ENZYMIC ASPECTS OF FATTY ACID UPTAKE AND ESTERIFICATION BY THE BOVINE MAMMARY GLAND

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ABSTRACT

ENZYMIC ASPECTS OF FATTY ACID UPTAKE AND ESTERIFICATION BY THE BOVINE MAMMARY GLAND

by

Eldon Wayne Askew

In-vitro assay systems were developed to allow the measurement of lipoprotein lipase (EC 3.1.1.3) and glyceride synthetase (EC 6.2.1.3, EC 2.3.1.15, EC 3.1.3.4, EC 2.3.1.2) activity in bovine mammary tissue. Certain aspects of fatty acid uptake and esterification were studied prior to investigating the involvement of these enzymes in a metabolic aberration of bovine lipid metabolism, milk fat depression.

Lipoprotein lipase activity in bovine mammary gland exhibited characteristics similar to those reported for other tissues. The majority of the subcellular lipolytic activity was associated with the particulate fraction of the cell and was strongly dependent upon prior activation of the coconut oil substrate with serum. A lipase with properties similar to tissue lipoprotein lipase comprised the majority (80%) of milk lipase activity toward serum-activated coconut oil.

Lipoprotein lipase activity was present in lactating tissue, but absent in non-lactating tissue.

The majority of the subcellular fatty acid esterifying activity was associated with the particulate fraction of the cell. Fatty acid esterification was strongly dependent upon ATP, CoA, α-GP, and Mg^{++}. The system was also stimulated by NaF, dithiothreitol, and bovine serum albumin. Although palmitate, stearate, oleate, and linoleate were all esterified at rates consistent with their content in milk fat, butyrate was poorly esterified by this system. The poor rate of butyrate esterification plus the inability of this system to form greater than 58% triglyceride agreed with the suggestion that bovine mammary tissue may require a short chain fatty acid for a third acylation in milk fat synthesis. Certain combinations of fatty acids were partially additive in their combined esterifications. Stearic acid was particularly complimentary to the esterification of oleic and palmitic acids. Unlabelled *trans* vaccenic acid did not compete with palmitate-1-^{14}C in the esterification process as efficiently as unlabelled oleic acid, indicating that mammary gland enzymes may preferentially esterify the *cis* isomer of C-18:1. Linoleic acid behaved differently than the other acids tested. Although poorly esterified itself, linoleate also inhibited the *in-vitro* esterification of other fatty acids.

In-vitro mammary gland lipoprotein lipase and glyceride synthetase activities were not significantly different when cows were fed normal, restricted roughage-high grain or restricted roughage-high grain plus MgO rations. However, fatty acid compositional studies of mammary lipids and cream suggested that a much different array of long chain fatty acids was being presented to mammary enzymes of cows fed restricted roughage-high grain rations. Extention of *in-vitro* studies to *in-vivo* fatty acid compositional changes suggested three possible mechanisms whereby mammary gland fatty acid esterification might be decreased in cows fed restricted roughage-high grain rations: 1) A stearic acid deficiency may exist, resulting in reduced esterification of other acids; 2) An excess of the *trans* isomer of C-18:1 may be presented to the mammary gland. This isomer may not be esterified as well as the *cis* isomer; 3) An increase in the concentration of linoleic acid in mammary tissue FFA may be inhibitory to the esterification of other fatty acids.

The highly ordered structure of milk fat triglycerides and the marked shift in composition of the long chain fatty acids presented to the mammary gland under the conditions of milk fat depression together with observed *in-vitro* fatty acid specificities suggested that restricted roughage-high grain rations may impair fatty acid utilization by the mammary gland.

The net result may be reduced utilization of a non-ideal array of long chain fatty acids by the mammary gland for milk fat synthesis.

ENZYMIC ASPECTS OF FATTY ACID UPTAKE AND ESTERIFICATION BY THE BOVINE MAMMARY GLAND

By

Eldon Wayne Askew

A THESIS

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and
Institute of Nutrition

1969

DEDICATION

This thesis is dedicated to the author's grandmother, Mrs. Carrie Askew, who fostered in the author an early desire to read and fish.

ACKNOWLEDGEMENTS

The author wishes to express appreciation to Dr. J. W. Thomas and Dr. R. S. Emery for guidance in conducting this research. Appreciation is also extended to guidance committee members Dr. H. A. Tucker, Dr. L. Dugan and Dr. W. W. Wells. The assistance of Dr. W. D. Oxender in obtaining surgical biopsy samples for this study is appreciated. Various aspects of this study were greatly facilitated by the cooperation of colleague J. D. Benson.

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VITA

E. W. Askew was born August 23, 1942, in Pontiac, Illinois, the son of Eldon and Elizabeth Askew. He was raised on a dairy and grain farm in central Illinois. He was graduated from Fairbury-Cropsey Community High School, Fairbury, Illinois, in 1960. He was awarded the Bachelor of Science degree in Agricultural Science in 1964 and the Master of Science degree from the University of Illinois in Feburary 1966. Research for the Master of Science degree in dairy nutrition was conducted under the guidance of Dr. K. E. Harshbarger.

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The author is a member of Alpha Zeta, Gamma Sigma Delta, and Sigma Xi.

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CHAPTER I

INTRODUCTION

The ruminant mammary gland exhibits a unique type of lipid metabolism. Such characteristics as accelerated lipid metabolism at parturition, synthesis of large quantities of short chain fatty acids, and the synthesis of a product containing > 98% triglycerides make the mammary gland an ideal tissue for investigating the regulation of glyceride synthesis.

Alterations in mammary gland lipid metabolism in response to dietary manipulation of ration components also provides a further method for studying control of lipid metabolism. A dietary manipulation that influences the yield and composition of milk fat is the feeding of restricted roughage-high grain rations. Although not all animals respond the same, the percent and yield of fat in the milk usually begin to decline within days following the feeding of such rations. The biochemical mechanism responsible for decreased lipid secretion by the mammary gland under the conditions of milk fat depression is unknown.

