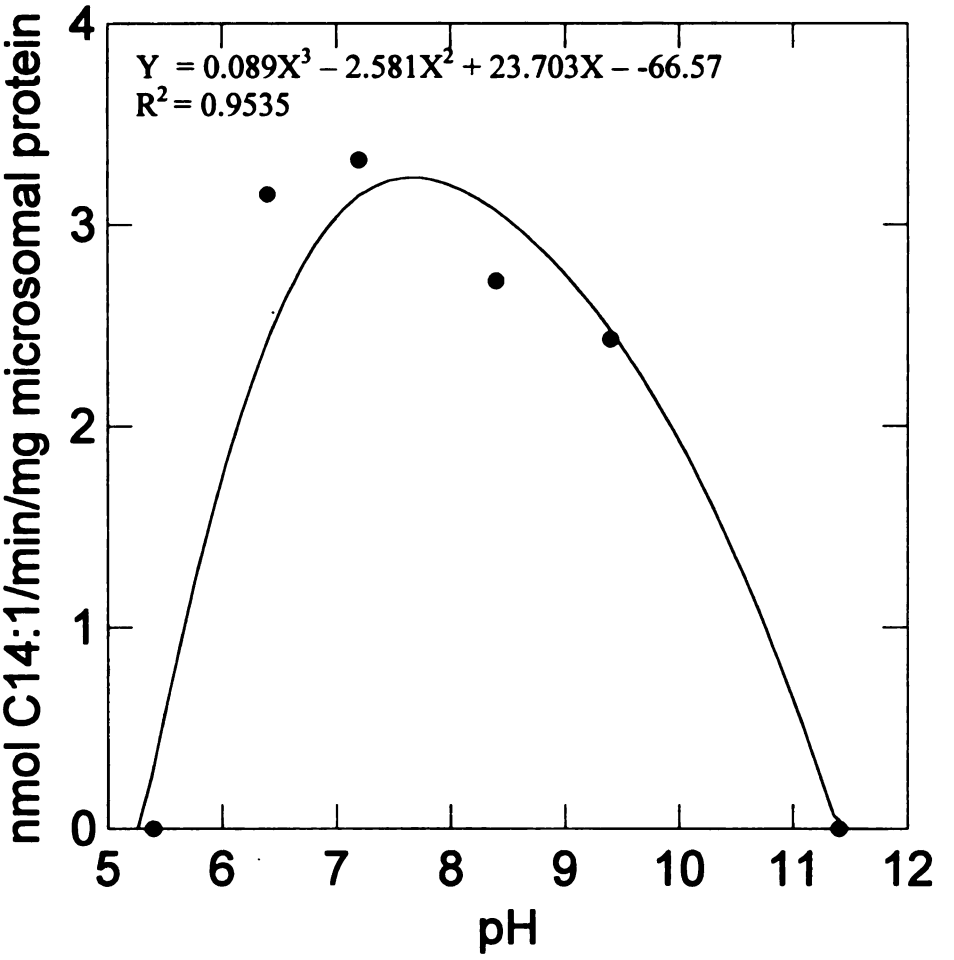


Figure 6- Effect of pH on the desaturation of myristoyl-CoA by the Δ^9–desaturase of hen liver microsomes. The assay was performed as described in Figure 1 with different pH values. The complete system contained 8 mg hen liver microsomal protein, 133 μM C14:0 CoA, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 60mg BSA, 1.16 mM NADH, 1.31 mM $MgCl_2$, in a total volume of 7.5 mL. Incubations were at 37°C for 5min.

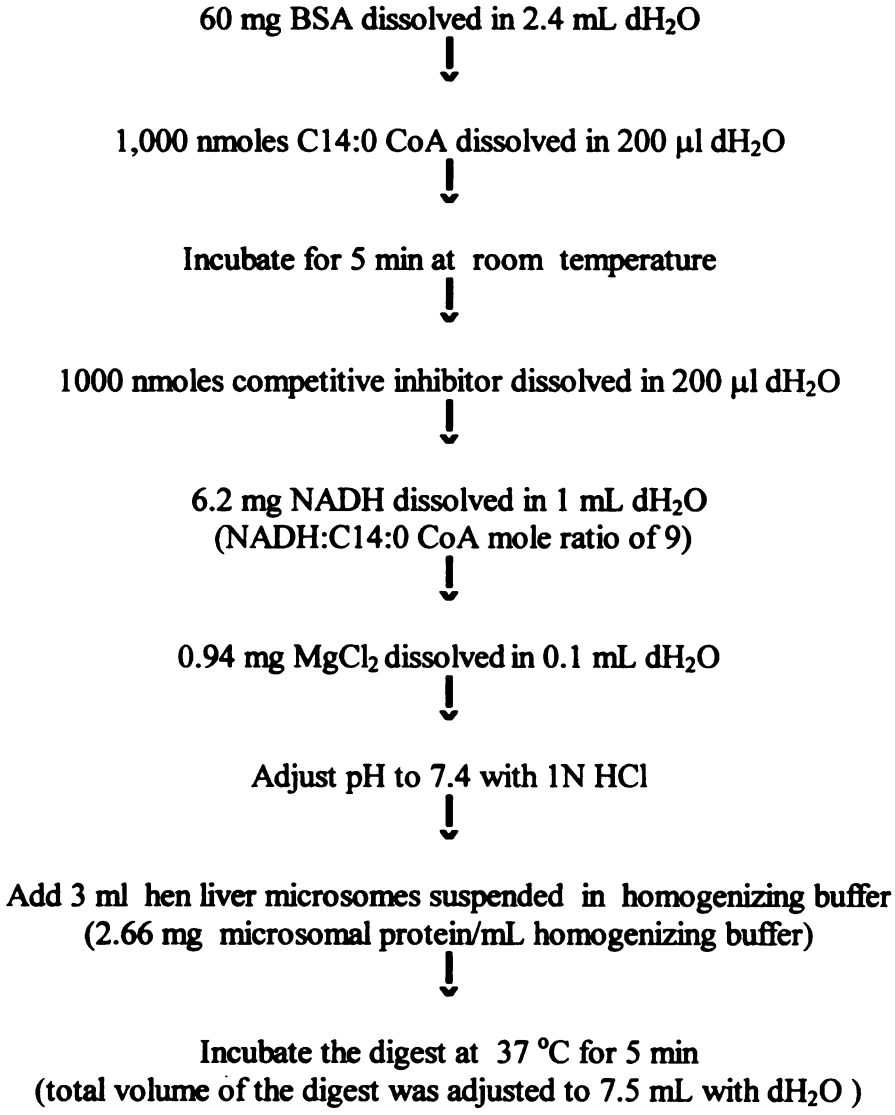


Figure 7- Effect of competitive inhibitors on the desaturation of myristoyl-CoA by the Δ^9-desaturase system of hen liver microsomes. The total volume of the digest was adjusted to 7.5 mL by the addition of 0.6 mL distilled water. The total number of acyl-CoA derivatives (substrate and competitive inhibitor) in the incubation medium did not exceed 2000 nmoles as over this concentration the substrate itself was inhibitory.

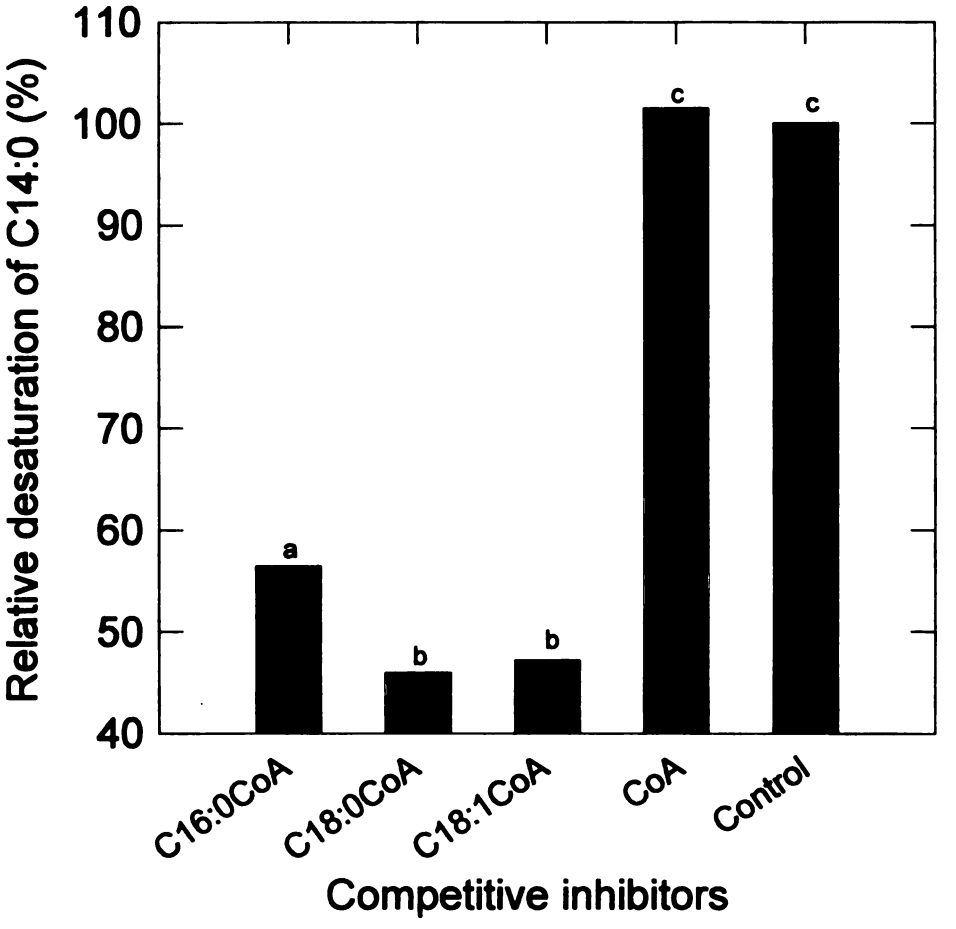


Figure 8- Effect of palmitoyl-, stearoyl-, and oleoyl-CoA, and free CoA on the activity of the Δ^9-desaturase of hen liver microsomes (normalized to the control = 100%) using myristoyl-CoA as a substrate. Incubations were carried out under conditions of initial velocity described in Figure 7. The concentration of the myristoyl-CoA in the incubation medium (133.3μM) was equal to the concentration of the competitive inhibitor. The total number of acyl-CoA derivatives (substrate and competitive inhibitor) in the incubation medium should not exceed 2000 nmoles (266.66 μM) since over this concentration the substrate itself was detrimental to the enzyme activity.

[a-c] Means with no common superscript are significantly different ($p<0.05$).

The desaturase enzyme was not inhibited by free CoA. Again, this is consistent with the results reported by Enoch et al., (1976). The inhibition is competitive with stearoyl-, oleoyl-, and palmitoyl-CoA . Stearoyl- and oleoyl-CoA were more effective in inhibiting the desaturase activity than palmitoyl-CoA. In the presence of stearoyl- and oleoyl-CoA, the desaturation rate decreased by 52.3 and 54.1%, respectively. In the presence of palmitoyl-CoA the decrease in the desaturation rate was 43.4% (Figure 8).

The fact that inhibition of enzymatic desaturation has been shown to be dependent on the chain length of the acyl-CoA derivatives indicates that the number of methylene groups of the fatty acid may regulate the desaturase activity via London-Van der Waal's forces that bind the fatty acid to the enzyme. As the hydrocarbon tail of the fatty acid is essentially hydrophobic, London-Van der Waals dispersion forces would be able to provoke binding with the enzyme. Because the dispersion forces vary as $1/_D{}^7$, D being the distance between the interacting molecules, the substrate and the lipoproteic enzyme must be close enough to make the binding effective. These forces increase with the number of carbon units of the molecule and in consequence may be quite large. Salem (1962) calculated the effect of each $-CH_2-$ group at an interaction distance of 5 A° to provoke a London-Van der Waals dispersion (Wdisp.) energy of: W disp./N= 0.4 Kcal/mole, where N is the number of identical units. This strengthening of the London-Van der Waals forces with the number of $-CH_2-$ may explain the high affinity of the enzyme toward stearoyl- and oleoyl-CoA.

2.4.1.7 Effect of bovine serum albumin and cytosolic proteins

BSA has been shown to bind fatty acyl-CoA derivatives and protect them from acylthiolester hydrolases (Jeffcoat et al., 1976). The effect of BSA on the desaturase system was investigated and results clearly show a stimulation of enzyme activity under the experimental conditions used (Figure 9). The specific enzyme activity, expressed as nmol C14:1 produced per min per mg protein, was found to be 3.49 and 1.93 in the absence and presence of the microsomal supernatant, respectively. When BSA was not added to the incubation medium, the specific activity dropped to 3.09 and 1.73 in the absence and presence of microsomal supernatant, respectively. Figure 9 also reveals that the microsomal supernatant has the capacity to reduce the specific activity of the desaturase system whether BSA was present or not.

The effect of heat and trypsin digestion on the capacity of the microsomal supernatant to decrease the desaturation activity was investigated. The microsomal supernatant obtained as described earlier is heat labile. The activity of the microsomal supernatant which contain the cytosolic proteins, was completely lost after 30 min of boiling using the assay conditions described in Figure 10. Boiling induced precipitation of the proteins. The increase in the desaturase specific activity due to the heat precipitation of cytosolic proteins was 1 nmol per min per mg microsomal protein compared to the control (Figure 11).

The effect of trypsin digestion on the microsomal supernatant is shown in Figure 11. Desaturase specific activity was increased by 1.04 nmol per min per mg microsomal protein compared to the control. The control experiment was performed

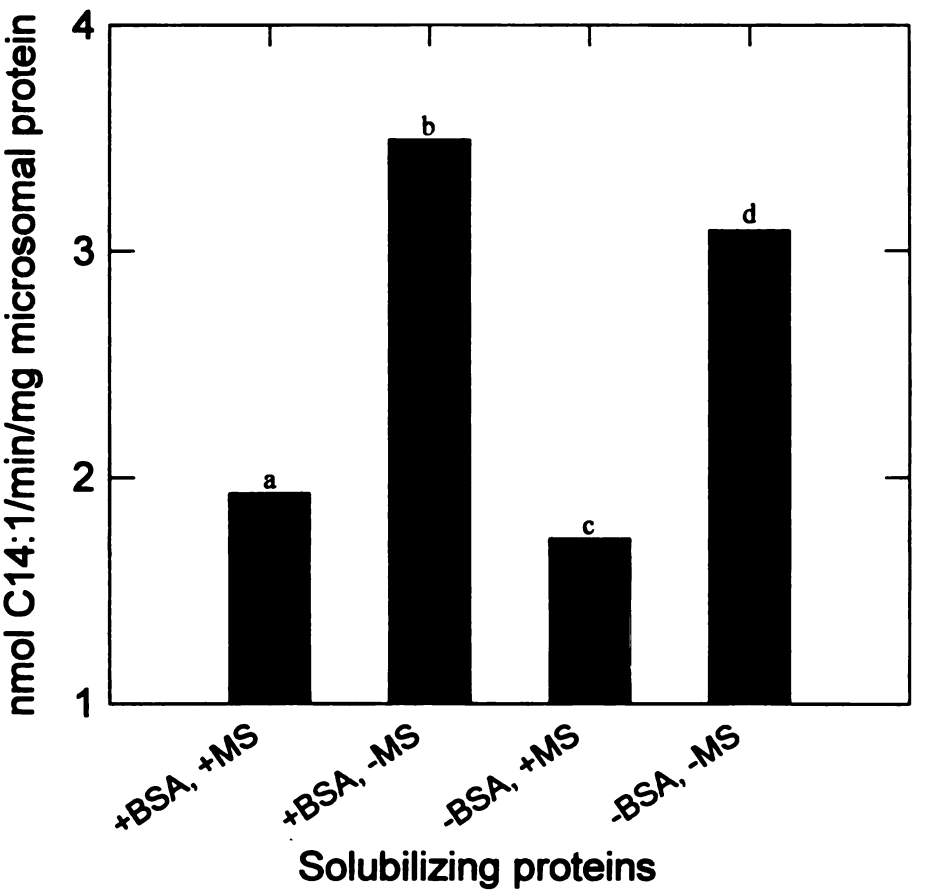


Figure 9- Effect of bovine serum albumin (BSA) and microsomal supernatant (MS) on the desaturation of myristoyl-CoA under the experimental conditions described in Figure 1. The complete system contained 8 mg hen liver microsomal protein suspended in the homogenizing buffer, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 133 μM C14:0 CoA, 60mg BSA, 1.16 mM NADH, 1.31 mM $MgCl_2$, pH 7.4, in a total volume of 7.5 mL. Incubations were at 37°C for 5min. When MS was added, the 8 mg microsomal protein were suspended in MS instead of the homogenizing buffer. + BSA indicates the presence of bovine serum albumin; - BSA indicates the absence of bovine serum albumin; +MS indicates the presence of microsomal supernatant; -MS indicates the absence of microsomal supernatant.
[a-d] Means with no common superscript are significantly different ($p<0.05$).

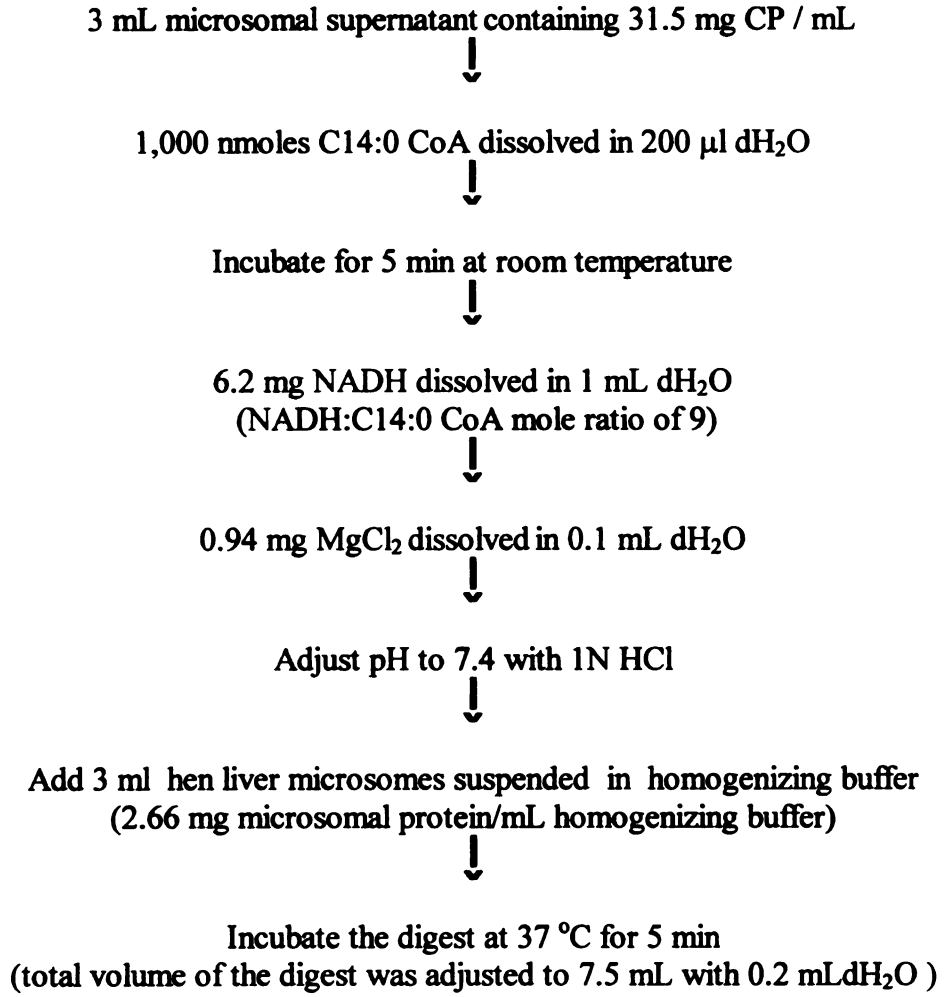


Figure 10- Design of study to establish the effects of heat treatment and trypsin digestion of the microsomal supernatant containing the cytosolic proteins (CP) on the desaturation reaction. The microsomal supernatant was trypsin digested in one set of experiments and boiled for 30 min in another set.