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In addition to decreasing the yield of milk fat, the feeding of restricted roughage-high grain diets causes major changes in the fatty acid composition of blood and milk fat. The proportion of long chain unsaturated fatty acids (oleic, linoleic) increases while saturated fatty acids (palmitic, stearic) decrease (Beitz and Davis 1964, Davis and Sachan 1966). A major (40-60%) portion of the fatty acid content of milk fat is provided by the long chain fatty acids of blood lipids. These fatty acids serve as substrates for the enzyme lipoprotein lipase and the enzymes of the triglyceride synthetase complex. Although substrate specificity for these enzymes has not been demonstrated, the non-random distribution of fatty acids in milk fat triglycerides suggests a highly ordered biosynthetic pathway (Breckenridge and Kuksis 1967). Alterations in long chain fatty acids presented to the mammary gland may preclude normal uptake and/or esterification of these fatty acids into milk fat. Thus a ration induced alteration of substrate presented to the mammary gland may be responsible for the reduction in milk fat yield observed under the conditions of milk fat depression.

Enzymic aspects of fatty acid uptake and esterification have not been characterized in bovine mammary tissue. It was therefore necessary to devise *in-vitro* assay systems that would permit measurement of enzyme response to ration. Basic

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biochemical data provided by such studies should further the understanding of glyceride biosynthesis by the bovine mammary gland, even if these enzymes could not be implicated in milk fat depression. The results reported herein bear upon:

1) Devising *in-vitro* assay systems to measure the activity of lipoprotein lipase and triglyceride synthetase,

2) Partial characterization of some of their biochemical properties,

3) Measurement of their activities in response to restricted roughage-high grain rations, and

4) Relating *in-vitro* observations to *in-vivo* metabolic occurrences.

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CHAPTER II

REVIEW OF LITERATURE

This review will be introduced by a brief discussion of lipid digestion and absorption. Characteristics of two enzymes active in the metabolism of absorbed lipids, lipoprotein lipase and triglyceride synthetase, will be discussed. The metabolism of long chain fatty acids (LCFA) by a specific organ, the mammary gland, will be discussed, integrating the previous aspects of the review. Finally the topic of milk fat depression will be introduced, presenting some of the current theories of mechanisms involved.

A. LIPID ABSORPTION AND DIGESTION

The daily intake of dietary fat by the cow is of the same order of magnitude as the daily output of fat in the milk (Moore and Steele 1968). Although lipids of common feedstuffs are high in unsaturated C-18 fatty acids, lipids of ingesta leaving the rumen are markedly more saturated (Garton 1961) indicating extensive ruminal hydrogenation. Micro-organisms of the rumen can effect extensive changes in dietary lipids (Garton 1961, 1969), including hydrolysis of glycerides and

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phospholipids, hydrogenation of unsaturated fatty acids, and fermentation of glycerol to volatile fatty acids (VFA). Hydrolysis of glyceride fatty acid can proceed rapidly to completion in the rumen as a result of bacterial lipase action (Garton 1969). Fatty acids must be free in the rumen before they can be hydrogenated (Patton and Kesler 1967). Evidence cited by these authors was the higher degree of unsaturation of the neutral lipids of feed and rumen ingesta compared to free fatty acids (FFA) of rumen ingesta.

Hydrogenation of Dietary Lipids by the Rumen

Measurement of the iodine numbers of dietary and ruminal lipids (Shorland et al. 1955) provided the first direct evidence that dietary lipids were hydrogenated by the rumen. Shorland et al. (1955) reported that more than 50% of dietary linolenic acid was hydrogenated to stearate. These investigators also reported that *trans*-unsaturated fatty acids were formed in the rumen. These *cis-trans* fatty acid isomers are characteristic of ruminant fats (Garton 1961) and can comprise approximately 10% of ruminant depot fat as well as 35% of milk fat (Tove 1965). Shorland et al. (1957) incubated oleic, linoleic, and linolenic acids with sheep rumen contents and found that 20% of each acid was completely saturated to stearate, while 17, 48, and 67% of each acid, respectively was converted to *trans* isomers.

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Although ruminant bacteria and protozoa are both involved in hydrogenation of dietary lipids (Garton 1965), protozoa are believed to be especially effective in this respect (Gutierrez et al. 1962). *Trans* isomers are believed to be intermediates in the metabolic sequence of events of ruminal hydrogenation (Ward et al. 1964, Kemp and Dawson 1968). Biohydrogenation apparently does not occur in any portion of the digestive tract except the rumen (Bath and Hill 1967).

Post Ruminal Lipid Digestion

Once liberated from ester form and hydrogenated, long chain fatty acids pass from the rumen without further alterations. Negligible degradation of long chain fatty acids (LCFA) occurs in the rumen. No evidence exists for absorption from the rumen of fatty acids having greater than sixteen carbon atoms (Garton 1969).

Little change takes place in the fatty acid composition of digesta lipids during passage through the abomasum (Bath and Hill 1967). Microbial disintegration occurs in the abomasum, liberating their structural lipids. The pattern of lipid composition and distribution changes as abmosal digesta enters the upper part of the small intestine (Leat and Hall 1968). Digestive secretions of bile lipids having a high phospholipid content is responsible for these changes (Garton 1969). Due primarily to the high content of unsaturated C-18

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fatty acids, jejunal contents have a higher degree of unsaturation than rumen or abomasal digesta (Lennox et al. 1968). The absorption of long chain fatty acids, hydrolysis and subsequent uptake of esterified fatty acids has been shown to occur from the middle and lower jejunum (Lennox and Garton 1968). Fat absorption by the ruminant may be different from that occurring in the monogastric. The monogastric utilizes monoglycerides, important products of fat digestion, to promote the solubility of LCFA in bile salt micelles (Senior 1964). However, monoglycerides have not been detected in the intestinal contents of the sheep (Leat and Harrison 1967) or the cow (Leat and Hall 1968). Lysolecithin from biliary sources is present in high concentrations in ruminant intestinal contents and may replace the function of monoglyceride in promoting fat solubility (Leat and Harrison 1967, Leat and Hall 1968). Although re-esterification of absorbed fatty acids by intestinal mucosa occurs predominantly via the monoglyceride pathway in non-ruminants (Mattson and Volpenhein 1964), the absence of monoglycerides in the small intestine of the cow and sheep implies that this pathway is of minor significance in the ruminant (Leat and Hall 1968). Skrdlant et al. (1969) have found evidence for the existence of both pathways in calf intestinal mucosa. Leat and Cunningham (1968) found an active monoglyceride pathway in gut loops of the sheep, but Bickerstaffe and Annison (1968) found monoglycerides to be ineffective precursors of triglycerides in sheep intestinal

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Transport and Removal of Absorbed Lipids

Lipid transport in the ruminant is believed to occur similarly to lipid transport in the non-ruminant. Long chain fatty acids enter the circulatory system via the thoracic duct in the form of lymph chylomicrons. These chylomicrons consist principally of triglycerides (Felinski et al. 1964, Leat and Hall 1968, Wadsworth 1968). Phospholipids of chylomicrons play an important role in transport of unsaturated fatty acids (Leat and Hall 1968). Lymph cholesterol esters are quantitatively unimportant in fatty acid transport (Hartman and Lascelles 1966, Leat and Hall 1968, Wadsworth 1968) which is contrary to earlier reports (Duncan and Garton 1962). Although precise quantitative evidence is lacking in the bovine, about one-third of chylomicron triglyceride is absorbed by the liver, one-third by adipose tissue, and the rest by other tissues including the mammary gland (Felinski et al. 1964, Di Luzio 1960, Robinson 1963b). Fatty acids either taken up from chylomicron triglyceride or FFA mobilized from adipose tissue are incorporated into lipoproteins by the liver. These relationships have been summarized by Tove (1965). The majority of triglycerides synthesized by the liver re-enter the plasma as low density ($d < 1.019$) lipoproteins (Robinson 1963b).

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Triglycerides of chylomicron and low density lipoproteins are removed from the circulating blood lipids by the action of the enzyme lipoprotein lipase.

B. LIPOPROTEIN LIPASE

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Robinson (1963b) proposed that the lipase released into blood after the injection of heparin be termed "clearing factor lipase", referring to its ability to "clear" the turbidity of lipemic plasma. Korn (1959) favored the term "lipoprotein lipase", since the action of this enzyme is upon protein-bound triglycerides in plasma.

The presence of lipoprotein lipase (LPL) in tissues was first demonstrated by Korn (1959) when he found that acetone powders of rat heart tissue contained a lipase with the clearing properties of post-heparin plasma. Lipoprotein lipase has since been found in extracts of adipose tissue, spleen, lung, kidney medulla, aortic-wall tissue, diaphram, and lactating mammary gland (Robinson 1963b). The passage of chylomicron triglycerides from the bloodstream into extra-hepatic tissues (Robinson 1965) is believed to be facilitated by their hydrolysis to free fatty acids which are known to leave the blood at an extremely rapid rate (Fredrickson and Gordon 1958). This hydrolysis is thought to be due to the action of the enzyme lipoprotein lipase acting at a site close to the blood capillary wall.

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Robinson (1959) suggested that since LPL functions in the uptake of lipoprotein triglycerides from circulation, localized changes in concentration of this enzyme at the tissue level might play an important regulatory role in fat transport. If uptake of triglyceride fatty acids by tissues is dependent upon their prior hydrolysis, then LPL must control, at least locally, the distribution of fatty acids to the tissue. A number of situations have been described relating LPL activity to triglyceride fatty acid uptake (Bragdon and Gordon 1958, Bezman et al. 1962, McBride and Korn 1963, Robinson 1963a, Rodbell and Scow 1965, Brown and Olivecrona 1966, Garfinkel et al. 1967, Otway and Robinson 1968). Lipoprotein lipase is one of the most adaptive of animal enzymes (Nikkila and Pykalisto 1968) and has been used as a model for studies of enzyme regulation. The activity of this enzyme in certain tissues has been shown to decrease upon fasting (Cherkes and Gordon 1959), acute exercise (Nikkila et al. 1963), in experimental diabetes (Kessler 1963, Schnatz and Williams 1963), and cessation of lactation (McBride and Korn 1963, Robinson 1963a). The activity of LPL has been shown to increase in certain tissues upon refeeding after starvation (Robinson 1963b) and at parturition (McBride and Korn 1963, Robinson, 1963a).

Lipoprotein lipase has an extremely rapid turnover (Wing, Salaman, and Robinson 1966, Wing, Fielding, and Robinson 1967,

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Nikkila and Pykalisto 1968, Wing and Robinson 1968), and under suitable conditions the enzyme can be synthesized *in-vitro* (Salaman and Robinson 1966).

Although lipoprotein lipase is believed to be the same enzyme in all tissues, its activity in a particular organ or tissue within an animal can vary independently of its activity in other tissues of the same animal. The reason for differential tissue or organ LPL activity is not known but probably is related to hormonal and/or metabolite effectors which in turn may be related to the physiological and nutritional state of the animal. An illustration of differential LPL activity is provided by the lipemia of pregnancy. The concentration of triglyceride fatty acid in the plasma of the pregnant rat increases shortly before parturition (lipemia of pregnancy) and then declines rapidly to near normal values at parturition. This is not due to increased rates of entry of triglyceride fatty acid into the circulation but to a decrease in adipose tissue LPL activity coincident with the rise in lipemia (Otway and Robinson 1968). McBride and Korn (1963) and Robinson (1963a) have observed a marked increase in mammary gland LPL activity immediately prior to parturition. Otway and Robinson (1968) have suggested that the lipemia of pregnancy may be due to diminished uptake of triglyceride fatty acids by adipose tissue and that the disappearance of the lipemia may be due to increased uptake of triglyceride fatty acids by the mammary gland.

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Physiological Function of Lipoprotein Lipase

Fat transfer hepatically and extra-hepatically is thought to occur via different mechanisms. Chylomicra are believed to pass intact from the blood through gaps in the endothelial linings of liver sinusoids (Robinson 1965), while extra-hepatic triglyceride transport from blood to tissue is believed to involve hydrolysis of triglyceride fatty acids and subsequent re-esterification in the tissue.