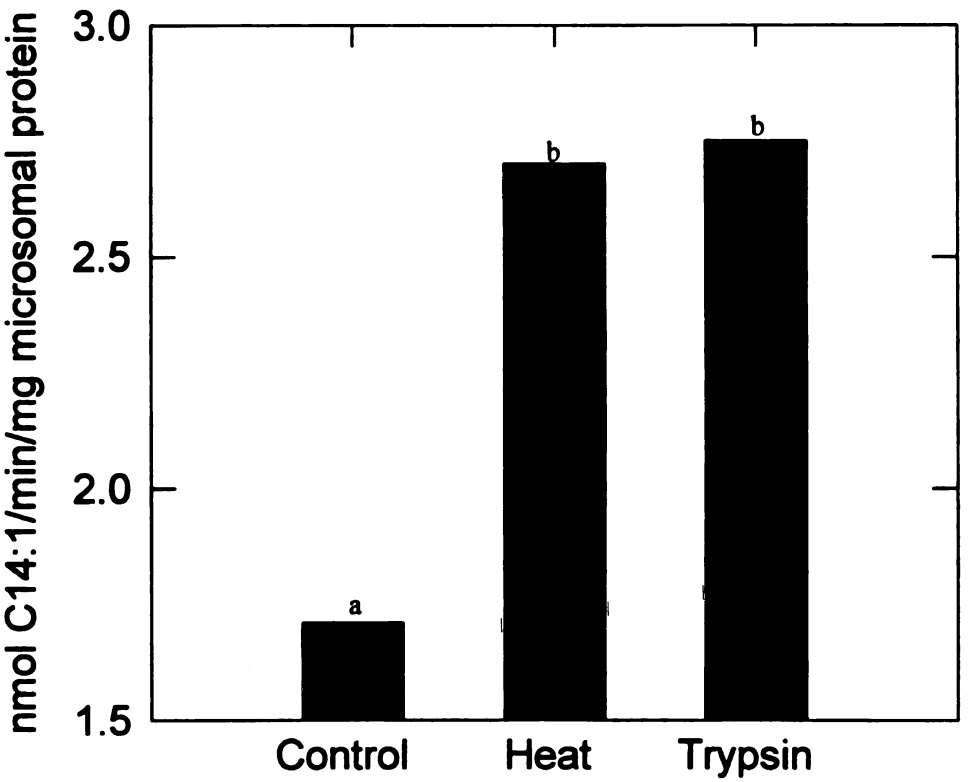


Figure 11- Effect of heat and trypsin digestion of the microsomal supernatant on the desaturation of myristoyl-CoA using the Δ^9–desaturase system of hen hepatic microsomes. The experimental conditions are described in Figure 10.
[a-b] Means with no common superscript are significantly different ($p<0.05$).

with the native microsomal supernatant. The marked decrease in desaturase activity in the presence of cytosolic proteins could be due to: (1) the incorporation of myristoyl-CoA into complex lipids by the acyltransferase, (2) the cleavage of myristoyl-CoA to free myristic acid by acylthiolester hydrolases, and (3) elongation of myristoyl-CoA to palmitoyl-CoA by the fatty acid synthetases. Cook and Spence (1973) reported that the rate of incorporation of stearoyl-CoA into phosphoglycerides used as a measure of acyltransferase activity greatly exceeded its rate of desaturation. A comparison of the desaturation rate in the presence and absence of BSA (Figure 9) indicates that the albumin in some way protects the acyl-CoA from the action of endogenous competing enzymes such as acylthiolester hydrolases and acyltransferase, but not from the desaturase. Cytosolic proteins and BSA showed no effect on the desaturation rate when partially purified stearoyl-CoA desaturase was used (Jeffcoat et al., 1976).

2.5 Conclusion

The current research effort is directed at an understanding of the regulation of myristoyl-CoA desaturation by the Δ^9-desaturase system of hen liver microsomes. Experimental evidence has been presented to show that the assay described here gives a true measure of the initial velocity of the reaction under pseudo first order reaction conditions. The inhibition of the desaturation of myristoyl-CoA by oleoyl-, stearoyl-, and palmitoyl-CoA suggests that all these acyl-CoA derivatives are probably catalyzed by the same enzyme, stearoyl-CoA desaturase.

The involvement of cytosolic proteins which reduce the desaturation of myristoyl-CoA has been established. Heat treatment and trypsin digestion were

effective means to abolish the capacity of cytosolic proteins to reduce the desaturation of myristoyl-CoA.

Although all the available evidence implicates the CoA ester of the fatty acid as the substrate for desaturation, direct desaturation of the fatty acid without prior activation to the CoA derivative is possible using the experimental conditions described in Chapter 3.

CHAPTER 3

DIRECT DESATURATION OF FREE MYRISTIC ACID BY HEN LIVER MICROSOMAL Δ^9-DESATURASE WITHOUT PRIOR ACTIVATION TO MYRISTOYL-CoA DERIVATIVE.

3.1 ABSTRACT

Direct desaturation of free myristic acid by hen liver microsomal Δ^9-desaturase without prior activation to myristoyl-CoA by the addition of adenosine triphosphate (ATP) and CoA was observed when the incubation medium was mixed at mixing speeds greater than 250 rpm in the presence of fatty acid-binding proteins (FABP). Desaturation was linear with time and proportional to the microsomal protein concentration. Desaturation was maximal at pH 7.9. The greatest desaturation rate was observed at a mixing speed of 500 rpm in the presence of FABP. Desaturation decreased at mixing speeds greater than 500 rpm. Data suggest that when free myristic acid is bound to FABP in the form of protein-monomer complexes, its activation to the CoA derivative is not necessary for it to be desaturated by the Δ^9-desaturase when using mixing rates greater than 250 rpm. Myristic acid:FABP complexes serve as substrates for the Δ^9-desaturase at mixing rates greater than 250 rpm. Desaturation was reduced by bovine serum albumin (BSA) and α-bromohexadecanoate, and no desaturation was observed in the absence of FABP. These findings suggest that FABP may regulate the accessibility of fatty acids in the desaturation reaction to the active site of the desaturase rather than just

protecting the membrane-bound desaturase from the cytotoxic effect of free fatty acids.

3.2 INTRODUCTION

Enzymatic desaturation of saturated fatty acids to form monounsaturated fatty acids is known to occur in various aerobic organisms including yeast and several animal species (Brett et al., 1971). Early experiments using liver homogenates established that the desaturation of fatty acids involves two enzymatic reactions, the catalyzed activation of the fatty acid to the acyl-CoA derivative by thiokinases in the presence of CoA, ATP, and Mg^{++}, followed by desaturation of the derivative by the acyl-CoA desaturase system (Masoro, 1968).

It has been reported by Holloway (1971) that mammalian Δ^9- fatty acyl-CoA desaturases are bound to the endoplasmic reticulum and have an obligatory requirement for reduced nicotinamide adenine dinucleotide (NADH), molecular oxygen, cytochrome b_5, and NADH cytochrome b_5 reductase (EC 1.6.2.2). These components have been isolated and characterized by Spartz and Strittmatter (1973) and Strittmatter et al. (1974). The purified cytochrome b_5 reductase (EC 1.6.2.2) and cytochrome b_5 consist of a hydrolytic catalytic segment exposed to the aqueous environment and a hydrophobic portion involved in binding to the microsomal membrane. Stearoyl CoA desaturase is completely submerged in the hydrocarbon region of the membrane with the catalytic domain exposed to the aqueous environment to permit reduction of its non-heme iron by cytochrome b_5 and interaction with the hydrophilic portion of substrate molecules (Enock et al., 1976).

Using purified stearoyl-CoA desaturase, the latter investigators reported that 9,10-desaturation of fatty acyl-CoA derivatives with chain lengths of 12 to 19 carbon atoms is catalyzed by the same enzyme, with stearoyl-CoA being the best substrate. These experiments, coupled with those of Raju and Reiser (1972) who demonstrated the lack of desaturation of lipid bound stearate, established fatty acyl-CoA as the true substrate for stearoyl CoA desaturase. The same has since been demonstrated to be true for the Δ^6 and Δ^5 fatty acyl CoA desaturases (Brennner, 1971).

The first evidence suggesting the possibility of a direct desaturation of oleoyl phosphatidycholine to linoleoyl phosphatidylcholine by a membrane –bound enzyme system was presented by Gurr et al. (1969). Pugh and Kates (1977) presented evidence for direct Δ^5 desaturation of eicosatrienoyl lecithin to arachidonoyl lecithin by rat liver microsomes.

We have undertaken studies to show whether free myristic acid could be directly desaturated without first being activated to the CoA derivative. This question has previously been considered by other investigators (Masoro, 1968; Raju and Reiser, 1972; Cook and Spence, 1973; Pugh and Kates, 1973; Brenner, 1974; Catala et al., 1975; Cook, 1978; Voet, 1990), but no evidence for the direct desaturation of free fatty acids without being first activated to the CoA derivative was presented. The data to be presented here will clearly show direct desaturation of free myristic acid in the absence of CoA and ATP, when the incubation medium is mixed at speeds greater than 250 rpm in the presence of fatty acid-binding protein (FABP). As described in the previous chapter, we developed an enzyme assay to quantitate the direct desaturation of free myristic acid by Δ^9-desaturase system in hen liver microsomes.

This procedure is now being used to study several properties of the enzyme system including specific activities, substrate specificity, and interaction of substrate with BSA and FABP.

3.3 MATERIALS AND METHODS

3.3.1 Materials

Tetradecanoic, *cis*-9-tetradecenoic, pentadecanoic acids, bovine serum albumin (BSA), trypsin, trypsin inhibitor, sodium lactate, NADH, and oxidized nicotinamide adenine dinucleotide (NAD) were obtained from Sigma Chemical Co. (St Louis, MO). The standards were of greater than 98 % chemical purity according to the manufacturers. Magnesium chloride and potassium hydroxide were obtained from Aldrich Chemical Co. (Milwaukee, WI). A Lightnin Lab Master (SI) mixer (model L1U03 and an impeller type A100 (dia. 1")) were obtained from Matoon and Lee Equipment, (Farmington, MI). Laying hens (White Leghorn) were obtained from the MSU Poultry Farm.

3.3.2 Preparation of hen liver microsomes

For each set of experiments, three laying white Leghorn hens were sacrificed. The whole livers were excised and cooled immediately over ice. The livers were minced and mixed together to decrease possible variations in desaturation yield from one single liver preparation to another. A 20g sample of the ground livers was immediately homogenized in a Waring blender for 30 sec with 60 ml of chilled 0.1 M potassium phosphate buffer (pH 7.4) containing 0.3 M sucrose. The temperature of

the buffer solution was 0 to 2 °C. The resulting homogenate was centrifuged at 15,000 x g for 30 min at 2 °C. The sedimented cellular debris and mitochondria were discarded. Microsomes containing the Δ^9-desaturase system were either used directly in the enzyme assay as mitochondrial supernatant or sedimented from the postmitochondrial supernatant fraction by centrifuging at 105,000 x g for 1 hr at 2 °C. The resulting microsomal pellet was either suspended in the homogenizing buffer or in the microsomal supernatant (containing the cytoplasmic proteins) to provide a final concentration of 2.66 mg of microsomal protein/ml. The protein concentration was determined by the method of Lowry et al. (1951). The remaining ground livers were stored at -20 °C but for no longer than 10 hours. Independent studies indicated that there was no loss in enzymatic activity over the duration of the holding period.

3.3.3 Enzyme assays

The preliminary assay to convert free myristic acid to myristoleic acid using the Δ^9-desaturase system of hen liver microsomes is outlined in Figure 1. The rationale for each subsequent step in the process is described below.

3.3.4 Lipid extraction and fatty acid methyl esterification

The enzymatic reaction was stopped by adding 20 ml of hexane: isopropanol (3:2, v/v). Samples were extracted three times with a mixture of hexane: isopropanol (3:2, v/v) and the combined solvent extracts evaporated under nitrogen. The internal fatty acid standard C15:0 (0.02 mg) was added to each of the samples which were then derivatized to their fatty acid methyl esters by the boron trifluoride:methanol method of Morrison and Smith (1964).

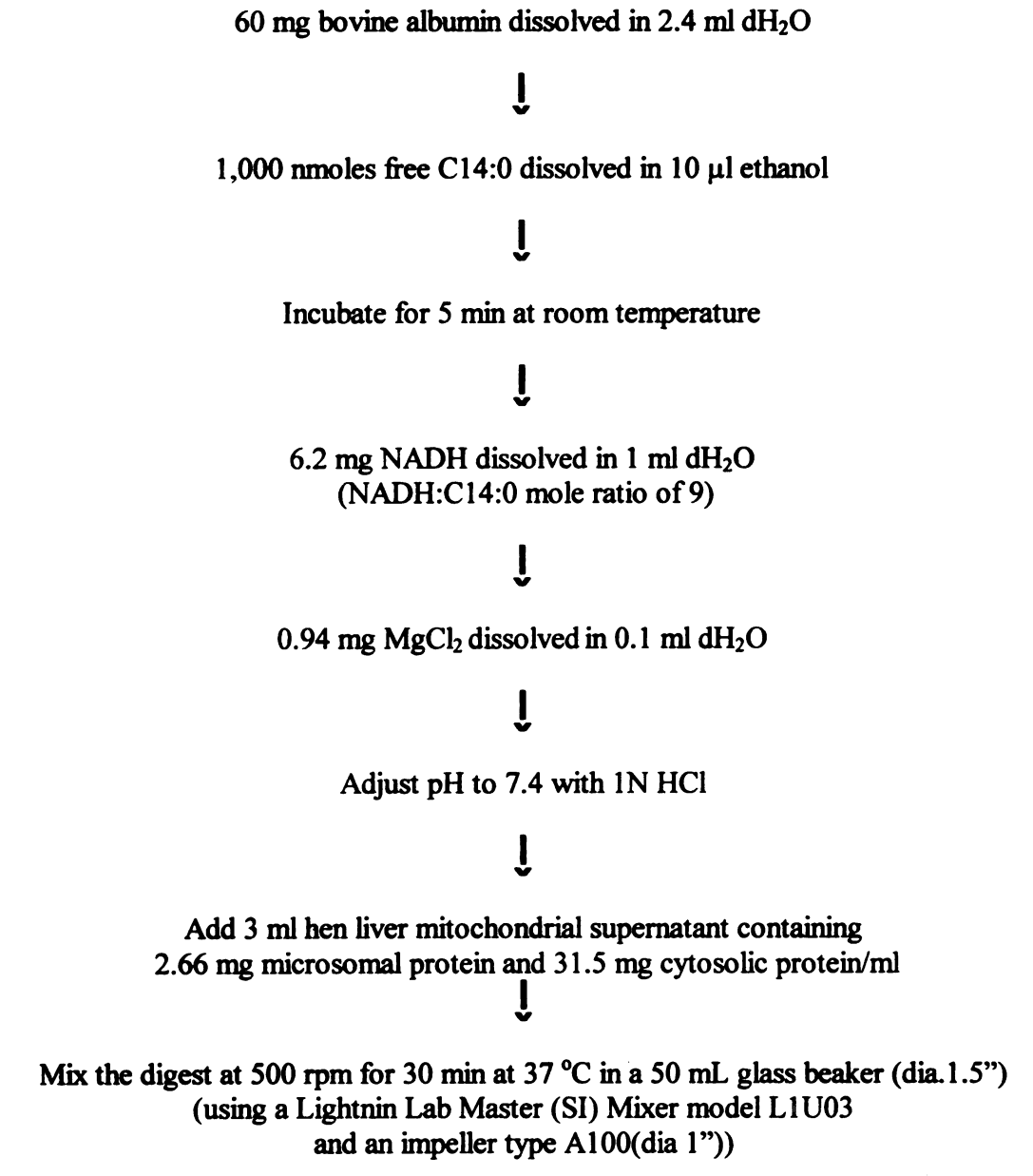


Figure 1- Preliminary assay for the direct desaturation of free myristic acid to myristoleic acid, in the absence of CoA and ATP, by the Δ^9-desaturase system of hen liver microsomes. The total volume of the digest was adjusted to 7.5 mL by the addition of 0.99 mL distilled water.

3.3.5 Gas chromatographic (GC) and GC-MS analyses

Gas chromatographic analyses were carried out on a Hewlett-Packard 5890 A gas chromatograph (Avondale, PA) equipped with a flame ionization detector (FID), split-mode(1/12) and a fused silica capillary column (30 m x 0.25 mm) coated with 0.25 μm film of DB-225 (J & W Scientific, Folsom, CA). Operating conditions of the gas chromatograph were as follows: temperatures - injector 275° C, detector 300° C, column 165° C; hold 5 min; increase oven temperature 0.5° C/min to 180° C, hold 20 min, increase 10° C to 215° C, hold 10 min. Fatty acid methyl esters were identified by comparing their relative gas chromatographic retention times with those of known standards. Standard curves were generated from known concentrations of pure methyl ester standards of C14:0, *cis*-9-C14:1 ranging from 7.8 to 62.5 pg and containing 0.01 pg of C15:0. The peak area corresponding to C14:0, *cis*-9-C14:1, and C:15:0 were determined using a digital integrator. The C14:0 and *cis*-9-C14:1 peak areas were divided by the internal standard area to obtain the response ratios for both standards and samples. The standard response ratios for C14:0 and *cis*-9-C14:1 were plotted against their corresponding concentrations. Standard response ratio plots should bracket the sample response ratio. The C14:0 and *cis*-9-C14:1 concentrations in the sample extracts were quantified from the standard response ratio plots and corrected for the dilution factor (df = 2). Initial analysis indicated that there was no endogenous *cis*-9-C14:1 in the medium. Thus, all the *cis*-9-C14:1 observed was derived as a result of the desaturation reaction *per se*. The identity of *cis*-9-C14:1 was confirmed by comparing the mass spectra of authentic *cis*-9-C14:1 standard and that derived from the desaturation of C14:0 (Appendix 1). The GC-MS analyses were carried out on a

JEOL A × 505H double focusing mass spectrometer equipped with a Hewlett-Packerd 5890 J GC. The fatty acid methyl esters, dissolved in hexane, were injected using a splitless injection technique onto a 30m x 0.25 mm i.d., 25μm coated DB 225 capillary column interfaced directly into the ion source. The GC oven temperature was programmed from 100°C to 165 °C at 5 °C per min and held for 5 min then to 172 °C at 0.5 °C per min and finally to 230 °C at 10°C per min. The injector and transfer line temperatures were maintained at 230 °C and 230 °C, respectively. Fatty acids were analyzed in the m/z range of 45 – 600 for the methyl ester derivatives in positive electron ionization mode (EI).

3.3.6 Trypsin digestion

To each 6 mL of microsomal supernatant containing 31.5mg/mL of cytosolic proteins, 340 mg of trypsin were added, and the mixture was incubated for 1 hr at 37°C. Proteolysis was stopped with the addition of 680 mg trypsin inhibitor.

3.3.7 Statistical analysis

All treatments were performed in duplicate (n=2). The statistical analyses were based on the linear and polynomial regressions and analysis of variance (ANOVA) procedures of SYSTAT 7.0 (SPSS Inc., 1997). ANOVA was also used to analyze the data and included NADH, competitive inhibitors, heat treatment, trypsin digestion, BSA and microsomal supernatant (MS) as main effects and BSA x MS as interaction. The Tukey multiple comparison procedure was used for mean separation

(Berk, 1998). If levels within a factor were significantly different, they were examined further using the student t-test for differences among individual means. Results were judged to be statistically significant based on type I error rate of 5%.

3.4 RESULTS AND DISCUSSION

Mammalian desaturases have been known for many years to be bound to the endoplasmic reticulum and require oxygen and NADH (Oshino et al., 1966; 1971). In mammalian tissues, the formation of monoenes from free fatty acids is a two-stage reaction: activation of the saturated fatty acid to fatty acyl-CoA by acyl-CoA synthetase, followed by desaturation of the acyl-CoA derivative (Masoro, 1968). In the activation reaction, the energy is generally supplied by adenosine triphosphate (ATP) conversion to adenosine monophosphate (AMP) and pyrophosphate (Pande and Mead, 1968). In the present study with Δ^9-desaturase in hen liver microsomes, the results indicate that direct desaturation of free myristic acid without prior activation to myristoyl-CoA by the addition of ATP and CoA is possible when the incubation medium is mixed at mixing speeds greater than 250 rpm in the presence of fatty acid - binding proteins.

3.4.1 Factors that affect the desaturation free myristic acid

In Chapter 2, it was demonstrated that hen liver microsomes catalyzed the oxygen-and reduced pyridine nucleotide-dependent desaturation of myristoyl-CoA to myristoleoyl-CoA. In the current study, we used essentially the same enzyme assay as for myristoyl-CoA with two exceptions: (1) free myristic acid was used as the

substrate instead of myristoyl-CoA and (2) the reaction mixture was mixed at 500 rpm to provoke direct collision between substrate and the Δ^9-desaturase. In preliminary experiments (Figure 1), the reliability of the assay conditions was tested by measuring the effect of incubation time, microsomal protein concentration, pH, temperature, reducing agent, substrate specificity, inhibition, effect of BSA, and microsomal supernatant on the enzyme activity.

3.4.1.1 Effect of incubation time

Under the assay conditions, the amount of myristoleate formed was directly proportional to the incubation times. Incubations were stopped after 30 min, over which time the response of the enzyme was linear (Figure 2). Linearity of response did appear to be consistent for 60 min. Longer incubation times were used with free myristic acid than with myristoyl-CoA in order to have sufficient monoene to be accurately determined. Desaturation of myristoyl-CoA was also linear with time but slowed after 30 min (Chapter 2). The decrease of monoenes formation with time when myristoyl-CoA was used as a substrate, may be due to the decrease in substrate concentration as a result of its utilization in other enzymatic reactions (Chapter 2). In contrast, the direct desaturation of free myristic acid was not affected by competing enzymic reactions.