Role of Heparin

Patten and Hollenberg (1969) have recently shown that heparin stimulated the activity of adipose LPL in solution by binding the enzyme to its chylomicron substrate, as suggested by Korn (1959). Heparin had no effect on either the stability of the extracted enzyme or on enzyme activity after the enzyme-chylomicron complex had formed. These authors suggested that exogenous heparin activates rat adipocyte LPL by forming additional binding sites on the enzyme molecule. Endogenous heparin may also be responsible for the binding of lipoprotein lipase at the capillary wall. Heparin in tissue has been shown to exist as a molecular complex with protein (Serafini-Fracassini and Durward 1968). The appearance of LPL in the blood in response to heparin injections may be due to injected heparin competing with endogenous heparin for the enzyme, causing its release into the blood (Robinson and French 1960).

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Robinson (1967) has attempted to determine which LPL activity (that released by heparin vs. that retained by the tissue) is concerned with triglyceride fatty acid uptake and utilization. Research on the LPL of rat heart supported the view that only a proportion of the tissue enzyme (that which can be released by intravenous heparin injections) is concerned in the uptake and utilization of chylomicron triglyceride fatty acid. This LPL might be the proportion that is associated with the capillary endothelial cells. Robinson (1967) concluded that measuring LPL activity in response to heparin injections will not provide a valid estimate of total tissue enzyme may indicate the portion of the enzyme that is physiologically active.

Locus of Lipoprotein Lipase

The locus of LPL under physiological conditions has not been definitely established although it is assumed to be located at the surface of the capillary endothelial cells. Chylomicra in the blood have been observed to apply themselves closely to the luminal surface of endothelial cells of tissue rich in the enzyme (Robinson 1963b). The rapid release of LPL upon heparin administration suggests that LPL is located in or very close to the vascular bed (Ho et al. 1967). Recent evidence has shown that adipose tissue LPL is found in the adipocytes themselves and not in the surrounding stromal vascular components (Rodbell 1964, Pokrajac et al. 1967,

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Patten and Hollenberg 1969, Nestel et al. 1969). However, in the previously mentioned studies collagenase was used to separate adipocytes from stromal vascular components and collagenase has been shown to inactivate stromal vascular LPL (Pokrajac et al. 1967, Cunningham and Robinson 1969). Cunningham and Robinson (1969) found that 80% of adipose tissue LPL was located outside the adipocyte and was inactivated by collagenase. Perhaps intracellular LPL represents newly synthesized enzyme prior to transport out of the cell to the stromal vascular network. Schoefl and French (1968) have examined by electron microscopy the small blood vessels of lactating mammary glands of rats, mice, and guinea pigs after intravenous injections of chyle or artificial fat emulsions. Chylomicra and the artificial particles were concentrated against the luminal surface of the endothelium. These particles could be seen in the vessel lumen but not in the pinocytotic vesicles or intracellular junctions. The hydrolytic action of LPL was also demonstrated histochemically by light and electron microscopy.

Specificity of Lipoprotein Lipase

Lipases in general are associated with the degradation of typical triglycerides. However, lipoprotein lipase is unlike normal lipases in that it does not, or if so, very slowly, hydrolyze triglyceride emulsions unless a lipoprotein complex is also present (Korn 1955). Lipases preferentially hydrolyze esters of long chain fatty acids (Desnuelle and

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Savary 1963). The unique characteristic of LPL is its specificity for triglycerides in the form of a natural or synthetic complex (Korn 1961). Emulsions of uncomplexed triglycerides are hydrolyzed slowly, if at all, in the presence of lipoprotein lipase.

Lipoprotein lipase is unable to hydrolyze the ester bonds of pure coconut oil. The triglycerides of coconut oil can interact with serum to form a complex that is enzymically indistinguishable from chylomicrons (Korn 1955). Although serum activated artificial triglyceride emulsions are hydrolyzed at similar rates to chylomicron triglycerides, some investigators (Rodbell and Scow 1965) have shown chylomicron triglyceride to be hydrolyzed at a faster rate. Serum albumin is not able to activate coconut oil. Serum lipoproteins are responsible for a major portion of the activation of coconut oil when it is preincubated with serum (Korn 1955). The necessity for activation of artificial triglyceride emulsions in an *in-vitro* assay system has not been adequately explained. Some investigators have reported significant *in-vitro* substrate activation by sonication without the presence of serum (Data 1963, Doizaki and Zieve 1966).

Determination of substrate specificity for LPL has been complicated by the requirement for a "lipoprotein like" substrate. Use of triglyceride emulsions of specific fatty acid compositions

is subject to many variables, such as solubility (Doizaki and Zieve 1966), degree of emulsification (Eiber et al. 1966), and extent of activation (Doizaki and Zieve 1966, Desnulle and Savary 1963) that tend to complicate the determination of fatty acid or positional specificity. Eiber et al. (1966) demonstrated a high degree of dependence of LPL upon degree of emulsion of substrate. Specificity may also be obscurred by the presence of other lipases such as tributyrinase (Bradford et al. 1968) or β-monoglyceride lipase (Biale and Shafir 1969, Payza et al. 1967, Greten et al. 1969). Another factor preventing accurate determination of fatty acid specificity is the degree of fatty acid exchange occurring between glycerides and the surrounding medium (Borgstrom and Carlson 1957, Payza et al. 1967). Some lipases will even esterify fatty acids into glyceride molecules under the proper conditions (Borgstrom 1964). Payza et al. (1967) incubated ^{14}C-stearic and oleic acids with post heparin plasma and found that both acids were incorporated into already existing di- and triglycerides. Certain triglycerides were better acceptors than others. Oleic acid exchanged faster than stearic.

Early work (Borgstrom and Carlson 1957) indicated that fatty acids esterified at the α-position of the glycerol skeleton were preferentially cleaved and those at the β-position were acted upon more slowly. Engleberg (1959) found that lipoprotein lipase hydrolyzed vegetable fats more

rapidly than animal fats. Coconut oil, safflower oil, and corn oil were all hydrolyzed at the same rate, indicating no fatty acid specificity. However, emulsification was uncontrolled in these studies. The most definitive work in this area has been carried out by Korn (1961), who investigated the specificity of chicken adipose lipoprotein lipase with respect to chain length, degree of unsaturation and the position (α or β) of fatty acids in the triglyceride molecule. Korn reasoned that if the enzyme preferentially hydrolyzed certain bonds involving specific fatty acids one would then expect these fatty acids to comprise a greater percent of the free fatty acids than of the triglycerides. As a control comparison he also degraded the chylomicrons used as a substrate with pancreatic lipase which is known not to have a fatty acid specificity and to cleave preferentially fatty acids esterified at the α-position. No positional specificity was noted, and the free fatty acid (16:0, 18:0, 18:1, 18:2) molar percentages formed were found to be the same as those of triglycerides. He concluded that LPL is similar to pancreatic lipase in having no specificity among glyceride bonds involving palmitic, stearic, oleic, and linoleic acids. Indications were also the same for capric, lauric, myristic, and palmitoleic acids, but the concentration of these in the chylomicrons was too low to obtain reliable data. Unlike pancreatic lipase, LPL hydrolyzed all three ester bonds of a triglyceride molecule at very similar rates.

In reference to Borgstom and Carlson's (1957) findings that LPL preferentially cleaves fatty acids esterified at the α-position, Korn (1961) theorized that although LPL can hydrolyze all three bonds of a triglyceride at essentially the same rate, there may be a required sequence in which the α-esters are first hydrolyzed before hydrolysis can proceed at the other positions.

Some investigators have reported that lipoproteins containing unsaturated fats were hydrolyzed or "cleaved" from circulation faster than saturated fats (Engelberg 1959, 1966, 1967, Nestel et al. 1962). Engelberg (1966, 1967) suggested that polyunsaturated fats may either increase the activity or amount of LPL or increase the "sensitivity" of endogenously of endogenously synthesized lipoproteins to lipolysis. This author advanced a "steric" theory to explain facilitation of lipolysis by unsaturated fats. Steric factors are known to play a role in hydrolytic reactions at oil-water interfaces. Lipid micelles containing saturated fatty acids are tightly packed and rigid, whereas there is less cohesion in the packing of unsaturated fatty acids due to kinks brought about by double bonds. Such steric effects of unsaturated fatty acids would theoretically tend to facilitate enzyme-substrate contact, thereby increasing the rate of lipolysis (Engelberg 1967).

Other workers have not found unsaturated fats to be cleared faster than saturated fats (Nestel and Scow 1964, Eiber et al. 1966). Contrary to Korn (1961), Eiber et al. (1966) found that triglycerides containing di- and trienoic acids were hydrolyzed at slower rates by human plasma LPL than those containing saturated fatty acids. Using emulsions of pure triglycerides Doizaki and Zieve (1966) could find no preference of human plasma LPL in hydrolysis of saturated or unsaturated esters of fatty acids from C-8 to C-18. Contrary to Doizaki and Zieve (1966), Bradford et al. (1968) using emulsions of pure triglycerides and human plasma LPL found that C-4, C-8, C-10, and C-12 fatty acids were all liberated at equal rates. Short chain acids were all liberated at greater rates than longer chain acids, which were liberated in the order of C-18:1 > C-18:2 > C-18:3 > C-14 ~ C-16 ~ C-18.

In summary, LPL has been shown to possess fatty acid and positional specificity by some investigators but no specificity by others. Due to the inherent technical problems involved in substrate preparation and possible differences between tissues used, such studies should not be regarded as unequivocal proof either for or against LPL specificity. Rigorous studies about the specificity of LPL await its further purification and more suitable substrate preparation.

Other Factors Influencing Lipoprotein Lipase Activity

Lipoprotein lipase is activated by low concentrations of heparin and inhibited by higher amounts (Korn 1962b). Heparin may stimulate tissue LPL activity by extracting the enzyme from the tissue. Conceivably the removal of LPL from its tissue locus provides stimulus for the formation of new enzyme (Wing et al. 1966). In addition to heparin, LPL from tissues requires the addition of divalent or ammonium cations and fatty acid acceptors (Korn and Quigley 1957).

Adipose and plasma LPL are more stable and more active during incubations conducted at 27°C than 37°C (Greten et al. 1968). The stability of the enzyme has also been shown to be dependent upon the ionic strength of the medium surrounding it (Fielding 1968). Whole plasma, long chain fatty acids, and heparin all stabilize the enzyme (Fielding 1968).

Actinomycin D, known to interrupt DNA dependent RNA synthesis, causes an increase in LPL activity. Garfinkel et al. (1967) proposed that actinomycin D may destroy an RNA that codes for an enzyme responsible for the destruction of LPL.

Lipase hydrolysis occurs at maximum rates only when adequately interfacial area is maintained (Wills 1965). Therefore any substance that destroys the substrate emulsion and thus reduces the interfacial area of the substrate may be classed as an enzyme inhibitor, although this is not inhibition

in the usual sense of the word. Oxidizing agents that combine with enzyme sulfhydryl groups are all thought to cause inhibition of enzymatic activity by a steric blocking effect (Wills 1965). Bacterial heparinase depresses LPL activity of post heparin plasma (Korn 1957). Lipoprotein lipase is inactivated at low ionic strengths (Fielding 1968). Both polyanions and polycations inhibit LPL by interacting with the enzyme. Korn (1926b) has speculated that this interaction may be with the protein or with an acidic mucopolysaccharide prosthetic group. Sodium chloride, a potent inhibitor of LPL, may alter the interaction between LPL and its substrate (Data and Wiggins 1964). However, protamine sulfate and sodium chloride have recently been shown to inhibit enzyme activity after formation of the enzyme-chylomicron complex (Patten and Hollenberg 1969).