3.4.1.2 Effect of microsomal protein concentration

The rate of direct desaturation of free myristic acid was linear with microsomal protein concentrations over the range of 2 to 8 mg per 7.5 ml of final incubation

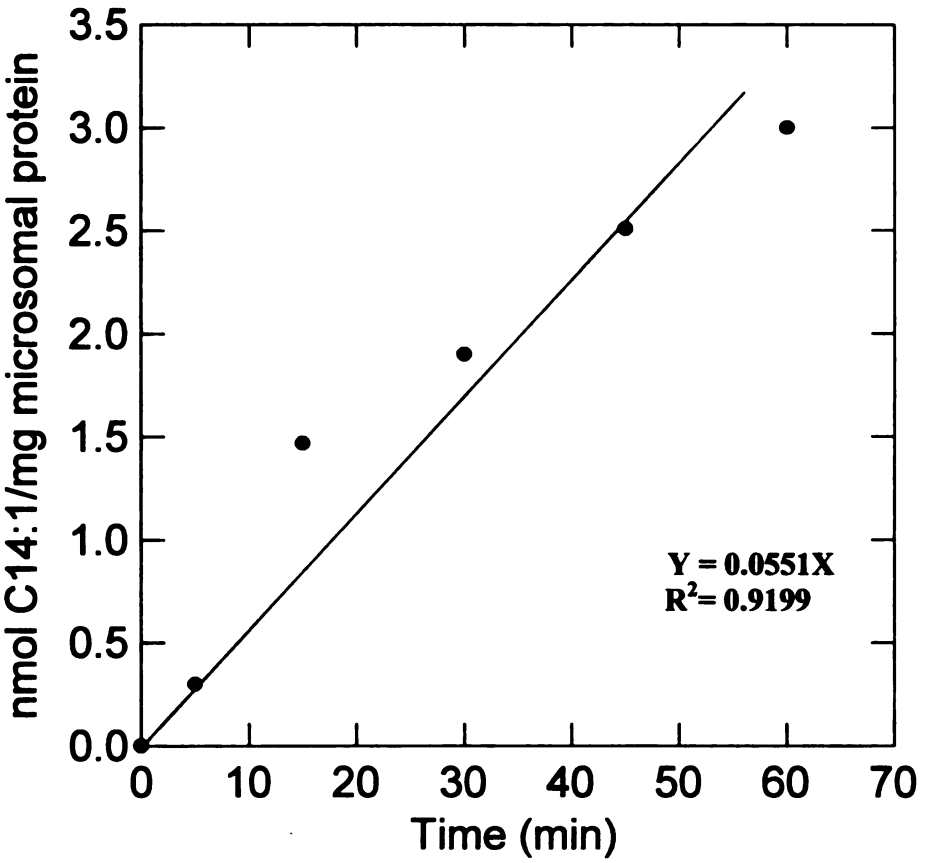


Figure 2- Direct desaturation of free myristic acid as a function of time by the Δ^9-desaturase system of hen liver microsomes. The desaturation activity was measured at 37°C under conditions described in Figure 1 with different incubation times. The complete system contained 8 mg hen liver microsomal protein, 94.5 mg cytosolic protein, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 133 μM free C14:0, 60mg BSA, 1.16 mM NADH, 1.31 mM $MgCl_2$, pH 7.4, in a total volume of 7.5 mL. The digest was mixed at 500 rpm.

volume (Figure 3). Inhibition occurred at protein concentrations somewhat greater than 8 mg. There was a marked decrease at the concentration of 10 mg per 7.5 ml of final incubation volume. A relatively high microsomal protein concentration of 8 mg per 7.5 ml incubation volume was purposely employed in all the assays, unless otherwise indicated, to assure there is enough production of myristoleate during the incubation time of the assay. At constant levels of substrate, monoene formation was inhibited at high protein concentrations. This finding was also observed when myristoyl-CoA was used as a substrate. The decrease in the desaturation rate with high protein concentrations could be due in part to the addition or accumulation of desaturation products.

3.4.1.3 Effect of pH

The pH of the reaction system influences the velocity of all enzyme-catalyzed reactions. The importance of pH in the desaturation reaction is due to its ability to: (1) change the ionization state of the Δ^9-desaturase system, (2) change the ionization state of the substrate which in turn affects the reactivity of the reaction, (3) and change the solubility of the substrate solubilizing protein. The Δ^9-desaturase was active within a pH range 6.5 to 8.5 (Figure 4). The optimal pH for the formation of monoenes from the direct desaturation of free myristic acid in hen liver microsomes was 7.9.

3.4.1.4 Effect of temperature

Temperature is a key factor in the desaturation reaction due to its ability to:

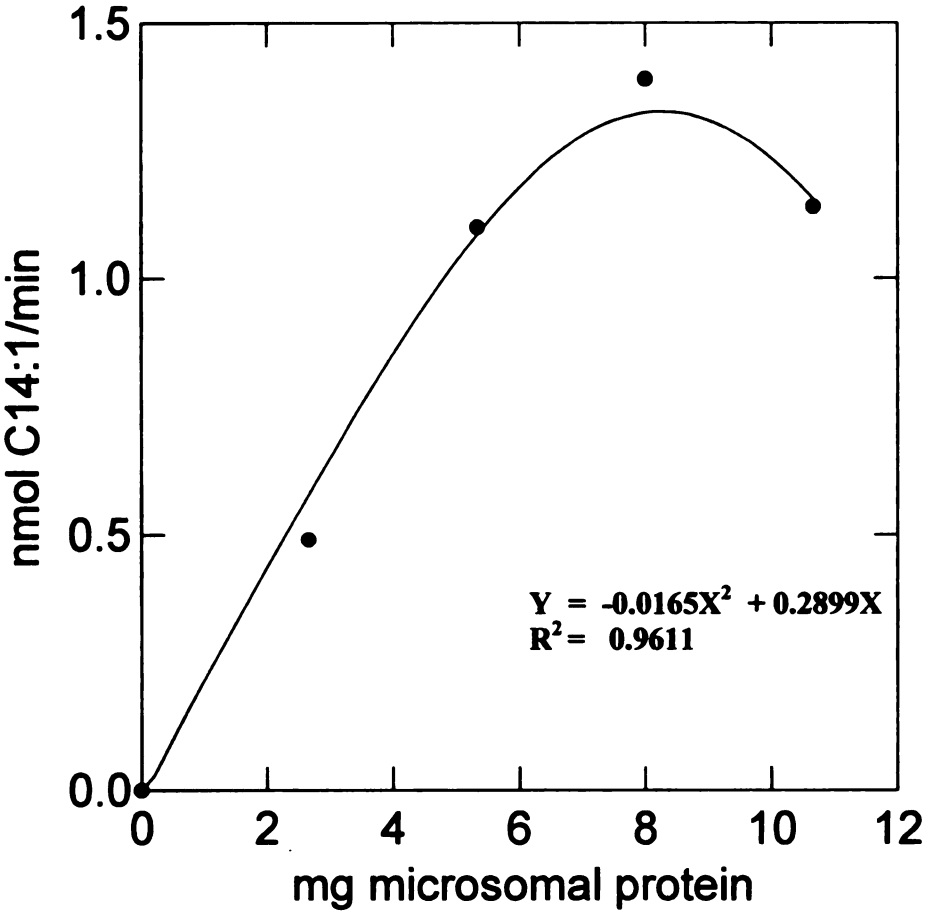


Figure 3- Direct desaturation of free myristic acid as a function of microsomal protein concentration by the Δ^9-desaturase system of hen liver microsomes. The desaturation activity was measured at 37°C under conditions described in Figure 1 with different microsomal protein concentrations. The complete system contained 94.5 mg cytosolic protein, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 133 μM free C14:0, 60mg BSA, 1.16 mM NADH, 1.31 mM mg $MgCl_2$, pH 7.4, in a total volume of 7.5 mL. The digest was mixed at 500 rpm for 30 min.

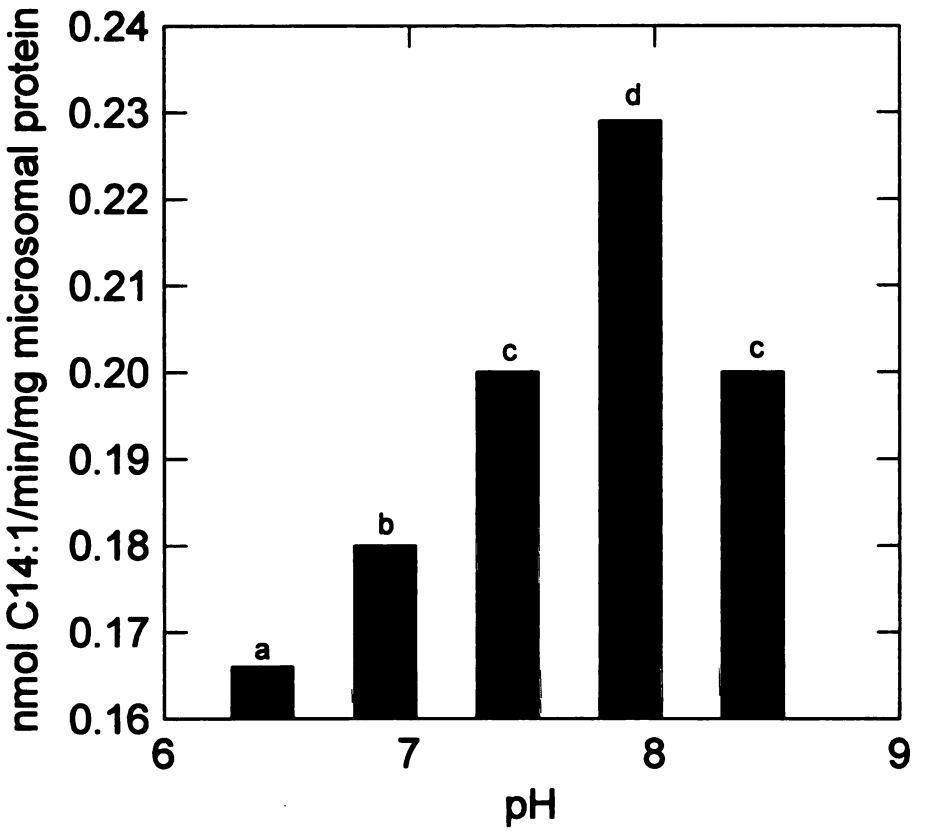


Figure 4- Effect of pH on the direct desaturation of free myristic acid by the Δ^9–desaturase system of hen liver microsomes. The desaturation activity was measured at 37°C under conditions described in Figure 1 at different pH values. The complete system contained 8 mg microsomal protein, 94.5 mg cytosolic protein, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 133 μM free C14:0, 60mg BSA, 1.16 mM NADH, 1.31 mM $MgCl_2$, in a total volume of 7.5 mL. The digest was mixed at 500 rpm for 30 min.

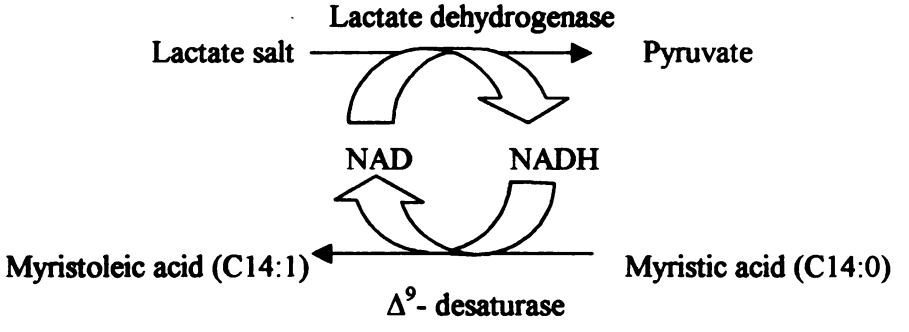
[a-d] Means with no common superscript are significantly different ($p<0.05$).

(1) change the rate of the reaction, (2) denature the desaturase system and the cytoplasmic proteins, and (3) change the fluidity and the crystalline-liquid crystalline phase transition of the membrane (Sackman and Träuble,1971).

The optimal temperature for the direct desaturation of free myristic acid by hen liver microsomes was 37 °C (Figure 5). At lower and higher temperatures, rates of the desaturation reaction were affected. No C14:1 formation was observed at 25 °C for the duration of the experiment (30min). Mixing time has no effect on the temperature as the temperature remained unchanged during the whole incubation period.

3.4.1.5 Effect of reducing agent

The generation of NADH *in situ* by adding NAD^+ and sodium lactate and taking advantage of the presence of lactate dehydrogenase (LDH) was investigated using the following coupled enzyme assay.



LDH is present in the mitochondrial supernatant of hen liver (Voet, 1990)). The effect

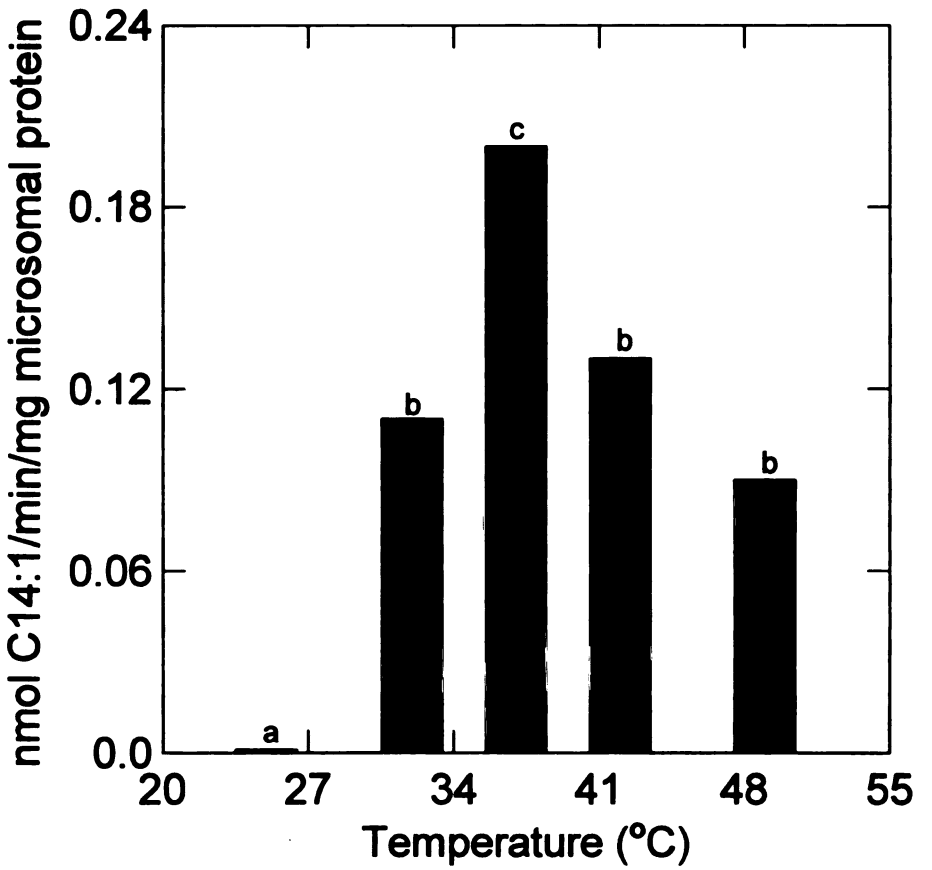


Figure5- Effect of temperature on the direct desaturation of free myristic by the Δ^9-desaturase system of hen liver microsomes. The desaturation activity was measured under conditions described in Figure 1 at different temperatures. The complete system contained 8 mg microsomal protein, 94.5 mg cytosolic protein, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 133 μM free C14:0, 60mg BSA, 1.16 mM NADH, 1.31 mM mg $MgCl_2$, pH 7.4, in a total volume of 7.5 mL. The digest was mixed at 500 rpm for 30 min.

[a-c] Means with no common superscript are significantly different ($p<0.05$).

of NADH and generated *in situ* NADH on the direct desaturation of myristic acid is shown in Figure 6. The difference between added NADH and that generated *in situ* one was not statistically significant. In subsequent experiments, NADH was generated *in situ*.

3.4.1.6 *Effect of bovine serum albumin (BSA)*

Free fatty acids (FFAs) are potent non-specific detergents that inhibit most enzymatic reactions (Bass, 1985). The latter reported that BSA protects many of the enzymes that are affected by FFAs, either partially or completely, by complexing the FFAs and abolishing their detergent properties. The capacity of BSA for tight binding of long chain fatty acids is well known. Studies of the structural interactions between FFA and BSA using ^{13}C NMR spectroscopy revealed multiple binding sites, hydrophobic interaction with the hydrocarbon chain, and electrostatic interaction with the carboxylate anion (Parks et al., 1983; Hamilton et al., 1984; Cistola et al., 1987 a). Brecher et al. (1984) reported that methyl esterification of the carboxyl group of long chain fatty acids decreased the binding of fatty acids to BSA by 40 to 50 % at pH 7.4. Results of this study show that the direct desaturation of myristic acid is decreased in the presence of BSA (Figure 8) when evaluated as described in Figure 7. Specific activity, expressed as nmol *cis*-9-C14:1 produced per min per mg protein, decreased by 12.9% in the presence of BSA. In the absence of the microsomal supernatant, direct desaturation of myristic acid did not occur whether BSA was present or not (Figure 8). These observations might be attributed to the tight binding BSA-C14:0 complexes (multiple binding sites) that make myristic acid sterically inaccessible to

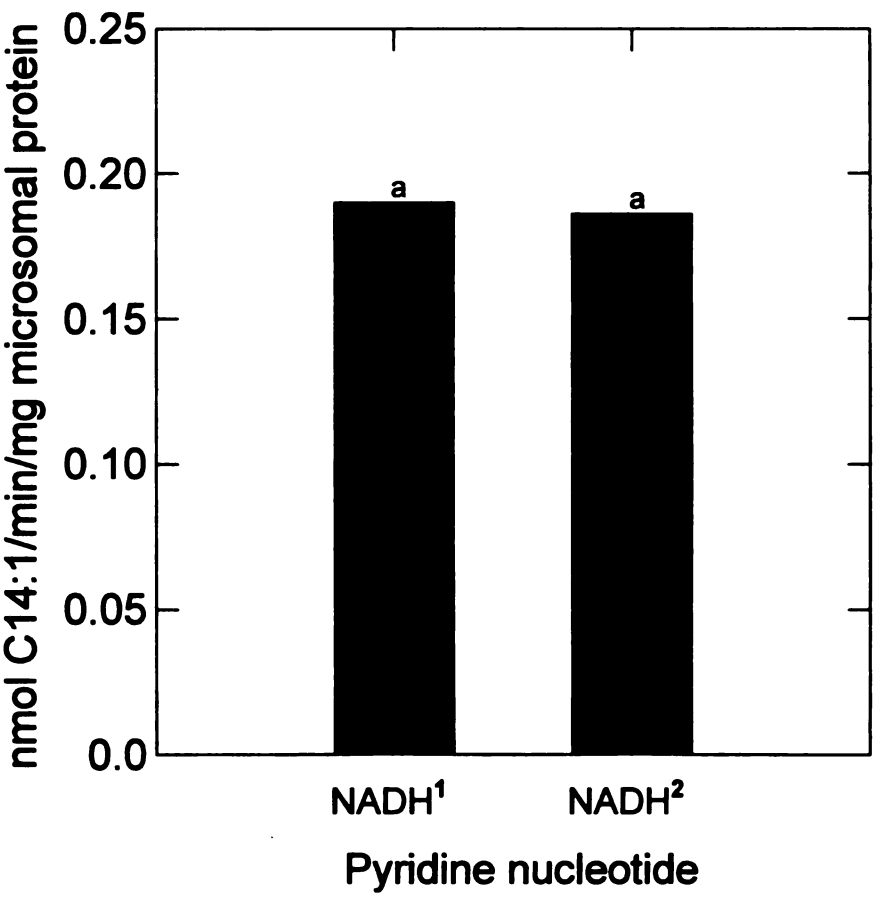


Figure 6- Effect of pyridine nucleotide on the direct desaturation of free myristic acid by the Δ^9-desaturase system of hen liver microsomes. The desaturation activity was measured at 37°C under conditions described in Figure 1.The complete system contained 8 mg microsomal protein, 94.5 mg cytosolic protein, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 133 μM free C14:0, 60mg BSA, 1.24 mM NAD, 3.09 mM sodium lactate, 1.31 mM $MgCl_2$, pH 7.4, in a total volume of 7.5 mL. The digest was mixed at 500 rpm for 30 min. $NADH^1$ was generated *in situ* and $NADH^2$ was added directly. When NADH was added directly, NAD and sodium lactate were not added to the incubation medium.
[a]Means with no common superscript are significantly different ($p<0.05$).

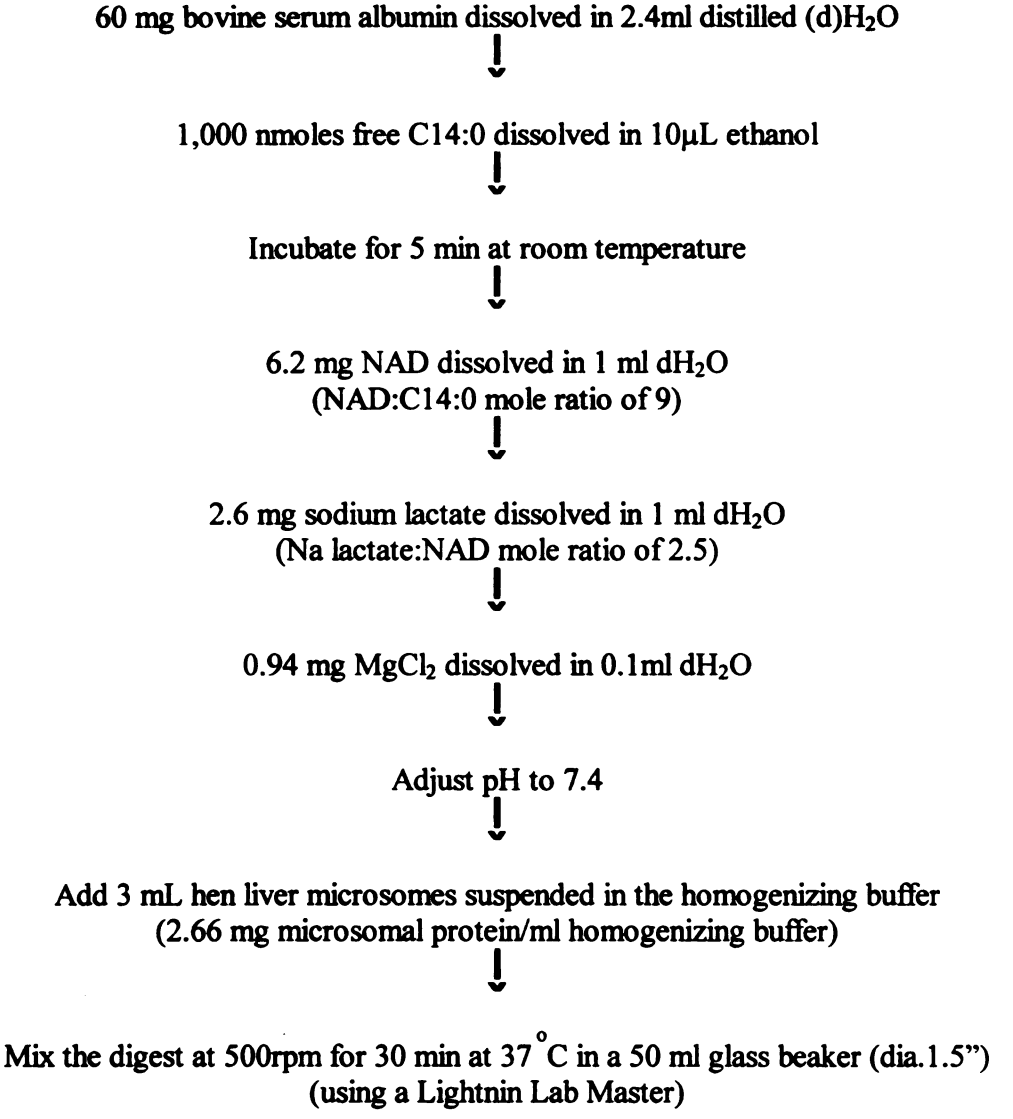


Figure 7- Assay for the effect of bovine serum albumin and microsomal supernatant (MS) on the direct desaturation of free myristic acid by the Δ^9-desaturase system of hen liver microsomes. When absent, the bovine serum albumin solution was replaced by 2.4 ml dH_2O. When the microsomal supernatant (MS) was used in the assay, the microsomes were suspended in the microsomal supernatant (2.66 mg microsomal protein / mL MS) instead of the homogenizing buffer. The MS is the source of cytosolic proteins and it contains 31.5 mg cytosolic protein (CP) / mL MS.
The final volume of the incubation medium was fixed at 7.5 ml to keep the mixing parameters constant.

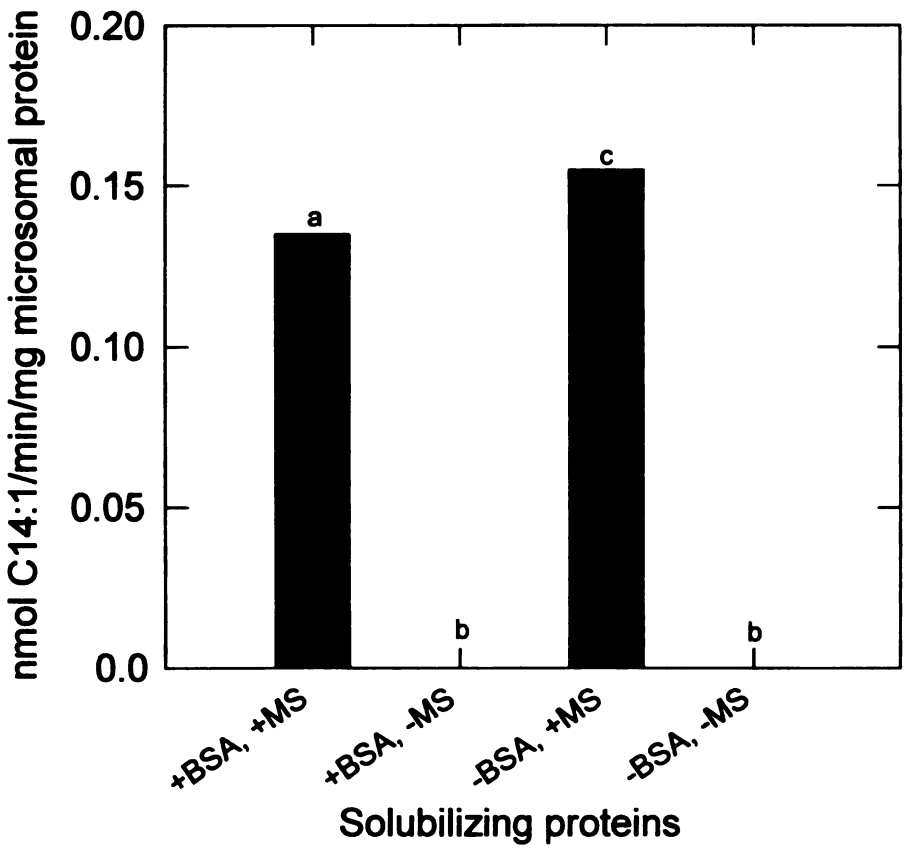


Figure 8- Effect of bovine serum albumin and microsomal supernatant on the direct desaturation of myristic acid by hen liver microsomal Δ^9-desaturase. All incubations were carried out under the experimental conditions described in Figure 7. In the absence of bovine serum albumin 2.4 ml distilled water were added to keep the final volume of the assay constant (7.5 ml). The microsomal supernatant is the source of cytosolic proteins and it contains 31.5 mg cytosolic protein/ml. +BSA indicates the presence of bovine serum albumin, -BSA indicates the absence of bovine albumin. +MS indicated the presence of microsomal supernatant, -MS indicates the absence of microsomal supernatant.

[a-c] Means with no common superscript are significantly different ($p<0.05$).

the active site of the Δ^9-desaturase (Figure 9). By contrast, when myristoyl-CoA was the substrate, BSA increased the Δ^9-desaturase specific activity by protecting the substrate from the competing enzyme present in the microsomal supernatant, but not from the Δ^9-desaturase.

Direct desaturation of myristic acid differs from that of myristoyl-CoA in that it requires not only a mixing speed greater than 250 rpm but also the microsomal fraction containing FABP. Collins and Hargis (1989) reported the presence of FABP in the cytosolic fraction of chicken liver. Moreover, Scapin et al. (1990) determined the crystal structure of chicken liver basic FABP by X-ray crystallography.

3.4.1.7 Effect of microsomal supernatant

The omission of the microsomal supernatant containing FABP resulted in loss of desaturation activity regardless of whether BSA was present or not (Figure 8). The microsomal supernatant was examined for protein that may be involved in the regulation of the direct desaturation of myristic acid using heat inactivation and trypsin digestion. The effect of heat inactivation and trypsin digestion on the capacity of microsomal supernatant to increase the desaturation activity revealed the involvement of such proteins. The microsomal supernatant is unstable to heat and undergoes major denaturation when boiled for 30min. The activity of the microsomal supernatant proteins was lost after 30 min of boiling when evaluated as described in Figure 10. At the same time, boiling produced precipitation of the cytosolic proteins. The desaturase specific activity was completely lost as a result of heat precipitation of the cytosolic proteins (Figure 11).

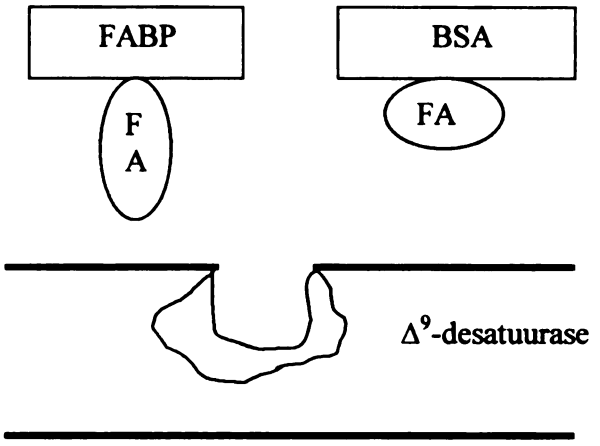


Figure 9- Qualitative scheme for the movement of C14:0-protein complexes in the incubation medium.

* FA: Fatty Acid (in this example the fatty acid is myristic acid).
* BSA: bovine serum albumin
* FABP: fatty acid binding protein

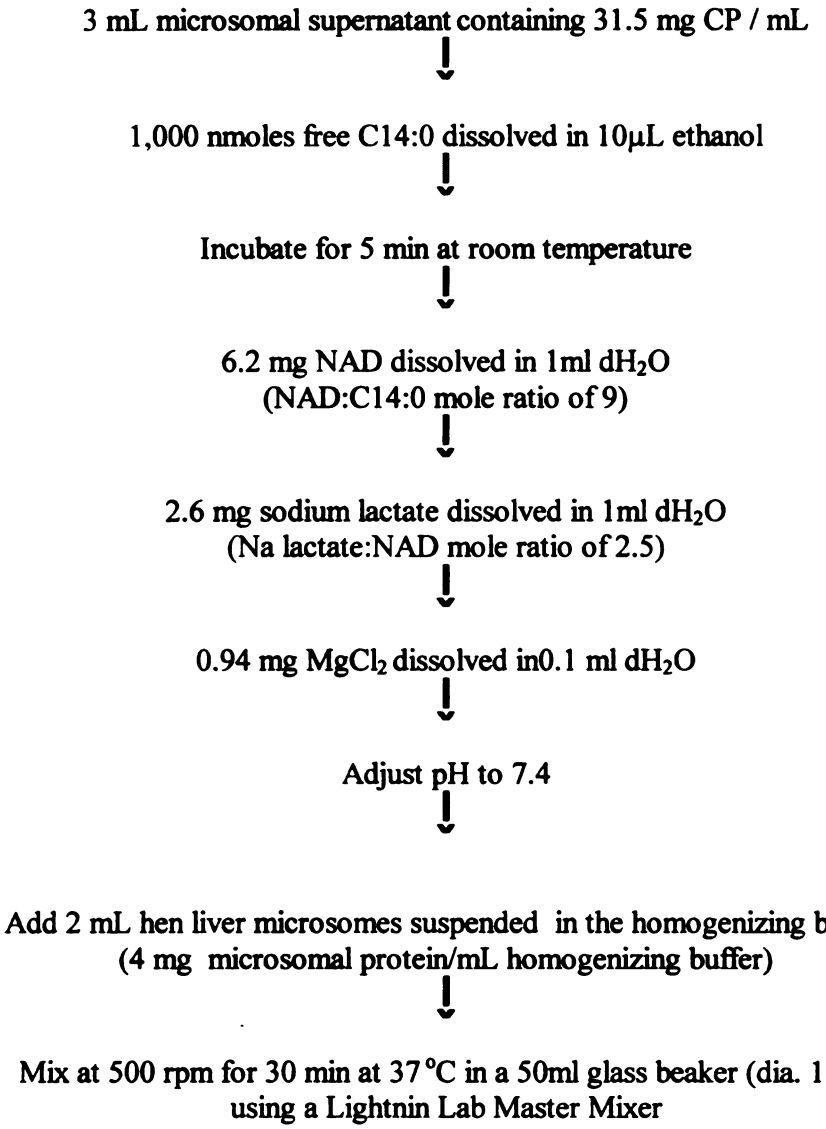


Figure 10- Heat treatment and trypsin digestion of the microsomal supernatant used in the direct desaturation of free C14:0 by the Δ^9–desaturase system of hen hepatic microsomes. The microsomal supernatant was trypsin-digested in one set of experiments and boiled for 30 min in another set. The final volume of the digest was adjusted to 7.5 ml with 0.39 ml dH_2O. Bovine serum albumin was omitted from the incubation medium, since its effect was detrimental to the desaturase activity.

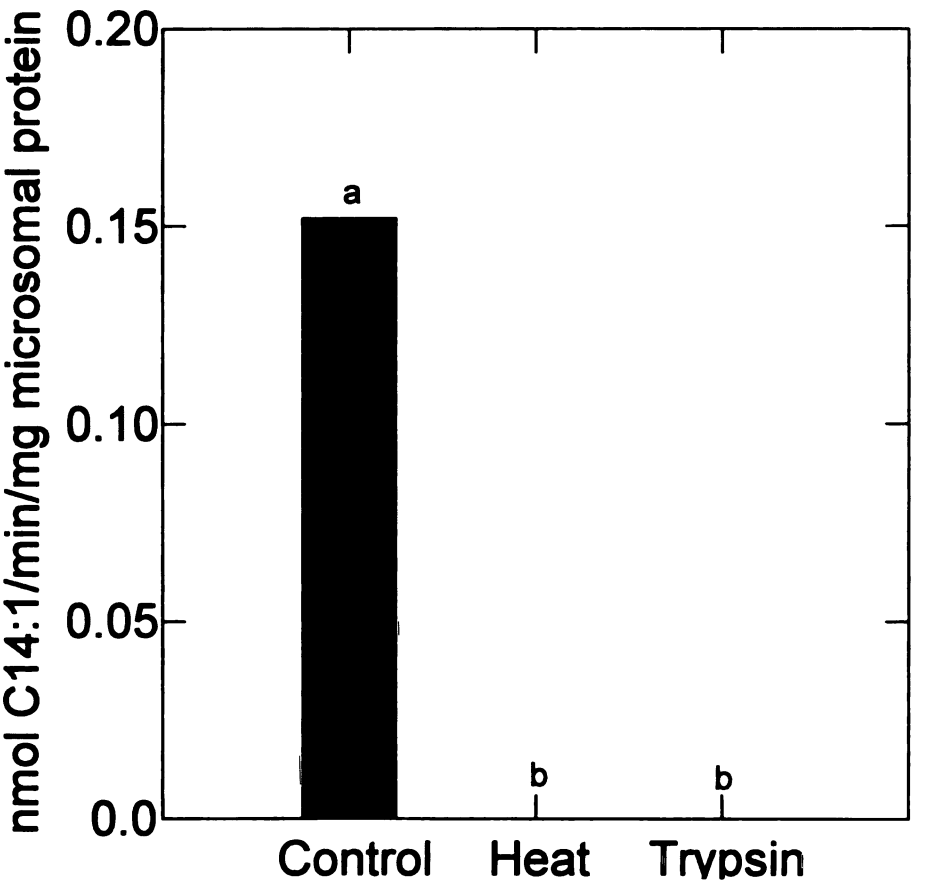


Figure 11- Effect of heat treatment and trypsin digestion of the microsomal supernatant on the direct desaturation of free myristic acid by the Δ^9–desaturase system of hen hepatic microsomes. All incubations were carried out using the experimental conditions described in Figure 10.
[a-b] Means with no common superscript are significantly different ($p<0.05$).

The effect of trypsin digestion on the microsomal supernatant proteins is shown in Figure 11. It was found that proteolysis of the microsomal supernatant containing FABP and other cytosolic proteins resulted in a complete loss of the desaturase specific activity compared to the control (Figure 11). The control experiment was performed with untreated microsomal supernatant. In contrast to free myristic acid, heat treatment and trypsin digestion of the microsomal supernatant increased the specific activity of the Δ^9-desaturase when myritoyl-CoA was the substrate. These results suggest the presence of a protein factor in the microsomal supernatant that activates the direct desaturation of myristic acid under the experimental conditions described in Figure 10. The protein factor is rather specific as it cannot be replaced by BSA. The question to be considered now is whether this protein factor binds the free myristic acid. If so, then the substrate for the direct desaturation by the hen microsomal Δ^9-desaturase would be in the form of a myristic acid:binding protein complex.

3.4.1.8 Effect of α-bromohexadecanoate

It has been established that α-bromohexadecanoate competes with free fatty acids for the same binding site of fatty acid-binding proteins (Ockner and Manning, 1976). The effect of α-bromohexadecanoate on the direct desaturation of myristic acid by hen hepatic microsomal Δ^9-desaturase was examined using different mole ratios of α-bromohexadecanoate: myristic acid (Figures 12 and 13). The linear decrease in specific activity observed with free myristic acid as a substrate in the presence of varying concentrations of α-bromohexadecanoate probably reflects the

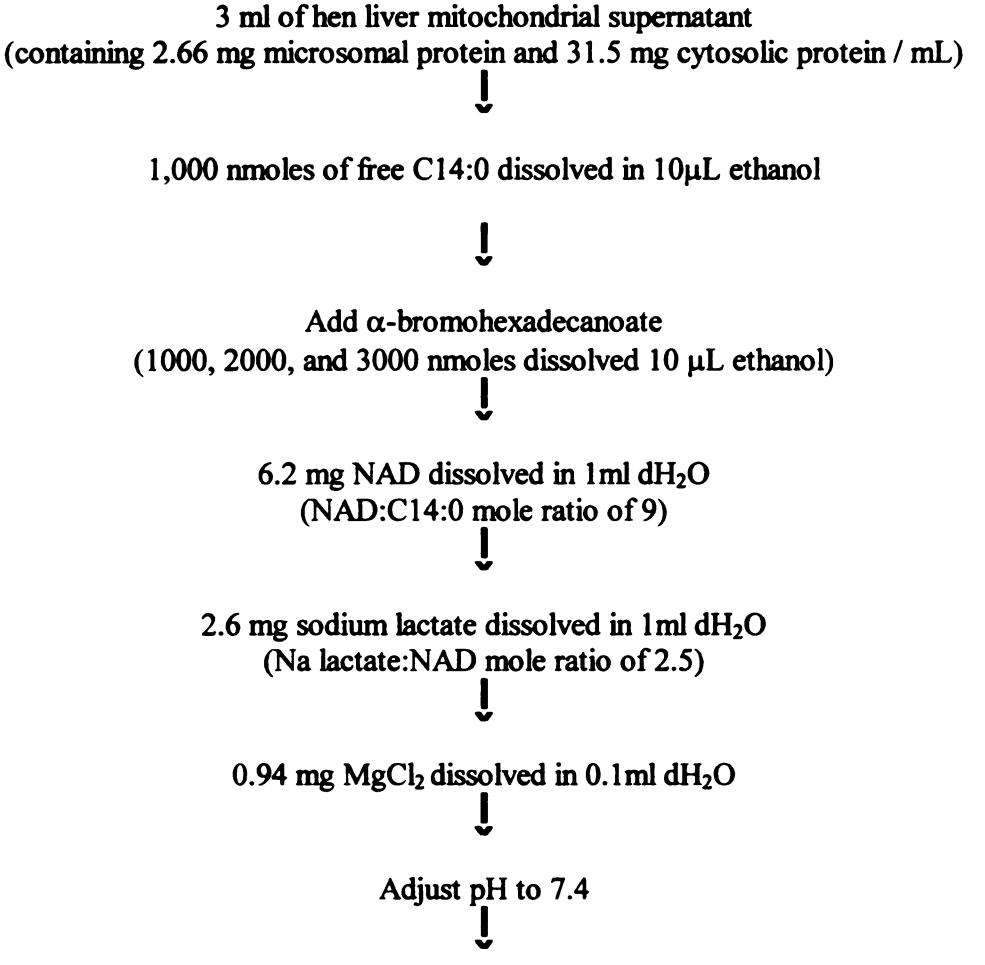


Figure 12- Assay for the effect of α-bromohexadecanoate on the direct desaturation of free myristic acid by the Δ^9-desaturase system of hen liver microsome. The final volume of the incubation was adjusted to 7.5 ml with 2.38 ml dH_2O. Bovine serum albumin was not added due to its detrimental effect on the desaturation yield.

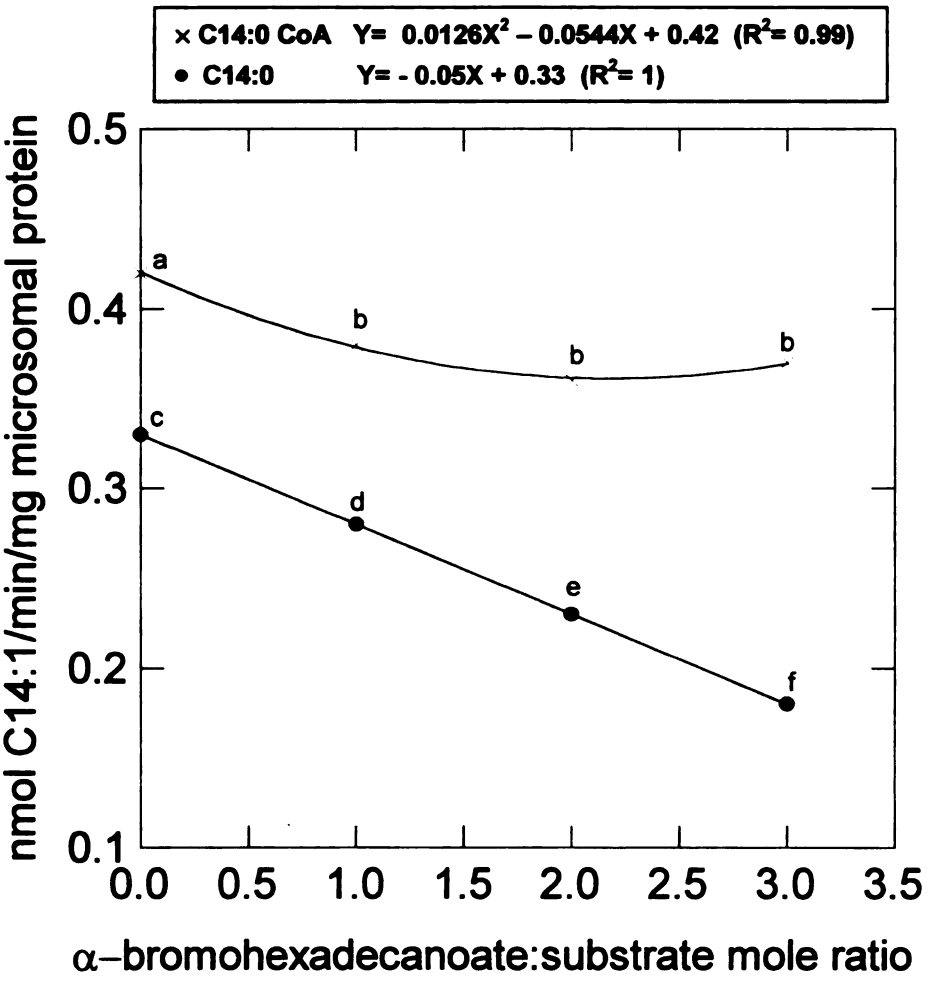


Figure 13- Effect of α-bromohexadecanoate:substrate mole ratios on the direct desaturation of myristic acid by the Δ^9–desaturase system of hen hepatic microsomes. The desaturation activity was measured under conditions described in Figure 12. When myristoyl-CoA was the substrate, 1000 nmol myristoyl-CoA were dissolved in 200µl distilled water.