Platelets contain antagonists to heparin, and may indirectly (through heparin) inhibit the enzyme (Mitchell 1959). Serum is known to contain an inhibitor of LPL which is not present in either citrated or oxalated plasma (Robinson 1963b). This serum inhibitor reduces the rate of hydrolysis as well as clearing the chylomicron triglycerides by post-heparin plasma. Certain phospholipids (phosphatidyl serine, phosphatidyl choline, cephalin) inhibit LPL (Berger et al. 1968). Following intravenous injection, cycloheximide, known to stop protein synthesis by blocking amino acid incorporation,

rapidly decreased LPL activity of heart, diaphram, lung and adipose tissue (Wing et al. 1967).

Lipoprotein lipase can be distinguished from pancreatic lipase by its sensitivity to strong salt solutions, protamine sulfate, pyrophosphate, its requirement for activated substrates, and its lack of positional specificity (Robinson 1965). Lipoprotein lipase can be distinguished from monoglyceride lipase similarly. Monoglyceride lipase is less heat sensitive, unaffected by NaCl, protamine sulfate, pyrophosphate, and is non-adaptive to radical changes in fat or carbohydrate content of the diet (Greten et al. 1969). Lipoprotein lipase can be distinguished from epinephrine sensitive lipase by cellular localization and response to heparin and epinephrine. The time period required for FFA release from adipose tissue stimulated by epinephrine is much longer than for the lipase released in response to heparin (Ho et al. 1967). Epinephrine sensitive lipase is localized in the intracellular compartment of fat cells, whereas LPL is associated with the stromal-vascular beds (Ho et al. 1967, Cunningham and Robinson 1969). Robinson (1967) has speculated that the hormonal responses of these two lipases are physiologically opposed. Insulin inhibited while noradrenaline, adrenaline, and ACTH activated the adipose lipase responsible for mobilizing stored triglycerides (Robinson 1967). These same hormones may be involved in controlling the extent of

deposition of triglyceride fatty acids in adipose tissue by exerting an opposite effect upon adipose lipoprotein lipase.

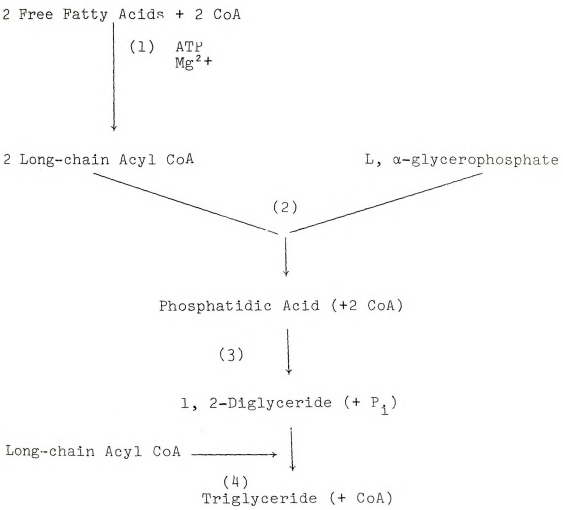
Recently LPL has been demonstrated to exist in two temperature dependent states in adipose tissue (Cunningham and Robinson 1969, Wing and Robinson 1968). Eighty percent of the total LPL activity was unstable at 37°C and existed at a site in the tissue outside the cell. Twenty percent was stable at 37°C and was associated with the fat cell itself. The finding of 80% of the activity outside the cell agrees well with the concept that LPL functions in the uptake of triglyceride fatty acid from the blood. Furthermore, the extra-cellular LPL was responsive to dietary changes whereas the cellular LPL was not (Cunningham and Robinson 1969). Nestel et al. (1969) found that increasing body weight in rats altered lipid metabolism in fat cells. Lipoprotein lipase activity per cell diminished as the weight of the fat cells increased. Diminished esterification of fatty acids was also observed. This author concluded that increasing adiposity interferes with the capacity of the tissue to take up triglyceride fatty acids. The findings of Cunningham and Robinson (1969) that collagenase (used by Nestel et al. 1969) destroys 80% of adipose LPL activity casts reservations upon studies (Nestel et al. 1969, Rodbell 1964, Patten and Hollenberg 1969) in which adipose LPL was measured in fat cells isolated by the collagenase procedure.

Once fatty acids have been hydrolyzed from lipoprotein triglycerides and pass into tissue cells, they become available for tissue specific re-esterification into new triglyceride molecules.

C. TRIGLYCERIDE SYNTHETASE

The biosynthesis of glycerides can proceed by either or both of two pathways depending upon the species and tissue being investigated. The classical pathway proposed by Kennedy (1961) is shown in Scheme 1. This pathway is also referred to as the α-glycerophosphate (α-GP) or phosphatidic acid (P.A.) pathway. An alternate pathway, not shown, utilizes monoglyceride as the acyl acceptor instead of α-GP. This pathway is termed the monoglyceride pathway (Clark and Hubscher 1960, Johnston and Brown 1962, Senior and Isselbacher 1962) and is believed to account for the major portion of triglycerides synthesized in the intestinal mucosa (Mattson and Volpenhein 1964, Kern and Borgstrom 1965).

In the α-GP pathway fatty acids are first activated to their CoA derivatives and subsequently esterified to the 1 and 2 (α and β) positions of glycerol-3-phosphate, forming phosphatidic acid. Phosphatidic acid is converted to diglyceride by the action of the enzyme phosphatidate phosphohydrolase. The newly formed diglyceride may be acylated, forming triglyceride. The triglyceride synthetase



Scheme 1. Pathway for the biosynthesis of triglyceride (Kennedy 1961). (1) acid-CoA ligase EC 6.2.1.3, (2) acyl-CoA-L-glycerol-3-phosphate O-acyltransferase EC 2.3.1.15, (3) L-α-phosphatidate phosphohydrolase EC 3.1.3.4, (4) acyl-CoA-1, 2-diglyceride O-acyl transferase EC 2.3.1.20.