[a-d] Means with no common superscript are significantly different ($p<0.05$).

reduction of myristic acid binding to FABP rather than the inhibition of the desaturation reaction *per se*. This study revealed that α-bromohexadecanoate concentrations did not affect the desaturation of myritoyl-CoA under the experimental conditions reported in Figure 12.

These results suggest that the specific protein factor in the microsomal supernatant is a FABP because it binds free fatty acid and the binding was completely inhibited by α-bromohexadecanoate. These results also suggest that the substrate for the direct desaturation by the hen microsomal Δ^9-desaturase is in the form of a myristic acid:FABP complex. The decrease in the desaturase specific activity was directly related with the decrease in concentration of the myristic acid:FABP complex. Previous studies reported that hepatic fatty acid binding protein (hFABP) may specifically direct the utilization of fatty acids toward either esterification (Wu-Rideout et 1976; Iritani et al., 1980) or oxidative paths(Appelkvist et al., 1980). Bass (1985) suggested that hFABP effects the transfer of fatty acids to the active site of the enzyme. Wu-Rideout et al. (1976), Ockner and Manning (1976) found that albumin was an ineffective replacer of hFABP in stimulating the activity of some microsomal enzymes utilizing fatty acids. This suggested the role of FABP in providing fatty acids to their enzymes may be more than a substrate solubilizer. Information in Figures 8 and 9 reveals that FABP cannot be replaced by BSA due to the tight binding of BSA-C14:0 complex (multiple binding site) (Parks et al., 1983; Hamilton et al., 1984; Cistola et al., 1987 a) making the C14:0 inaccessible the active site of the Δ^9-desaturase. The FABP-fatty acid complex has one binding site (Glatz et al., 1983), thus allowing easier access of C14:0 to the enzyme active site (Figure 9).

Glatz et al. (1983), using a radiochemical procedure, reported that FABP possesses only one binding site per protein molecule for long-chain fatty acids. Cistola et al.(1987a) using ^{13}C NMR spectroscopy reported that oleic acid binds hFABP through the carboxyl group. Moreover, these investigators suggested that the anionic carboxyl group of oleic acid bound to the hFABP experiences only one type of binding environment (the aqueous milieu adjacent to the protein surface) free of specific ion-pair electrostatic attractions with cationic residues on hFABP. Brecher et al. (1984) found that the incubation of hFABP with labelled oleate reached an apparent equilibrium at a fatty acid to protein mole ratio of about 1. Moreover, they reported that the methyl esterification of the carboxyl group of long chain fatty acids inhibit binding of the fatty acids to FABP. These findings lead to the conclusion that the FABP may regulate the accessibility of fatty acids in the desaturation reaction to the active site of the desaturase rather than just protecting the membrane-bound desaturase from the cytotoxic effect of free fatty acids.

3.4.1.9 Effect of mixing rate

The effect of mixing rate on the desaturation of the myristic acid:FABP complex by hen hepatic microsomal Δ^9-desaturase was examined using mixing speeds from 0 to 1800 rpm. Under the conditions outlined in the assay procedure (Figure 14), no desaturation was detected in the absence of mixing despite the presence of the microsomal supernatant containing FABP. This is in agreement with previously reported research (Masoro, 1968; Raju and Reiser, 1972; Cook and

Spence, 1973; Pugh and Kates, 1 973; Brenner, 1974; Catala et al., 1975; Cook, 1978; Voet, 1990) where no evidence was found for the direct desaturation of free fatty acids without being activated to the CoA derivatives. Production of myristoleic acid was observed at mixing speeds greater than 250rpm. The optimal mixing rate for maximum product formation was 500 rpm (Figure 15). The decline in myristoleic acid production at mixing speeds greater than 500 rpm could be due to the rupturing of the microsomal membrane.

To investigate the presence of endogenous myristoyl-CoA, an assay was performed as described in Figure 14, but with no substrate added. Any formation of product under these conditions would be due to an endogenous source of myristoyl-CoA. Myristoleic acid formation was not observed under these conditions (Figure 16). The possibility that free myristic acid was activated to its CoA derivative prior to the desaturation reaction in the presence of a thiokinase, CoA, and ATP in the microsomal supernatant is unlikely in view of the following observations: (1) desaturation was not observed at mixing rates lower than 250 rpm despite the presence of the microsomal supernatant, knowing that the activation reaction does not require mixing of the incubation medium; and (2) in the absence of BSA, the microsomal supernatant itself was detrimental to the desaturase activity when myristoyl-CoA was the substrate (2.4.1.8).

Different mixing rates were used to facilitate the transfer of the myristic acid:FABP complex (substrate) to the active site of the desaturase by direct collision to form productive enzyme-substrate complexes. Kim and Storch (1992) showed that

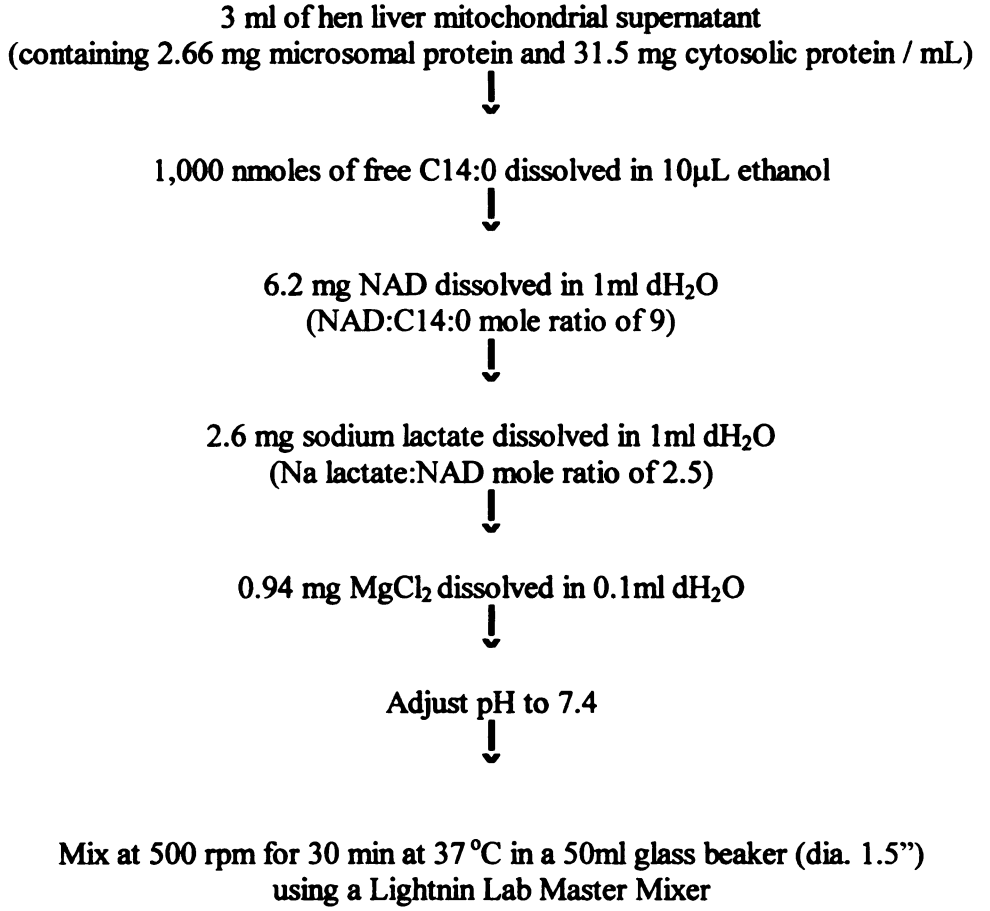


Figure 14- Final assay for the direct desaturation of myristic acid:FABP complexes by the Δ^9-desaturase system of hen liver microsomes in the absence of CoA and ATP. The final volume of the assay was adjusted to 7.5 ml with 2.39 ml distilled water.

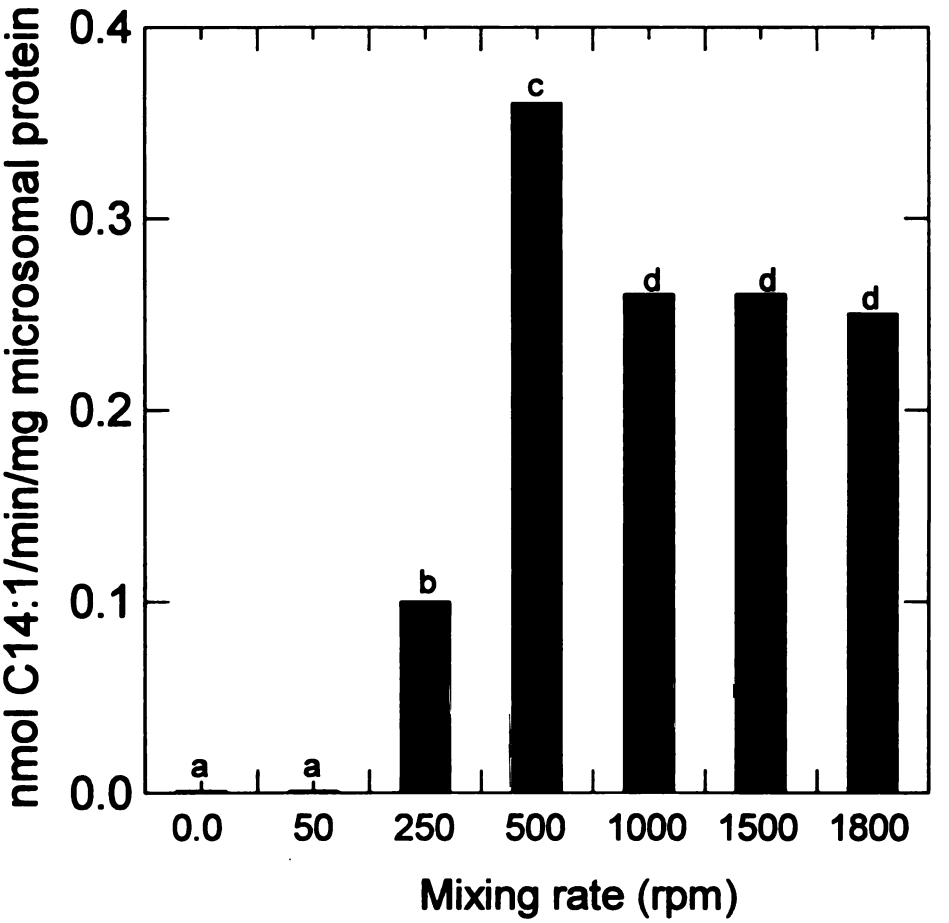


Figure 15- Effect of mixing rate on the direct desaturation of myristic acid: FABP complexes by the Δ^9-desaturase system of hen hepatic microsomes. The desaturation activity was measured under conditions described in Figure 14 using different mixing speeds. The complete system contained 8 mg microsomal protein, 94.5 mg cytosolic protein, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 133 μM free C14:0, 1.24 mM NAD, 3.09 mM sodium lactate, 1.31 mM $MgCl_2$, pH 7.4, in a total volume of 7.5 mL. The digest was mixed at 37°C for 30 min.

[a-d] Means with no common superscript are significantly different ($p<0.05$).

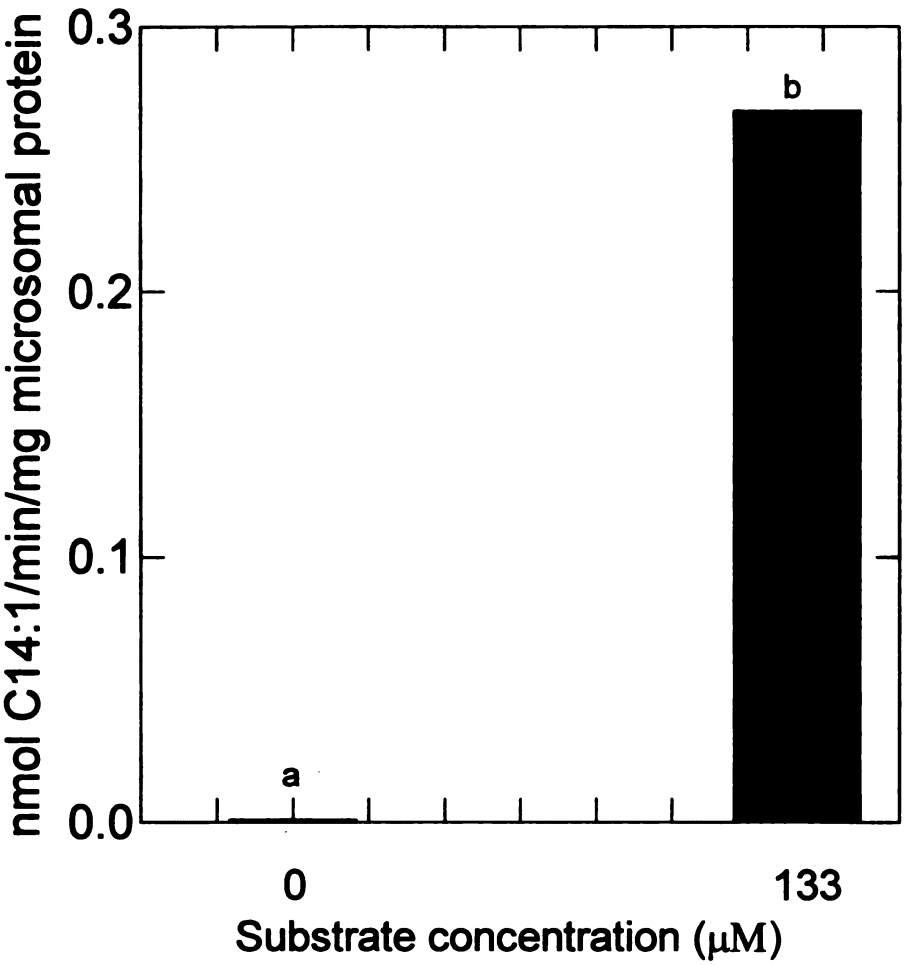


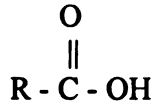
Figure 16- Effect of substrate concentrations on the direct desaturation of myristic acid:FABP complexes by the Δ^9-desaturase system of hen liver microsomes. The desaturation activity was measured at 37°C under conditions described in Figure 14 with different substrate concentrations. The complete system contained 8 mg microsomal protein, 94.5 mg cytosolic protein, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 1.24 mM NAD, 3.09 mM sodium lactate, 1.31 mM $MgCl_2$, pH 7.4, in a total volume of 7.5 mL. The digest was mixed at 500 rpm for 30 min.
[a-b] Means with no common superscript are significantly different ($p<0.05$).

the rate of transfer of the free fatty acid from hFABP to egg phosphotidycholine membranes is influenced by both the structure of the free fatty acid ligand and the properties of the aqueous phase (pH, ionic strength, temperature) through which the free fatty acid must travel. Working with purified stearyl-CoA desaturase and acyl-CoA derivatives as substrates, Enoch et al. (1976) reported that free CoA and free fatty acids did not bind to the Δ^9 -desaturase enzyme and did not inhibit the desaturation reaction. Brudett and Zeikus (1994) reported that "the secondary alcohol dehydrogenase provides an NADPH-dependent route to ethanol from acetyl CoA and acetyldehyde ". They did not use mixing.

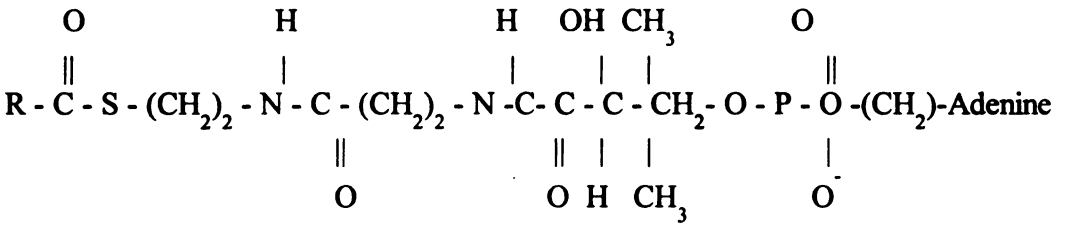
The data presented here lead to the conclusion that when free myristic acid is bound to FABP in the form of protein-monomer complexes, its activation to the CoA derivative is not necessary in order to be desaturated by the Δ^9-desaturase when using a mixing rate greater than 250 rpm. Myristic acid:FABP complexes probably serve as substrates for the Δ^9-desaturase at high mixing rates.

3.4.2 Substrate specificity studies

The variation in molecular structure between fatty acyl CoA and the free fatty acid gives rise to variation in reactivity. The only difference between the two molecules is the CoA esterified to the acyl group.



Free fatty acid



Fatty acyl CoA

This observation might be explained by the guiding and reaction side hypothesis:

"The formation of productive enzyme-substrate complexes occurs if S1 is complementary in shape, size, and chemical nature to E1, and S2 when needed, is properly guiding S1 to E1 without any steric hindrance".

Let us consider S = S1 + S2 and E = E1 + E2 (Figure 17)

S : substrate

S1: reaction side, determines the type of products.

S2: guiding side, determines the reactivity.

E : enzyme

E1: active site, determines the type of reaction

E2: non-catalytic side, maintains the enzyme in its tertiary structure.

The guiding side (S2) affects reactivity in two general ways:

(1) by the effect of long range forces between S2---E2 (mainly electrostatic and ion-dipole interaction) that facilitate the trafficking of the reaction side (S1) toward the active site (E1) and that activate the short range forces (London-van der Waal's

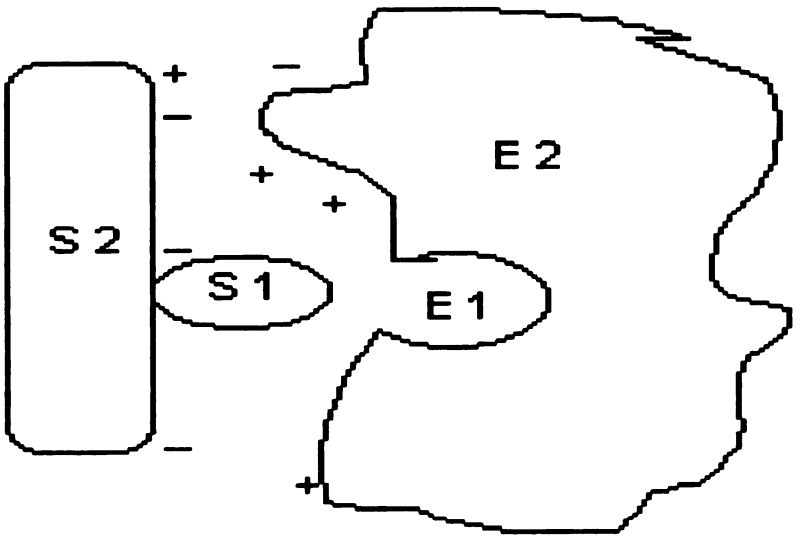


Figure 17- An enzyme-substrate complex illustrating both the geometrical and the physical complementarity between active site (E1) and reaction side (S1). Where E1 is the active site of the enzyme, E2 is the no-catalytic side of the enzyme, S1 is the reaction side of the substrate, and S2 is the guiding side of the substrate.

dispersion) between S1---E1 to induce a precise fit; and (2) by its steric effect which prohibits the formation of active site-reaction side (E1—S1) complexes , which lead to the formation of product. The steric effect of S2 is dependent on the configuration of E2. E2 is not a dead weight, it plays an important in the catalytic activity of E.

We investigated several scenarios to verify the validity of this hypothesis.

First scenario: fatty acyl CoA derivative and Δ^9-desaturase

S : C14:0CoA (substrate)	E : Δ^9-desaturase
S1: C14:0 (reaction side)	E1 : active site
S2: CoA (guiding side)	E2 : non-catalityc side

The transfer of C14:0 (S1) to the catalytic domain of the Δ^9-desaturase (E1) was accomplished via direct collision interaction through the electrostatic attractions between the water-soluble CoA moiety (S2) and the hydrophilic part (E2) of the membrane bound Δ^9-desaturase which exposes the catalytic domain to the aqueous environment. Enoch and Strittmatter (1978) reported that positively charged arginyl residues of the stearyl-CoA desaturase may be involved in the binding of the CoA moiety of the negatively charged substrate, as the substrate (stearyl-CoA) has three phosphate groups and bears three to four negative charges.

The guiding side CoA (S2) facilitates the trafficking of the hydrophobic myristic acid (S1) to traverse the aqueous phase to reach the membrane bound Δ^9-desaturase. There was no steric effect observed with CoA. Myristoyl CoA did meet the conditions of the proposed concept. The Δ^9-desaturase specific activity expressed as nmol C14:1/min per mg microsomal protein was 0.50. Enoch et al. (1976) showed that liposome bound stearyl-CoA is the true substrate for the purified Δ^9–desaturase

in the absence of BSA. Bound stearyl-CoA undergoes lateral diffusion in the bilayer to reach the active site of the enzyme. However, in the presence of BSA, the possibility that myristoyl-CoA is reaching the enzyme by lateral diffusion is unlikely due to the ability of the albumin to remove the fatty acyl-CoA from the liposomes. The transfer of substrate by direct collision is more likely.

Lichtenstein et al. (1982) reported that the addition of albumin to unilamellar vesicles at a molar ratio equivalent to that of oleoyl CoA effectively removed oleoyl CoA from the vesicle and result in oleoyl CoA-albumin complex. Jeffcoat et al. (1977) reported that albumin is not necessary for the desaturatioon reaction *per se.* The role of albumin is to protect the acyl CoA derivatives from the acyl transferase present in the cytosol. In agreement with the reported literature, our data show that BSA is not necessary for the desaturation myristoyl-CoA by hen liver microsomal Δ^9-desaturase (Figure 9, Chapter 2).