complex with the exception of one enzyme, is a multienzyme complex (Rao and Johnston 1966) existing in the particulate fraction of the cell (Brindley and Hubscher 1965). The enzyme phosphatidate phosphohydrolase exists primarily in the soluble portion of the cell (Smith et al. 1967, Johnston et al. 1967a). In the liver and mammary gland the localization

of the constituent enzymes (except phosphatidate phosphohydrolase) of both pathways is in the mitochondrial and microsomal fractions (Smith and Hubscher 1966, Pynadath and Kumar 1964, McBride and Korn 1964b) whereas in the intestine it is almost exclusively in the microsomal fraction (Brindley and Hubscher 1965). Rao and Johnston (1966) have purified the synthetase complex 70 fold from hampster intestinal mucosa. The enzymes of the complex are purified simultaneously indicating a high degree of structural organization. The substrates, intermediates, and products of the multienzyme complex remain enzyme bound during the course of the reaction. It is not clear if a separate synthetase exists for the acylation of monoglycerides and α-glycerophosphate. Johnston et al. (1967b) have shown that the diglyceride intermediates of the two pathways in the intestinal mucosa do not equilibrate. This suggests complex organization and partitioning of pathways. Most of the investigations of glyceride synthesis to date have been conducted on slices, crude homogenates, or microsomal preparations without further purification.

A characteristic of glyceride synthesis by particulate cell preparations utilizing the phosphatidic acid pathway is the stimulation of glyceride synthesis by the addition of supernatant. The formation of glycerides by the monoglyceride pathway is not stimulated by the supernatant fraction (Hubscher et al. 1967). The stimulation of glyceride

synthesis by the particle free supernatant has been shown to be due to several factors in this fraction. The first factor is a non-enzymatic protein that probably functions similarly to albumin and some lipoproteins (Smith and Hubscher 1966, Hubscher et al. 1967). A second factor is unsaturated fatty acids that may enhance glyceride synthesis from α-GP and palmitate by allowing synthesis of a more balanced product (Brindley et al. 1967). A third supernatant factor is phosphatidate phosphohydrolase (Smith et al. 1967, Johnston et al. 1967a). Although this enzyme is in the particulate portion of the cell it exists primarily in the soluble portion (90%) and accounts for most of the stimulation ascribed to the supernatant fraction (Smith et al. 1967, Johnston et al. 1967). The existence of further stimulatory factor(s) in the supernatant fraction has been indicated by Farstad (1967) who found a soluble factor that stimulated the formation of palmityl CoA by subcellular particulate fractions of rat liver.

New Developments in Glyceride Synthesis

Classical pathways of glycerolipid synthesis have recently come under closer scrutiny, largely due to the investigations of Lands, Goldfine, Vagelos, Agranoff and their co-workers. Three intermediates related to glycerolipid synthesis, acyl-glycero-3-phosphorylcholine (acyl-GPC), acyl-dihydroxyacetone phosphate (acyl-DHAP), and acyl carrier protein (ACP) will be discussed.

For a number of years many investigators have been concerned with finding a biochemical explanation for the non-random positioning and specific fatty acid composition of glycerolipids. Generally speaking, phospholipids and to a lesser extent triglycerides in natural compounds contain saturated fatty acids at position 1 and unsaturated fatty acids at position 2 of the glycerol molecule (Lands 1965a). Enzymic esterification of the 1 and 2 positions of α-GP leading to the formation of phosphatidic acid (diacyl-glycero-3-phosphate) lacked sufficient specificity to account for the distribution of acids that occur in tissue glycerolipids (Lands 1965a). Lands and Merkel (1963) described an acyl-glycero-3-phosphorylcholine (lyso-lecithin) that can act as an acceptor of fatty acids. This evidence points to formation of phosphatidyl choline via standard pathways with subsequent fatty acid deacylations and transacylations (Hill et al. 1968a). Whereas the acyl transferases that esterified α-GP to form phosphatidic acid were found to be relatively non specific (Lands and Hart 1964), the acyl-CoA:acyl-GPC acyl transferases in liver and erythrocytes have marked specificity for the particular acyl-CoA involved (Lands and Hart 1966, Reitz et al. 1968, Hill et al. 1968a). In some cases the observed distributions of fatty acids in naturally occurring lecithins, diglycerides, and triglycerides were similar to distributions predicted by the specificity of acyl transferases (Lands and

Hart 1966). Partial equilibration of triglyceride pools with lecithin pools via diglyceride intermediates may explain positional and fatty acid distributions observed in naturally occurring triglycerides (Lands 1965a). Although no direct evidence exists for this interchange, Slakey and Lands (1968) have observed that the composition of rat liver 1, 2 diglycerides is similar to that of the lecithins from that tissue. The distribution of fatty acids between the 1 and 3 positions of rat liver triglycerides is not random (Slakey and Lands 1968). Each position has a characteristic fatty acid composition. In rat liver triglycerides, fatty acid at the 3 position varies markedly from that at the 1 position, indicating that the diglyceride acyl-transferase does possess a specificity (Lands et al. 1966). Slakey and Lands (1968) proposed that the metabolic step by which the 3 position of triglycerides is acylated may influence the overall fatty acid composition of triglycerides in either of two ways: by preferentially incorporating certain 1, 2 diglycerides or by selecting particular acyl groups for esterification.

Dihydroxacetone phosphate (DHAP) has recently been implicated in phospholipid biosynthesis (Hajra and Agranoff 1968a). Guinea pig liver mitochondria formed acyl-DHAP from DHAP, acyl CoA, and NADPH (Hajra 1968a, 1968b). Hajra and Agranoff (1968b) suggested an alternate pathway for the biosynthesis of phosphatidic acid. Instead of two acylations of

α-GP to form phosphatidic acid, lyso-phosphatidic acid may be formed by the reduction of acyl-DHAP and then subsequently acylated to phosphatidic acid. The phosphatidate formed via acyl-DHAP had more saturated fatty acids in the 1 position than the 2 position, while the phosphatidate formed from glycero-3-phosphate had a more random distribution. The fatty acid distribution with DHAP as the acyl acceptor exhibited a pattern similar to that of natural glycerides (saturated acids at position 1, unsaturated acid at position 2).