Second scenario: free fatty acids and Δ^9- desaturase

S : C14:0 E : Δ^9-desaturase system

S1: C14:0 (reaction side) E1: active site

S2: absent (guiding side) E2: non catalytic side

Myristic acid as a free monomer did not meet the conditions of the hypothesis. The desaturation reaction did not occur because the guiding side (S2) was absent. The reaction is substrate-transfer limited, instead of being limited to the kinetics of the reaction. However, when the microsomal supernatant containing

FABP, was added to the incubation medium coupled with a mixing speed of 500 rpm, the specific activity observed was 0.268 nmol/min per mg microsomal protein. In this system, neither CoA nor ATP was added. Free myristic acid binds to FABP to form a myritic acid-FABP complex which ultimately dissociates in the aqueous phase. Brecher et al. (1984) reported that when exposed to protein (BSA or FABP), 50% of the fatty acids contained within liposomes could become protein-bound, and the newly formed fatty acid-protein complex dissociates from the bilayer surface to the aqueous space. They also reported that the movement of fatty acid from protein to liposomes is a much slower process.

The possibility that myristic acid reaches the active site of the Δ^9-desaturase by lateral diffusion in the bilayer is unlikely due to the effect of binding proteins. The transfer of myristic acid to the Δ^9-desaturase catalytic domain was accomplished via random collision between the myristic acid–FABP complexes and the membrane bound Δ^9-desaturase. The random collisions were generated using different mixing rates. A mixing rate of 500rpm was necessary to overcome diffusion obstacles due to the properties of the aqueous phase (pH, ionic strength, temperature), through which the myristic acid-FABP complexes has to travel. The desaturation reaction did not occur in the absence of FABP despite the use of different mixing rates. These results suggest that FABPs play a key role in the formation of enzyme substrate complexes probably via a physical effect on the myristate.

The collision between myristic acid–FABP complex and the membrane-bound Δ^9-desaturase activates the short-range forces between the myristic acid (S1) and the active site (E1) of the desaturase, therefore inducing a precise fit. The short-range

forces are London Van-der-Waal's dispersions and weak polar attraction due to the polarizable π electrons of the carbonyl group. In this case, the FABP is the guiding side (S2) and myristic acid is the reaction side (S1). This observed effect was specific for FABP and could not be duplicated by albumin (Figure 8), giving more weight to the argument that the effect of FABP is specific in regulating the desaturase activity. The reactivity of the reaction is determined by the nature of the guiding side (S2). Under the experimental conditions used, the guiding side CoA was more reactive than the guiding side FABP since specific activity of the desaturase was higher (Figure 18).

Third scenario: trimyristin and Δ^9-desaturase system

S : trimyristin	E : Δ^9-desaturase
S1: C14:0 (reaction side)	E1: active site
S2: glycerol backbone (guiding side)	E2: non catalytic side

The desaturation reaction did not occur even in the presence of FABP and the use of different mixing rates to overcome diffusion obstacles due to the properties of the aqueous phase (pH, Ionic strength, temperature) through which the substrate must travel. This lack of desaturation might be due to the steric effect exerted by the glycerol backbone (S2) on (E2). Trimyristin did not meet the conditions of the proposed hypothesis and thus no desaturation was observed. The same effect was observed with 1-monomyristoyl-rac-glycerol (C14:0), 1,2 dimyristoyl-rac-glycerol (C14:0), myristic acid methyl ester, and L-α-phosphotidyl choline dimyristoyl

(C14:0) (Figure18).

3.4.3 Desaturation of milkfat

As the Δ^9-desaturase is inactive on triacylglycerol substrates, the application of the desaturation process (outlined in Figure 14) on milkfat necessitates a lipase-catalyzed hydrolysis of milkfat triacylglycerols to liberate the free fatty acids (FFAs) prior to the desaturation reaction and finally re-esterify the FFAs. They would not all return to their original positions in the triacylglycerol moiety, hence the reaction of the final material to milkfat would be somewhat tenuous.

3.5 CONCLUSION

The current research effort was directed at understanding the regulation of the Δ^9-desaturase system of hen liver microsomes using myristoyl-CoA and myristic acid:FABP complexes as substrates. Experimental evidence has been presented to show that the variation in molecular structure between myristoyl-CoA and myristic acid:FABP complexes gives rise to variation in reactivity. CoA and FABP are guiding molecules. There are positive influences pulling reactants, but negative repulsion plays a major role in the way reactants are guided. Attraction and repulsion can act over short-and long-ranges. Short-range requires contacts with membrane bound Δ^9-desaturase, and long-range interactions occur by diffusible factors that set up gradients. Unraveling the mechanism of these interactions is complicated by the fact that guidance molecules can have bifunctional roles. The same molecule can be

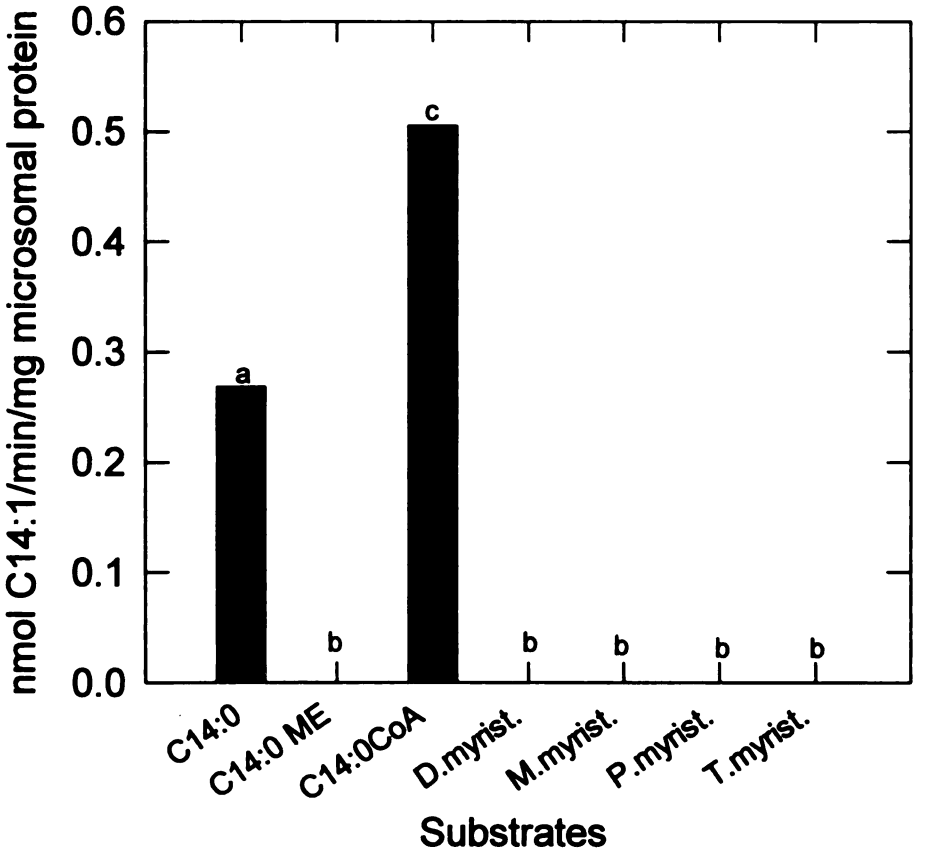


Figure 18- Effect of different substrates on the specific activity of the hen microsomal Δ^9-desaturase system. The desaturation activity was measured at 37°C under conditions described in Figure 14 using different substrates. The complete system contained 8 mg microsomal protein, 94.5 mg cytosolic protein, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 1.24 mM NAD, 3.09 mM sodium lactate, 1.31 mM $MgCl_2$, pH 7.4, in a total volume of 7.5 mL. The digest was mixed at 500 rpm for 30 min.

$^{a-c}$ Means with no common superscript are significantly different ($p<0.05$).

C14:0 : Free myristic acid
C14:0CoA : Myristoyl coenzyme A
T.myrst : Trimyristin
D.myrst : 1,2 dimyristoyl-rac-glycerol (C14:0)
M.myrst : 1-monomyritoyl-rac-glycerol (C14:0)
C14:0ME : Myristic acid methyl ester
P.myrst. : L-α- phosphotidyl choline dimyristoyl (C14:0)

attractive or repulsive, short- and long-range.

The frustrations of designing enzyme inhibitors are nothing new. This study shed some light on the effect of the substrate guiding side (S2) on the reactivity of enzymatic reactions. Every enzyme has an inhibitor and may be with a better understanding of the role of the substrate guiding side in enzymatic reactions researchers will be able to find a more efficient way to design enzyme inhibitors.

CHAPTER 4

REDUCTION OF THE HYPERCHOLESTEROLEMIC EFFECT OF ANHYDROUS MILKFAT

4.1 ABSTRACT

Alternatives to *trans*-rich fats are currently being sought that will allow a return to animal fat without eliciting deleterious health effects. Removing cholesterol from anhydrous milkfat to minimize its cholesterol-raising effect, has produced a viable alternative to native milkfat and *trans*-rich margarine spreads. A process to modify anhydrous milkfat to decrease its hypercholesterolemic effect was developed using β–cyclodextrin, KOH, $CaCl_2$, and mixing. The process was optimized by response surface methodology based on cholesterol and free fatty acid (FFA) reduction. Reductions in cholesterol and FFAs with this process were 95.8 and 92.4%, respectively. Gerbil feeding studies showed that modified milkfat (i.e., cholesterol-reduced milkfat) reduced serum total cholesterol by 28% and triacylglycerols by 47%.

4.2 INTRODUCTION

Multiple lines of evidence indicate that saturated fatty acids and cholesterol raise plasma cholesterol levels. Both animal (Hegsted and Gallagher, 1967) and human studies (Keys et al., 1957) have demonstrated elevation in serum cholesterol levels following increased saturated fat intake. These observations, coupled with epidemiological evidence of increased risk of coronary heart disease (CHD) in populations consuming excessive amounts of saturated fats and cholesterol, explain the emphasis on the reduction of saturated fat and cholesterol by most health agencies.

Milkfat plays an important role in the structure, mouthfeel, flavor and stability of dairy products and a host of foods in which milkfat is added as a functional ingredient. However, milkfat has a high proportion of hypercholesterolemic saturated fatty acids and cholesterol which has resulted in decreased consumption of dairy products (USDA, 1997).

The fat and oil industry is continuously looking at new techniques to alter the composition and functionality of fat molecules. Several methods for improving the nutritional and physical properties of milkfat have been developed over the past few years including changing the fatty acid composition by altering the diet of cows (Banks et al., 1990). Feeding protected soybean oil to cows has resulted in a 3.6 fold increase in linoleic acid (Banks et al., 1990) compared to the values obtained on feeding a basal diet. Milkfat fractionation using physical processes such as short-path distillation (SPD), melt crystallization, and supercritical fluid extraction has been applied in conjunction with cholesterol extraction. Arul et al. (1988) fractionated milkfat by SPD into four fractions at temperatures of 245 and 265 °C and pressures of 220 and 100 μm Hg. However, the

use of high temperature might decompose or polymerize the triacylglycerols even when the milkfat is distilled under vacuum. Makhlouf et al. (1987) fractionated milkfat into 7 fractions by melt crystallization at temperatures ranging from 26 to 9 °C. The major drawback of this technique is the significant overlapping of molecular weights of triacylglycerols. Bhaskar et al. (1993) fractionated milkfat using super critical fluid extraction into 5 different fractions (S1 – S5) with differing cholesterol levels at 3.5 - 24.1 MPa and 40 - 60 °C.

Dietary cholesterol is hypercholesterolemic (National Academy of Sciences, 1989b) and prone to oxidation. Increased attention has been focused on specific effects associated with certain cholesterol oxidation products and the need to eliminate or prevent their formation in the human diet. Peng et al. (1979) reported that oxides of cholesterol are toxic and cause degeneration of aortic smooth muscle cells in tissue culture, and may lead to the development of atherosclerosis (Peng et al., 1991). Epidemiological studies further suggest that dietary cholesterol increases the risk for coronary heart disease beyond its serum cholesterol-raising effect (Stamler and Shekelle, 1988).

Several investigators have used cyclodextrin (CD) to reduce the cholesterol levels in animal products. CD are oligosaccharides, consisting of seven molecules of glucose units arranged in a donut shaped ring (Szejtli, 1982a,b). Courregelongue et al. (1989) used β–CD to remove 41 % of the sterols from anhydrous milkfat. Bayol et al. (1989) reported the removal of 80 % Δ^4-cholesten-3-one from anhydrous milkfat, while Roderbourg et al. (1993) demonstrated that at least 37% of the cholesterol could be

removed from anhydrous milkfat in one single step. Graille et al. (1991) . . reported the simultaneous removal of 50% cholesterol and 52 % FFAs from cream. Okenfull et al. (1991) invented a new process called SIDOAK to reduce cholesterol in dairy products. The process consisted of adding CD to milk and mixing below 10°C. The maximum cholesterol reduction obtained was 80-90 %. Yen and Tsai (1995), using β-CD, indicated the removal of 95 % cholesterol simultaneously with 50 % FFA from lard.

The objectives of the present study were:

1) to develop a process to effectively remove cholesterol and FFAs from milkfat;

2) to test the effect of cholesterol-reduced milkfat on blood lipids

4.3 MATERIALS AND METHODS

4.3.1 Materials

Commercial grade unsalted butter was obtained from a local food store and converted into anhydrous milkfat (AMF) by melting at 55°C, centrifuging at 5000 x g for 10 min at room temperature, and filtering the top fat layer through Whatman No. 1 filter paper. The AMF was stored at -20°C for future treatments. Potassium hydroxide and calcium chloride were purchased from VWR Scientific Products (Batavia, IL), while β–CD was obtained from Cerestar (Hammond, IN).

4.3.2 Preliminary process

After several exploratory experiments, a preliminary process was developed. AMF (10g) containing 0.395% cholesterol and 0.293% FFA was mixed in a 50mL beaker with 10mL of a refining solution (0.0583 % (w/v) KOH and 0.763 % (w/v)

$CaCl_2$) at 1000 rpm using a Lightnin Lab Master (SI) mixer (model L1U03) and an impeller (type A100) (Matoon and Lee Equipment, Farmington, MI) until the temperature reached 50°C using a controlled temperature water bath. β–CD was added to the mixture to provide a β–CD:cholesterol mole ratio of 5.58. Mixing was continued for another 10 min. The resulting soap and β–CD :cholesterol complexes were immediately centrifuged at 8700 x g for 10 min at room temperature using a Sorvall RC 2-B centrifuge (Sorvall Inc., Norwalk). The modified AMF, i.e., reduced cholesterol and FFAs, was recovered in the upper supernatant phase. Percentage cholesterol and FFA reductions were 54 and 92 %, respectively.

4.3.3 Experimental design

When many factors and interactions affect desired responses, response surface methodology (RSM) is an effective tool for optimizing the process (Hunter, 1959). Thus, the optimization of cholesterol and FFA reduction was accomplished using response surface methodology.

Two responses were measured: percentage cholesterol reduction (Y_1) was defined as the ratio of the total amount of cholesterol in the sample to the total amount of cholesterol in the control multiplied by 100. Percentage FFA reduction (Y_2) was defined as the ratio of the total amount of FFAs in the sample to the total amount of FFAs in the control multiplied by 100. Based on preliminary experiments, the five independent variables to be evaluated were KOH:FFA mole ratio, $CaCl_2$:FFA mole ratio, mixing time, β–CD:cholesterol mole ratio, and mixing rate (Table 1). The other important independent variables were held fixed: temperature and centrifugation speed of 8700 x g

Table 1- Variables and their levels for central composite design used in optimizing the process for reducing cholesterol and free fatty acids in anhydrous milkfat.

Variable		Symbol	Coded-variable levels				
			-2	-1	0	+ 1	+2
KOH:FFAs	(mole ratio)	X1	0	0.5	1	1.5	2
$CaCl_2$:FFAs	(mole ratio)	X2	0	2.5	5	7.5	10
Mixing time	(min)	X3	5	7.5	10	12.5	15
CD:cholesterol	(mole ratio)	X4	4.72	5.15	5.58	6.01	6.4
Mixing rate	(rpm)	X5	200	600	1000	1400	1800

for 10 min at room temperature.

The experimental design adopted was a 5-factor, 5-level central composite (Box and Hunter, 1957). The coded values of the independent variables were -2 (lowest level), - 1.0 (medium level), 1.0, and 2 (highest level). For each independent variable studied, the central value (0) was chosen according to the preliminary explanatory preliminary experiments. The complete design shown in Table 2 consisted of 32 experimental points which included 6 replications of the center (0, 0, 0, 0, 0).

4.3.4 Extraction and determination of total cholesterol

Total cholesterol was extracted and quantified according to the AOAC Official Method 994.10 (1995). Total cholesterol was quantified in underivatized form using a Hewlett Packard 5890A gas chromatograph (GC) (Avondale, PA) equipped with a flame ionization detector and a fused silica capillary column (15 m x0.25mm i.d.) (J & W Scientific, Folsom, CA) coated with a 0.1 μm film of DB-1 (100% methyl polysiloxane).

4.3.5 Quantitation of free fatty acids (FFAs)

FFAs were quantified according to the AOAC Official Method 969.17 (1995).

4.3.6 Determination of the hypercholesterolemic effect of anhydrous milkfat

Feeding studies on gerbils to determine the hypercholesterolemic effect of anhydrous milkfat were done according to the protocol described by Hajri et al. (1998). Adult male Mongolian gerbils (*Meriones unguiculatus*) weighing 75±5 g were housed in

Table 2- Central composite design arrangement and responses used in optimizing the process for reducing cholesterol and free fatty acids in anhydrous milkfat.

	Variable levels[a]					Responses[b]	
Run	X1	X2	X3	X4	X5	Y1	Y2
1	-1	-1	-1	-1	1	61.78	69.53
2	-1	-1	-1	1	-1	28.58	58.07
3	-1	-1	1	-1	-1	46.46	68.06
4	-1	-1	1	1	1	77.3	75.3
5	-1	1	-1	-1	-1	38.53	69.16
6	-1	1	-1	1	1	63.66	79.12
7	-1	1	1	-1	1	68.22	77.6
8	-1	1	1	1	-1	40.5	70.79
9	1	-1	-1	-1	-1	20.41	92.42
10	1	-1	-1	1	1	65.41	92.28
11	1	-1	1	-1	1	65.98	92.38
12	1	-1	1	1	-1	42.84	92.21
13	1	1	-1	-1	1	54.28	96.16
14	1	1	-1	1	-1	38.21	90.36
15	1	1	1	-1	-1	32.28	92.35
16	1	1	1	1	1	74.23	92.48
17	-2	0	0	0	0	59.98	31.35
18	2	0	0	0	0	53.52	92.33
19	0	-2	0	0	0	38.03	91.92
20	0	2	0	0	0	53.32	92.38
21	0	0	-2	0	0	37.49	92.24
22	0	0	2	0	0	63.64	92.63
23	0	0	0	-2	0	46.85	92.64
24	0	0	0	2	0	55.7	92.45
25	0	0	0	0	-2	11.91	81.6
26	0	0	0	0	2	73.7	92.61
27	0	0	0	0	0	52.27	92.45
28	0	0	0	0	0	56.27	92.22
29	0	0	0	0	0	53.02	92.53
30	0	0	0	0	0	54.48	92.1
31	0	0	0	0	0	54.94	92.45
32	0	0	0	0	0	54.02	91.93

[a] Coded variables

[b] Y1 is cholesterol reduction (%). Y2 is FFA reduction (%)

individual cages in an air-conditioned room with a 12-h reversed light-dark cycle (lights on at 18:00h). The gerbils were assigned randomly to 6 groups (n=6 per group) and were fed two types of fats for 4 weeks: native anhydrous milkfat (control) and modified milkfat. Fat provided 40% of the total dietary energy. The basal composition of these diets (g/100g of total dry weight) was as follows: 22.2 casein, 23 cornstarch, 13.3 glucose, 15.0 cellulose, 20 fat, 5.0 mineral mix (Ausman-Hayes), 1.2 vitamin mix (Hayes-Cathcart), and 0.3 choline chloride. At the end of the feeding study, each gerbil was exsanguinated under anesthesia, the livers weighed and inspected for cholesterol, and plasma collected for total cholesterol, LDL-C, HDL-C, and plasma triacylglycerols. The feeding studies were done at Brandeis University under the direct supervision of Dr. K.C. Hayes.

4.3.7 Statistical analysis

Statistical analyses of the cholesterol and the FFA data were performed using software packages to fit the second order polynomial equation to the experimental data (SAS Institute, 1990). The model proposed for each response (Y) was:

$$Y = B_o + \Sigma B_i X_i + \Sigma B_{ii} X_i^2 + \Sigma B_{ij} X_i X_j$$

where B_o, B_i, B_{ii}, B_{ij} are constants and regression coefficients of the model, and X_i the independent variables in coded values. The criteria for eliminating a variable from the full regression were based on R^2 values and the significance of the F-test.

4.4 RESULTS & DISCUSSION

4.4.1 Process development and optimization

Response surface methodology was used to optimize the preliminary process for cholesterol and FFA reduction in anhydrous milkfat. Analysis of variance (ANOVA) for the regression was performed to test the goodness of fit. Results of the analysis of variance of each of the responses with their corresponding coefficient of multiple determination (R^2) and coefficient of variation (CV) are shown in Table 3. From the analysis of the F values and R^2, only four independent variables (KOH:FFA mole ratio, mixing time, CD:cholesterol mole ratio, and mixing rate) out of five appeared to influence cholesterol reduction (Y1). In fact, despite the exclusion of $CaCl_2$ from the model, the R^2 remained high (0.95) and the F-test significant to <0.001. The estimated regression coefficients for Y_1 and the results of the significance test on the coefficient for the four selected factors are indicated in Table 4. Also, from the analysis of the F values and R^2, only three independent variables (KOH:FFA mole ratio, $CaCl_2$:FFA mole ratio, and mixing rate) out of five appeared to influence FFA reduction (Y2). The exclusion of mixing time and CD did not affect the model, since the R^2 remained high (0.96) and the F-test was significant to <0.001. The estimated regression coefficients for Y2 and the results of the significance test on the coefficient for the three selected factors are indicated in Table 5. None of the models exhibited lack of fit. Both models were considered adequate and usable with satisfactory R^2 values and a significant F-test at <0.001. The predicted Y1, Y2, and the residual values using the model are reported in Tables 6 and 7.

Table 3- Parameter estimates and analysis of variance of the second order polynomial regression for five factors and two responses (cholesterol (Y1) and FFA (Y2) reduction)

	Y1		Y2	
Source	Coefficient	prob>|T|	Coefficient	prob>|T|
Intercept	-18.66	0.91	29.09	0.87
X1	-139.81	<0.01	160.62	<0.01
X2	12.67	0.07	-3.75	0.06
X3	10.3	0.14	-4.98	0.5
X4	9.97	0.85	-13.76	0.82
X5	-0.01	0.68	0.06	0.16
X1*X1	3.8	0.26	-30.68	<0.01
X2*X2	-0.29	0.04	-0.01	0.92
X3*X3	-0.09	0.48	<-0.01	0.98
X4*X4	-2.25	0.63	0.14	0.97
X5*X5	<-0.01	0.01	<-0.01	0.16
X1*X2	1.38	0.14	-2.26	0.04
X1*X3	0.85	0.35	-1.96	0.07
X1*X4	21.15	<0.01	-7.71	0.2
X1*X5	<-0.01	0.55	<-0.01	0.69
X2*X3	-0.55	<0.01	0.04	0.82
X2*X4	-0.94	0.37	1.37	0.25
X2*X5	<-0.01	0.94	<-0.01	0.38
X3*X4	-1.1	0.3	1.71	0.16
X3*X5	<0.01	0.19	<-0.01	0.1
X4*X5	0.01	0.05	<-0.01	0.7
R^2	0.97		0.95	
F-ratio	20.07		11.49	
prob F	<0.05		<0.05	
CV (%)	8.77		5.76	

$$Y = B_0 + \sum_{i=1}^{5} BiXi + \sum_{i=1}^{5} Bii\ XiXi + \sum_{i=1}^{5} Bij\ XiXj$$

Table 4- Parameter estimates and analysis of variance of the second order polynomial regression for four factors and one response (cholesterol reduction (Y1))

Source	Coefficient	prob>|T|
Intercept	51.91	<0.01
X1	-3.99	0.05
X3	10.76	<0.01
X4	4.97	0.01
X5	30.44	<0.01
X1*X1	5.23	0.14
X3*X3	-0.94	0.78
X4*X4	-0.06	0.98
X5*X5	-8.7	0.02
X1*X3	-0.72	0.87
X1*X4	12.86	0.01
X1*X5	2.31	0.62
X3*X4	0.26	0.95
X3*X5	1.06	0.82
X4*X5	4.36	0.35

$R^2 = 0.95$ F-ratio = 22.79 prob F <0.05 CV (%) = 9.1

$$Y = B_0 + \sum_{i=1}^{4} BiXi + \sum_{i=1}^{4} Bii\ XiXi + \sum_{i=1}^{4} Bij\ XiXj$$

$$Y1 = 51.91 - 3.99\ X_1 + 10.76\ X_3 + 4.97\ X_4 + 30.44 X_5 - 8.7\ X_5^2 + 12.86\ X_1X_4$$

Table 5- Parameter estimates and analysis of variance of the second order polynomial regression for 3 factors and one response (FFA reduction (Y2))

Source	Coefficient	prob>|T|
Intercept	92.11	<0.01
X1	24.58	<0.01
X2	2.39	0.07
X5	5.28	<0.01
X1*X1	-31.5	<0.01
X2*X2	-1.19	0.6
X5*X5	-6.24	0.01
X1*X2	-5.91	0.07
X1*X5	-7.37	0.02
X2*X5	0.99	0.75

$R^2 = 0.96$; F-ratio = 65.34 prob F <0.05 CV (%) = 3.70

$$Y = B_o + \sum_{i=1}^{3} BiXi + \sum_{i=1}^{3} Bii\ XiXi + \sum_{i=1}^{3} Bij\ XiXj$$

$$Y2 = 92.11 + 24.58\ X_1 + 5.28\ X_5 - 31.5\ X_1^2 - 6.24\ X_5^2 - 7.37\ X_1\ X_5$$

Table 6- Cholesterol reduction in anhydrous milkfat as predicted by the second order polynomial regression for 4 factors

Run	X1	X3	X4	X5	Y1 (actual)	Y1 (estimated)	Residuals (act - est)
1	-1	-1	-1	1	61.78	61.31	0.46
2	-1	-1	1	-1	28.58	30.82	-2.24
3	-1	1	-1	-1	46.46	45.15	1.3
4	-1	1	1	1	77.3	73.71	3.58
5	-1	-1	-1	-1	38.53	34.68	3.84
6	-1	-1	1	1	63.66	61.92	1.73
7	-1	1	-1	1	68.22	72.84	-4.62
8	-1	1	1	-1	40.5	41.55	-1.05
9	1	-1	-1	-1	20.41	23.61	-3.2
10	1	-1	1	1	65.41	66.33	-0.92
11	1	1	-1	1	65.98	63.36	2.61
12	1	1	1	-1	42.84	42.92	-0.08
13	1	-1	-1	1	54.28	52.55	1.72
14	1	-1	1	-1	38.21	32.92	5.28
15	1	1	-1	-1	32.28	33.35	-1.07
16	1	1	1	1	74.23	77.4	-3.17
17	-2	0	0	0	59.98	60.96	-0.98
18	2	0	0	0	53.52	53.57	-0.05
19	0	0	0	0	38.03	52.03	-14
20	0	0	0	0	53.32	52.03	1.28
21	0	-2	0	0	37.49	40.31	-2.82
22	0	2	0	0	63.64	61.85	1.78
23	0	0	-2	0	46.85	46.87	-0.02
24	0	0	2	0	55.7	56.81	-1.11
25	0	0	0	-2	11.91	12.77	-0.86
26	0	0	0	2	73.7	73.87	-0.17
27	0	0	0	0	52.27	52.03	0.23
28	0	0	0	0	56.27	52.03	4.23
29	0	0	0	0	53.02	52.03	0.98
30	0	0	0	0	54.48	52.03	2.44
31	0	0	0	0	54.94	52.03	2.9
32	0	0	0	0	54.02	52.03	1.98

Table 7- Reduction of free fatty acids as predicted by the second order polynomial regression for 3 factors

Run	X1	X2	X5	Y2 (actual)	Y2 (estimated)	Residuals (act - est)
1	-1	-1	-1	69.53	71.65	-2.12
2	-1	-1	-1	58.07	63.17	-5.1
3	-1	-1	-1	68.06	63.17	4.88
4	-1	-1	1	75.3	71.65	3.64
5	-1	1	-1	69.16	68.02	1.13
6	-1	1	1	79.12	77.5	1.61
7	-1	1	1	77.6	77.5	0.09
8	-1	1	-1	70.79	68.02	2.76
9	1	-1	-1	92.42	94.4	-1.98
10	1	-1	1	92.28	95.5	-3.22
11	1	-1	1	92.38	95.5	-3.12
12	1	-1	-1	92.21	94.4	-2.19
13	1	1	1	96.16	95.43	0.72
14	1	1	-1	90.36	93.34	-2.98
15	1	1	-1	92.35	93.34	-0.99
16	1	1	1	92.48	95.43	-2.95
17	-2	0	0	31.35	36.03	-4.6
18	2	0	0	92.33	85.19	7.13
19	0	-2	0	91.92	88.53	3.38
20	0	2	0	92.38	93.31	-0.93
21	0	0	0	92.24	92.11	0.12
22	0	0	0	92.63	92.11	0.51
23	0	0	0	92.64	92.11	0.52
24	0	0	0	92.45	92.11	0.33
25	0	0	-2	81.6	80.58	1.01
26	0	0	2	92.61	91.16	1.44
27	0	0	0	92.45	92.11	0.33
28	0	0	0	92.22	92.11	0.1
29	0	0	0	92.53	92.11	0.41
30	0	0	0	92.1	92.11	-0.01
31	0	0	0	92.45	92.11	0.33
32	0	0	0	91.93	92.11	-0.18

4.4.2 Optimization based on Y1 (Percentage cholesterol reduction)

The model Y1 is useful for indicating the direction-to-change variables to maximize cholesterol reduction. Judging from the significant regression coefficients (Table 4), the most important factors influencing cholesterol reduction are KOH:FFA mole ratio, mixing time, CD:cholesterol mole ratio, and mixing rate. They showed significant first order, quadratic, and two-factor interaction terms.

4.4.2.1 KOH:FFA mole ratio

KOH was used to neutralize the FFAs and remove them from the system. The range of KOH:FFA mole ratios examined is shown in Table 2. The negative slope B_1 = -3.99 (Table 4) indicated that cholesterol reduction decreased with increased KOH concentrations at any mixing time or rate (Figures 1 and 2). The negative effect of KOH on cholesterol reduction might be due to its ability to convert the FFAs present in the system to soap molecules. The soap molecules aggregate to form micelles because their concentration in the system (10.39 mM) surpasses the critical micelle concentration (cmc) which is reported to be 1.5 mM for sodium oleate (Small, 1995). The negatively charged carboxylate groups lie scattered over the surface of the micelle and form hydrogen bonds with the hydroxyl groups on the external rim of the β-cyclodextrin molecules, thus competing with cholesterol for the β-CD molecules.

The use of higher β-cyclodextrin:FFA mole ratios to counteract the negative effect of KOH (Figure 3), is not economically feasible. The shape of the response surface (Figure 3) and the slope of the two factor interaction terms (B_1B_4 = 12.86) are characteristic of the strong interaction between these two variables.

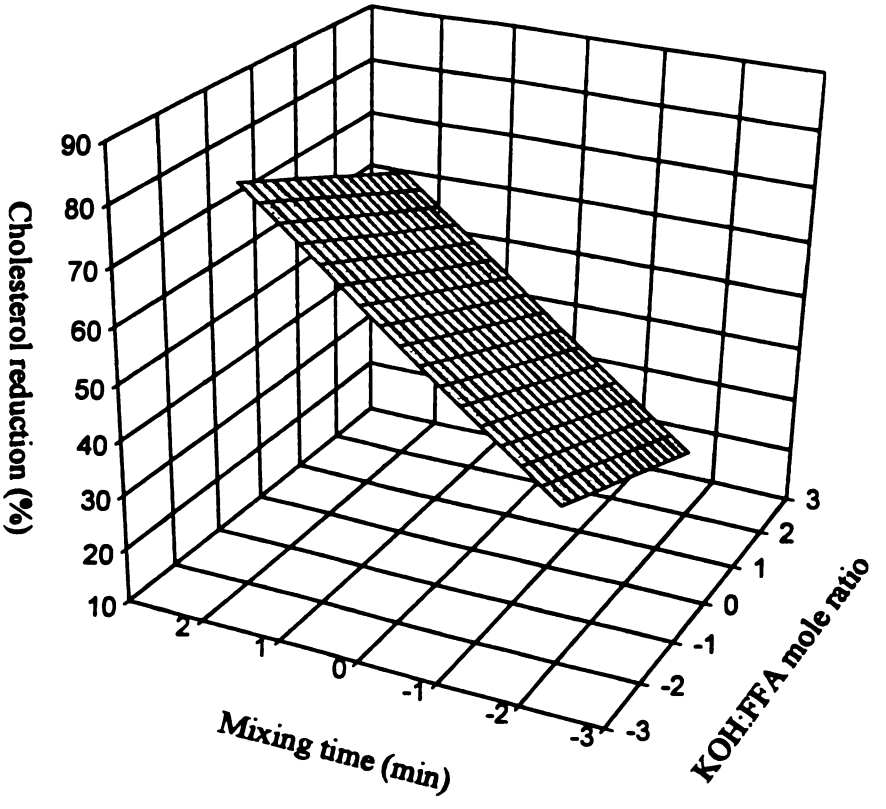


Figure 1- Response surface methodology for percent reduction of cholesterol (Y1) in anhydrous milkfat at a mixing rate of 1000 rpm and a β-cyclodextrin:cholesterol mole ratio of 5.58.

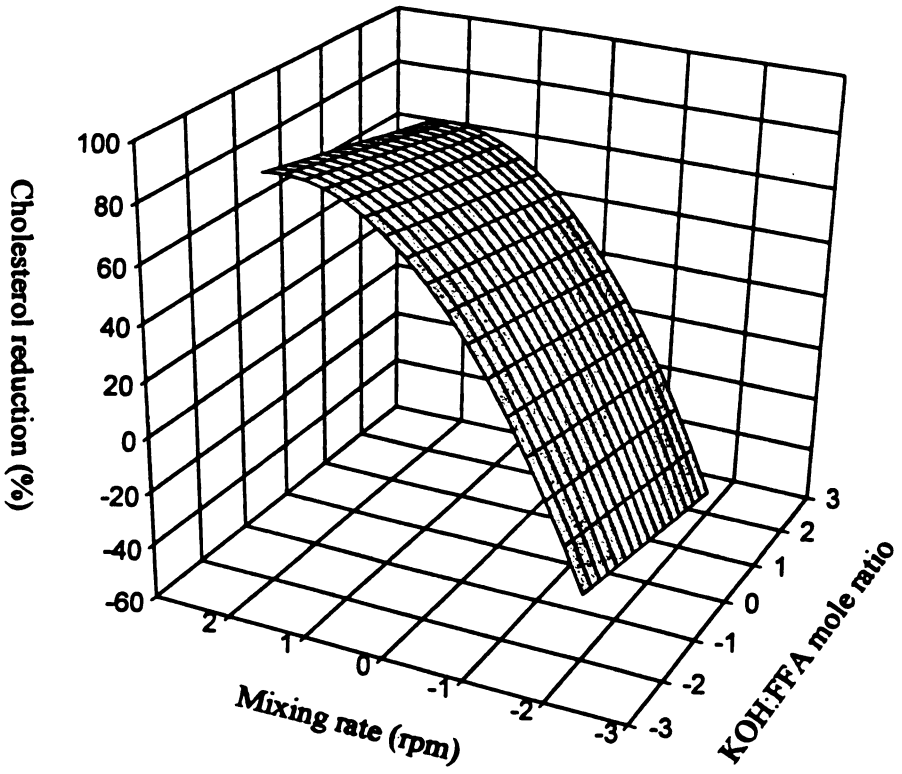


Figure 2- Response surface methodology for percent reduction of cholesterol (Y1) in anhydrous milkfat using a mixing time of 10 min and a β-cyclodextrin:cholesterol mole ratio of 5.58.

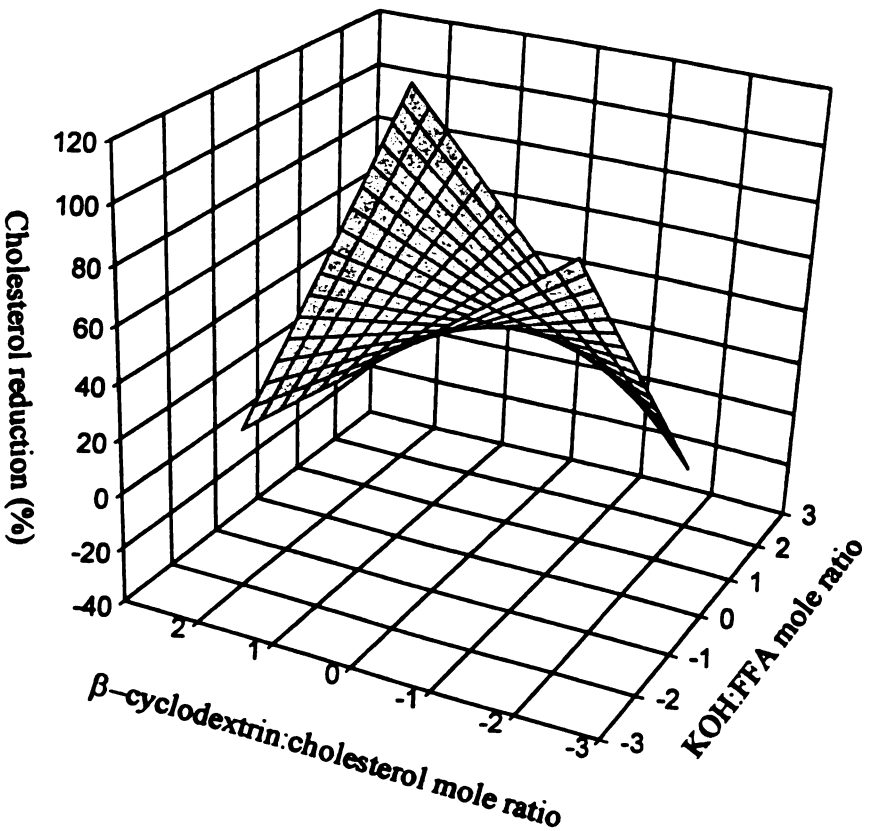


Figure 3- Response surface methodology for percent cholesterol reduction (Y1) in anhydrous milkfat using a mixing time of 10 min and a mixing rate of 1000 rpm.

Calcium salts are carboxylates acceptors. They react with the soluble potassium soaps to form insoluble calcium soaps, thus counteracting the detrimental effect of the soap micelles on cholesterol reduction as shown in runs 18 and 19 (Table 2).
The $CaCl_2$:FFA mole ratio used within the experimental domain had no significant effect on the model. The range of $CaCl_2$:FFA mole ratios tested is shown in Table 2.

4.4.2.2 Mixing time

Cholesterol reduction (Y1) was strongly dependent on mixing time. The positive slope, $B_3 = 10.76$, indicated that that cholesterol reduction increased with increased mixing time at any β-cyclodextrin:cholesterol mole ratio or any mixing rate (Figures 4 and 5). The range of mixing times examined is summarized in Table 2.

4.4.2.3 β-CD:cholesterol mole ratio

Theoretically, 1 mole of β-CD will complex 1 mole of compound (Szejtli, 1982a). Some larger molecular weight compounds may complex with more than one cyclodextrin molecule (Szjetli, 1982a). The range of β- CD:cholesterol mole ratios tested is shown in Table 2. Cholesterol reduction was strongly dependent on the β-CD:cholesterol mole ratio. The positive slope $B_4 = 4.97$, indicated that cholesterol reduction increased with increased β-cyclodextrin:cholesterol mole ratio (Figure 6).

4.4.2.4 Mixing rate

Mixing is necessary to increase the contact between the β-cyclodextrin molecules and cholesterol molecules. The greater the contact the greater the rate of complex

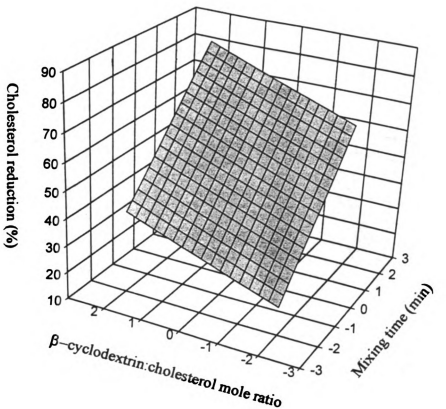


Figure 4- Response surface methodology for percent cholesterol reduction (Y1) in anhydrous milkfat using a KOH:FFA mole ratio of 1 and a mixing rate of 1000 rpm.

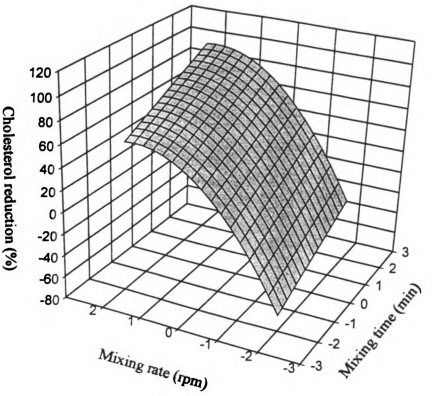


Figure 5- Response surface methodology for percent cholesterol reduction (Y1) in anhydrous milkfat using a KOH:FFA mole ratio of 1 and a β-cyclodextrin:cholesterol mole ratio of 5.58.

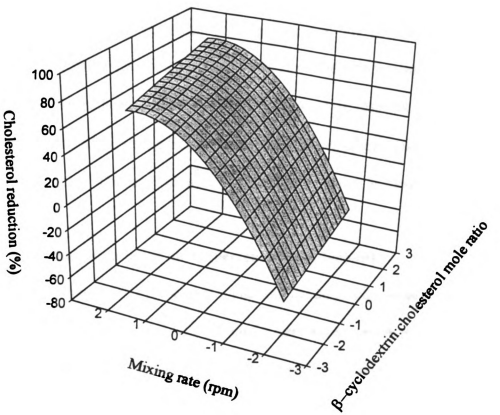


Figure 6- Response surface methodology for percent cholesterol reduction (Y1) in anhydrous milkfat using a KOH:FFA mole ratio of 1 and a mixing time of 10 min.

formation between cholesterol and β–cyclodextrin molecules (Awad et al., 1996). Moreover, mixing helps stabilize the oil-in-water emulsion. As soon as the temperature reached 50 °C, β-cyclodextrin was added and mixed for 10 min. The cholesterol reduction was strongly dependent on the mixing rate. The positive slope $B_5 = 30.44$ strongly indicated that cholesterol reduction increased with increased mixing rate at any β- cyclodextrin:cholesterol mole ratio . The shape of the response surface (Figure 6) was characteristic of the strong interaction between β-cyclodextrin:cholesterol mole ratio and the mixing rate. The range of mixing rates tested is shown in Table 2.

4.4.3 Optimization based on Y2(percent FFA reduction).

The model (Y2) is useful for indicating the direction-to-change variables to maximize FFA reduction. Judging from the significant regression coefficients (Table 5), the most important factors influencing FFA reduction are KOH:FFA mole ratio, $CaCl_2$:FFAs mole ratio, and mixing rate. They showed significant first order, quadratic, and two factor interaction terms.

4.4.3.1 KOH:FFA mole ratio

The diluted alkali metal hydroxide solution of known concentration (KOH:FFA mole ratio of 1) reacts with FFAs as follows:

$$RCOOH + KOH \longrightarrow RCOOK + H_2O$$

The amount of KOH used is a function of the FFA concentration in anhydrous milkfat. A KOH:FFA mole ratio of 1 is enough to neutralize all the FFAs present in the system

under the experimental conditions described in the preliminary process. An excess of alkali solution is detrimental to the yield due to loss of neutral fat by saponification (Braae, 1976) and to the flavor because of hydrolysis of lactones to their corresponding hydroxy acids. Reduction in FFA content was strongly dependent on the KOH:FFA mole ratio. The positive slope, $B_1 = 24.58$ (Table 5) indicated that FFA reduction increased with increased KOH:FFA mole ratio at all mixing rates (Figure 7). The range of KOH:FFA mole ratios examined is shown in Table 2.

4.4.3.2 Calcium chloride:FFA mole ratio

Calcium chloride was used as a carboxylate acceptor. Its addition to the refining solution is critical due its ability to:

(1) react with the carboxylates to form insoluble salts similar to limestone. Their separation from the system by centrifugation is therefore very efficient;

(2) react with the soluble potassium or sodium soaps to form insoluble soaps and minimize the losses of neutral fat by emulsification, thus improving the yield;

(3) destroy the soap micelles and minimize the floculation of the β-cyclodextrin on the electronegatively charged micelle surface, thus improving cholesterol reduction (Table 2);

(4) synergistically increase the reduction of FFA in conjunction with KOH;

(5) saturate the system with cations and thus improve FFA reduction by prohibiting the occurrence of the following reaction:

$$RCOO^- + H_2O \rightleftharpoons RCOOH + OH^-$$

The excess of cations should be provided by the calcium salts and not by the

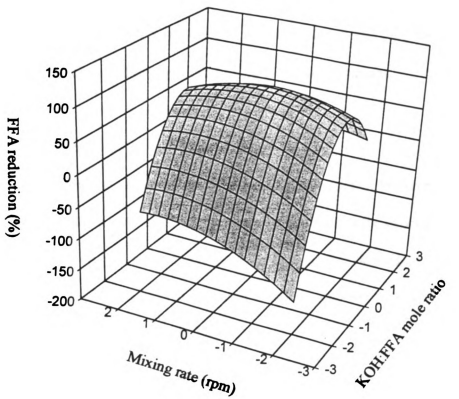


Figure 7- Response surface methodology for FFA reduction (Y_2) in anhydrous milkfat.

caustic solution due to the detrimental effect of excess hydroxyl groups on yield and flavor.

The range of $CaCl_2$:FFA mole ratios examined is shown in Table 2. The effect of calcium chloride on FFA reduction was confounded by the β-cyclodextrin present in the system, since β-cyclodextrin also reduce FFA concentrations.

4.4.3.3 Mixing rate

Mixing rate is an important factor to assure contact between the solutes of the refining solution and FFAs. The mixing rate used within the experimental range had a slight effect on FFA reduction (Figure 7). The positive slope $B_5 = 5.28$ (Table 5) indicated that FFA reduction increased with increased mixing rate. The range of mixing rates tested is shown in Table 2.

4.4.4 Process evaluation

The model for Y1 showed that the greater the β-cyclodextrin:cholesterol mole ratio, mixing time, and mixing rate, the greater the percentage of cholesterol reduction. The joint effect of these three independent variables used in runs 4 and 26 (Table2) reduced cholesterol in milkfat by 77.3 and 73.7%, respectively. Further optimization of these treatments may improve overall cholesterol reduction. The amount of β-cyclodextrin used in run 4 (Table 2) was greater than the amount used in run 26 (Table2).

Taking into account the cost-effectiveness of the process, further optimization was performed on run 26 (Table2) with regard to mixing time, as increasing the amount

of β-cyclodextrin is not economically feasible. In addition, use of higher mixing rates may increase the viscosity of the medium which could cause problems during centrifugation. It was found that the reduction of cholesterol could be further improved by increasing the mixing time. In fact, the theoretical mathematical model for cholesterol reduction showed that the cholesterol could be completely removed with 35 min of mixing. The observed cholesterol reduction with 35 min of mixing was 95.8% (Figure 8).

4.4.5 Effect of anhydrous milkfat on blood lipid levels

The effects of diet on circulating lipids in blood were among the earliest observations of factors influencing lipid metabolism and the atherogenic process. A voluminous research literature has accumulated over the past 75 years on the relationship between diet, lipoproteins, atherogenesis, and coronary heart disease (CHD). The major conclusions of this body of research are that (1) the higher the total blood cholesterol level, the greater the severity of atherosclerosis and the greater the risk for CHD, (2) dietary saturated fat and cholesterol raise total blood cholesterol and low density lipoprotein (LDL) cholesterol levels, and (3) polyunsaturated fat lowers total blood cholesterol and LDL cholesterol levels (Howell et al., 1997).

Although the synthesis of cholesterol by the liver is under feedback regulation from dietary cholesterol, a reduction of dietary cholesterol intake will cause a net plasma cholesterol decrease despite increased endogenous synthesis (Hegsted, 1986). The ability with which dietary cholesterol suppresses endogenous cholesterol synthesis varies greatly among individuals (Quintao and Speretto, 1987). Dietary cholesterol appears to suppress LDL receptor function in humans (Appelbaum-Bowden et al. 1984).

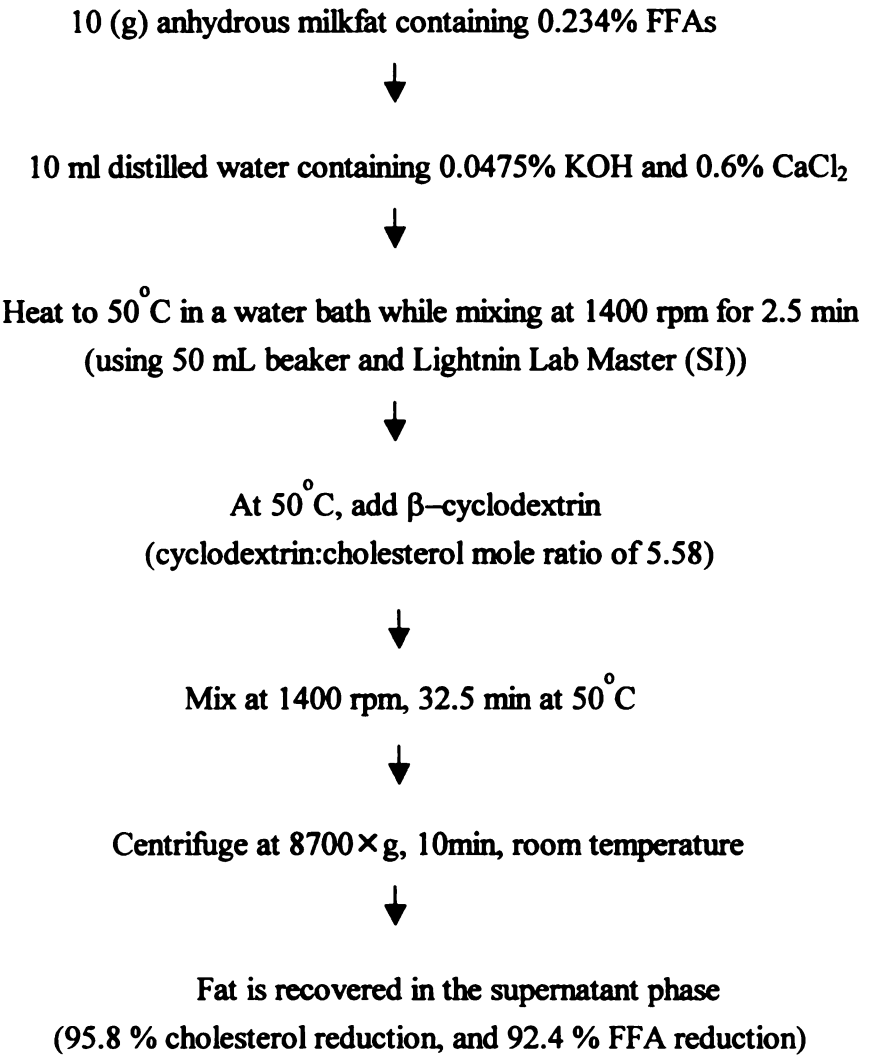


Figure 8- Optimized process to remove cholesterol and FFA in anhydrous milkfat. The number of moles of KOH and $CaCl_2$ added is function of the number of moles of FFA originally present in AMF. The mole ratio of KOH:FFA used is 1:1. The mole ratio of $CaCl_2$:FFA used is 5:1.

Most animal species are more sensitive than humans to dietary cholesterol and develop high plasma cholesterol concentrations and aortic lesions that resemble human atheroma (McGill, 1979a; 1979b).

The results of the present study confirm previous observations of Hegsted (1986) that dietary cholesterol does affect blood lipids. In fact, as can be seen from Figure 9, when gerbils were fed a modified milkfat, total plasma cholesterol and triacylglycerols were reduced by 28 and 47%, respectively.

There are side effects to any intervention. The purpose of dietary therapy is to reduce LDL cholesterol levels, but dietary therapy could have other effects that are undesirable. For example, a cholesterol-lowering diet, while lowering LDL cholesterol levels, will also lower HDL cholesterol levels (Denke, 1993). The decrease in HDL cholesterol observed in this study (Figure 9) is mainly due to the fact that HDL represents the major transport system for cholesterol in gerbils (Hajri et al., 1998).

4.5 CONCLUSION

The process proposed in this study has a number of attributes which could result in considerable practical advantages over the prior-art processes used for reducing cholesterol and FFAs in milkfat. The process is efficient since the cholesterol was almost completely (>98%) removed. Free fatty acid reduction was greater than 92.40 %, which was more than 45 % greater than the prior-art processes. The process is simple and efficient in removing cholesterol. The process can be done in a closed system to protect anhydrous milkfat from exposure to air to minimize oxidative deterioration. This process does not require expensive specialized equipment. It requires only heating and mixing

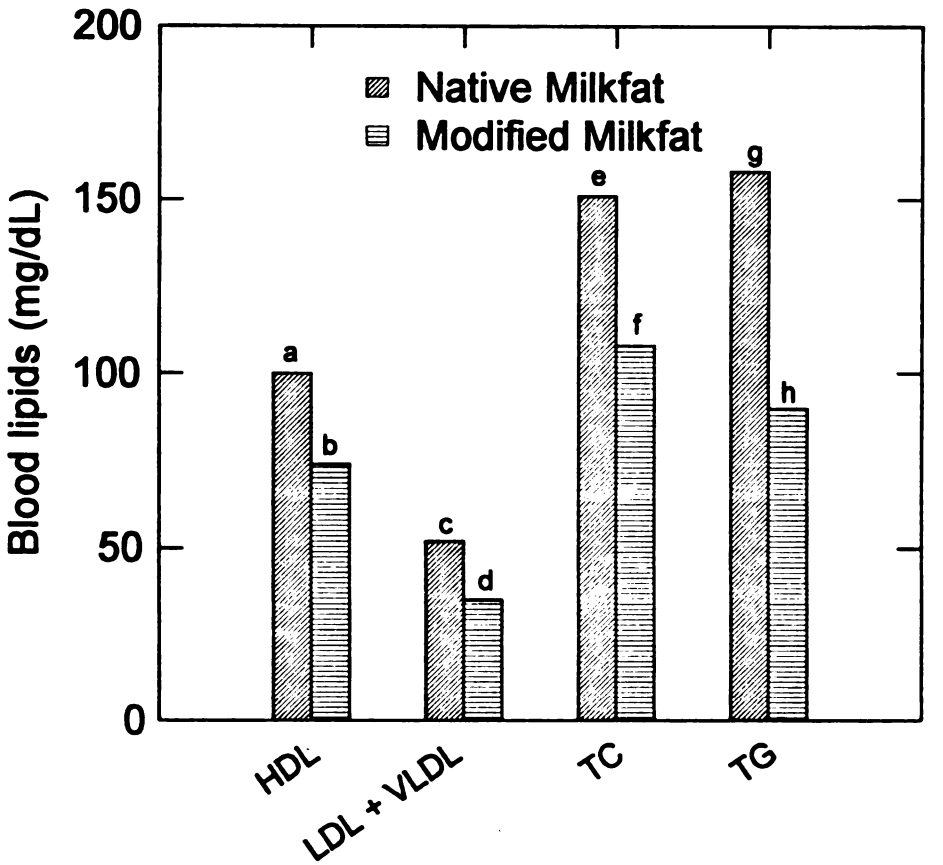


Figure 9- Blood lipid response of gerbils to modified milkfat.
[a-i] Means with no common superscript are significantly different ($p<0.05$).

TC: total cholesterol
HDL: high-density lipoprotein
LDL: low-density lipoprotein
TG: triglycerides

followed by centrifugation. Modified milkfat has all the benefits of native milk fat with one exception, it does not increase blood cholesterol and therefore decreases the risk of heart disease.

Modified milkfat is great for cooking, baking, toppings and spreading. Also, it could be used for whole milk reconstitution and consequently the whole spectrum of dairy products including ice cream, yogurt, and cheese.

SUMMARY AND CONCLUSIONS

It is in the area of consumer needs that we encounter some of the most difficult problems in fostering the rational development of food technologies. One key area of development is the modification of animal fats, particularly dairy products, to satisfy the changing dietary habits of consumers. Concerns about cholesterol and saturated fat originate from the fact that high serum cholesterol, especially LDL, is one of the risk factors associated with atherosclerosis. This study addressed alternatives to hypercholesterolemic anhydrous milkfat by: (1) developing an enzymatic process to convert the hypercholesterolemic myristic acid to myristoleic acid using the Δ^9-desaturase system of hen hepatic microsomes, (2) and reducing the cholesterol content of animal fats by chemical means, followed by their blending them with vegetable oils.

The first part of the study aimed at identifying the physiological role of the hepatic Δ^9-desaturase and the way in which this enzyme is influenced by solubilzing proteins such as BSA and FABPs, pH, temperature, enzyme concentration, and different mixing rates of the incubation system. Although most of the available evidence implicates the CoA ester of the fatty acid as the substrate for desaturation, direct desaturation of the fatty acid without prior activation to the CoA derivative was possible when the incubation medium was mixed at mixing speeds greater than 250 rpm in the presence of FABPs. Experimental evidence has been presented to show

that the variation in molecular structure between myristoyl-CoA and myristic acid:FABP complexes gives rise to variation in desaturation activity. CoA and FABP are guiding molecules. There are positive influences pulling reactants, but negative repulsion plays a major role in the way reactants are guided. Attraction and repulsion forces can act over short-and long-ranges. Short-range requires contacts with membrane bound Δ^9-desaturase, and long-range interactions occur by diffusible factors that set up gradients. Unraveling the mechanism of these interactions is complicated by the fact that guidance molecules can have bifunctional roles. The same molecule can be attractive or repulsive, short- and long-range.

The second part of this study aimed at developing a process to reduce cholesterol and free fatty acids in anhydrous milkfat. The process to reduce cholesterol in anhydrous milkfat is based on the specific affinity of β-cyclodextrin for free cholesterol and cholesterol esters. Other processes have been proposed in the U.S. and overseas, including diet alteration, solvent extraction, and adsorption with β-cyclodextrin. A major disadvantage of the solvent approach is the deterioration of the fine flavor components of milkfat. The process demonstrated in this study has a number of attributes that resulted in considerable practical advantages over other cholesterol-reduction technologies. The process is efficient since the cholesterol was almost completely (>98%) removed. Free fatty acid reduction was greater than 92.40 %, which was more than 45 % greater than other prior-art processes. The process can be done in a closed system to protect anhydrous milkfat from exposure to air to minimize oxidative deterioration. This process does not require expensive and

specialized capital equipment. It requires only heating and mixing followed by centrifugation. Modified milkfat has all the benefits of native milkfat and it does not increase blood cholesterol. Instead, it actually lowers it and therefore decreases the risk of heart disease. It is great for cooking, baking, topping and spreading. Also, modified milk fat could be used for whole milk reconstitution and consequently the whole spectrum of dairy products such as ice cream, yogurt, and cheese.

FUTURE RESEARCH

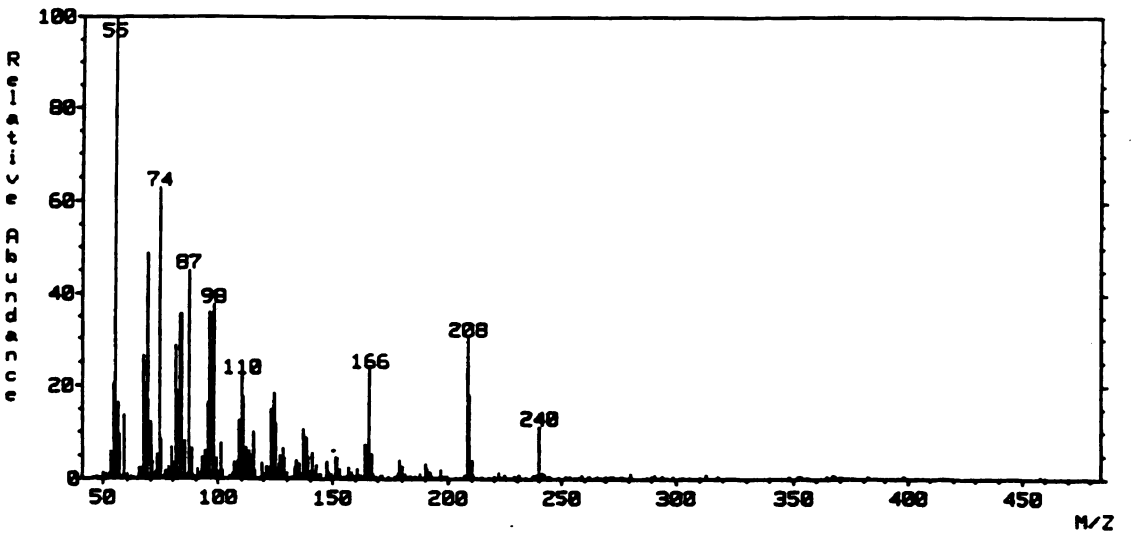
Some suggestions for future research are listed below:

1- Bridging the gaps in our understanding of unsaturated fatty acid biosynthesis, namely the detailed chemistry of desaturation reactions and the biochemical control mechanism that influence the activity of the enzymes involved.

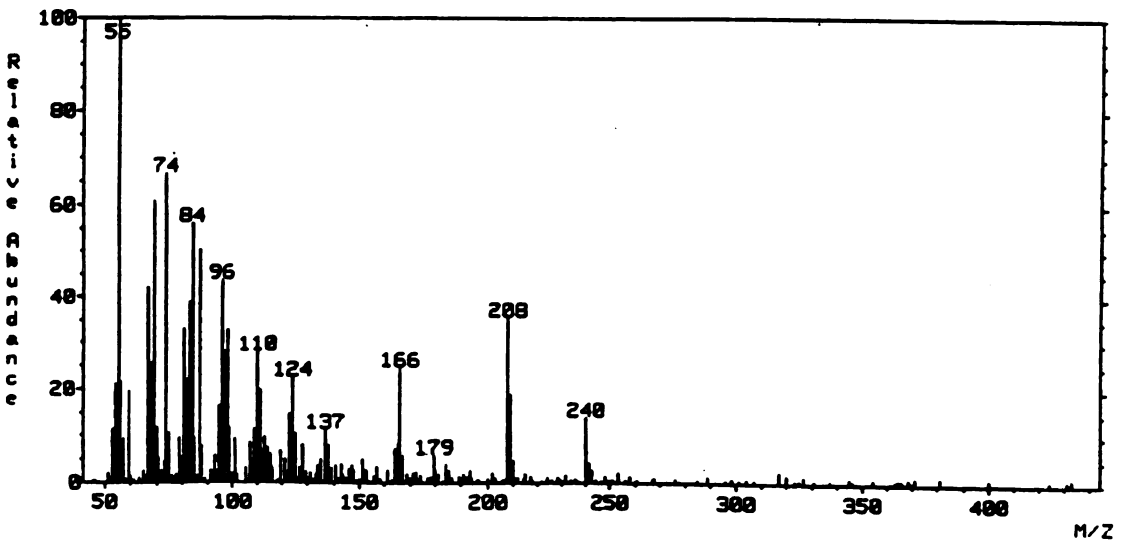
2- The mechanism by which alterations in dietary fatty acids affect atherogenesis and the risk of coronary heart disease, including degree of saturation, chain length, and fatty acid series.

3- Clarification of the role of dietary cholesterol and cholesterol oxide products in atherogenesis, including variability in response, effects on cholesterol metabolism in both fating and postprandial states, and interactions of postprandial lipoprotein remnants with cells of the arterial wall.

Appendix 1



Mass spectrum of authentic standard *cis*-9-C14:1



Mass spectrum of sample *cis*-9-C14:1 produced by the desaturation of C14:0 by hen liver desaturase system.

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