Acyl carrier protein, (ACP) well known for its role in fatty acid synthesis (Lynen 1967) has now been shown to function in the acylation of glycerol-3-phosphate by E. coli and clostridium butyricum (Goldfine 1966, Ailhaud and Vagelos 1966, Ailhaud et al. 1967, Goldfine et al. 1967). Since the ACP of plants and bacteria does not appear to be present as a component of a tightly associated synthetase complex such as that of yeast and mammals, acyl groups linked to acyl carrier protein in these systems may therefore be available for the direct acylation of glycerol-3-phosphate. Classically, the transfer of acyl groups from the soluble fatty acid synthetase complex to the particulate glyceride synthetase complex was thought to occur via the CoA intermediate. The significance of ACP mediated acylations in mammalian systems is not known. The lack of success to data in solubilizing an ACP from the mammalian fatty acid synthetase complex argues against such a mechanism. However, Rao and Johnston (1967)

have demonstrated the formation of a "protein-bound" form of CoA from hamster intestinal mucosa that participated in fatty acyl transfer reactions. The exact nature of this compound has not been determined.

The quantitative importance and distribution of the GPC, DHAP or ACP pathways of glycerolipid synthesis has not been established. They have primarily been implicated in phospholipid biosynthesis although the close relationship of phospholipids to triglycerides suggests possible involvement in triglyceride biosynthesis. Until further evidence is presented, the phosphatidic acid and monoglyceride pathways should be considered the main pathways for triglyceride synthesis in mammals.

Specificity of Glyceride Synthesis

The possibility that the type of fatty acid presented to a tissue or organ can exert an controlling effect on glycerolipid synthesis by that tissue is intriguing. The first tenet of this hypothesis is that fatty acids tend to be utilized differently depending upon the number of carbon atoms, and number and position of double bonds in their carbon skeleton. How the ratio of saturated:unsaturated fatty acids in the diet influences animal health is not easily explained on a biochemical level. The general classification of saturated and unsaturated fatty acids is not precise enough

to indicate the metabolic fate of an acid (Lands 1965b). Studies on the metabolism of glycerophosphatides show that the enzymes involved differ in their degree of selectivity toward substrates with differing degrees of unsaturation (Lands and Hart 1966, Waku and Lands 1968, Reitz et al. 1968). Acyl transferase reactions provide specific enzymic steps in lipid metabolism where the reactivity of the substrates can be dependent upon the degree of unsaturation of the fatty acid (Merkel and Lands 1963). Several examples of the fatty acid specificity of various tissues are listed in Table 1.

The most comprehensive investigations on acyl transferase specificity have been conducted on liver and erythrocyte phospholipid synthesis by Lands and co-workers from the University of Michigan. Prompted by the observation (Lands 1965a) that fatty acids are not distributed randomly between the 1 and 2 positions of phosphoglycerides in naturally occurring lipids, these workers have attempted to explain these observations by acyl transferase specificity. Lands (1965a) observed that acyl-CoA:acyl-GP acyl transferases of rat liver preferentially esterified stearate and palmitate (saturated fatty acids) at the 1 position and oleate and linoleate (unsaturated fatty acids) at the 2 position of the GPC molecule. Furthermore *trans*-isomers of oleate were sharply discriminated against in the esterification of position 1 whereas *cis* isomers were not (Lands 1965b).

Table 1

Summary of Some Specificities Observed in Glyceride Synthesis

Investigators	Tissue	Summary of Observations
Abou-Issa and Cleland 1969	Rat liver	Fatty acid specificity of the acyl transferase not responsible for the fatty acid distribution seen in phospholipids and triglycerides.
Brindley and Hubscher 1966	Cat and guinea pig intestinal mucosa	Observed species difference and fatty acid specificity in acyl CoA synthetase. Fatty acid specificity of α-GP pathway differed from monoglyceride pathway.
Brindley et al. 1967	Cat intestinal mucosa, rat liver	Presence of unsaturated fatty acids stimulated palmitate incorporation into glyceride.
Daniel and Rubinstein 1968	Rat adipose	Activated fatty acids of C-4 to C-22, exhibited greatest activity toward palmitate and linoleate.
Galton 1968	Human adipose	C-16:0 > C-18:0 > C-18:1 ~ C-14:0
Goldman and Vagelos 1961	Chicken adipose	Fatty acid composition of the diglyceride influenced the rate of esterification at position 3 but specificity of enzymes involved in the conversion of di- and triglycerides not adequate to explain composition of depot fat.
Hajra 1968b	Guinea pig liver	Unsaturated acyl-CoA's inhibited phosphatidic acid formation via the DHAP pathway.

Table 1 Cont.

Investigators	Tissue	Summary of Observations
Hill et al. 1968a	Pig liver, rat liver	Could find no fatty acid or positional specificity in phospholipid formation using cell free systems of pig liver, but found fatty acid and positional specificity when rat liver slices were used.
Hill et al. 1968b	Rat liver slices	Non-random synthesis of diglyceride followed by a random utilization of these diglycerides for triglyceride synthesis.
Johnston and Rao 1965	Hamster intestinal mucosa	No transacylase specificity for 1 or 2 position. No difference in triglyceride synthesis from C-16:0 or C-18:1.
Kuhn 1967a	Guinea pig mammary tissue	C-18:1 was esterified faster than C-16:0. C-18:1 favored dephosphorylation of phosphatidic acid more than C-16:0.
Lands and Merkel 1963	Rat liver	Acyltransfer occurred with a preferential esterification of saturated fatty acids at position 1 and unsaturated fatty acids at position 2.
Lands and Hart 1964	Rat liver, guinea pig liver	Acyl transfer to glycero-3-phosphate not specific enough to account for pattern observed in nature.
Lands and Hart 1965	Rat and guinea pig liver	Long chain fatty acids were preferentially esterified at position 1, whereas long chain unsaturated fatty acids were preferentially esterified at position 2.

Table 1 Cont.

Investigators	Tissue	Summary of Observations
Neptune et al. 1967	Rat skeletal muscle	Not much difference in the incorporation C-16 to C-18:3.
Pieringer et al. 1967	E. coli.	Various LCFA were incorporated at different rates into phospholipids but not consistent with cell phospholipid composition.
Prottey and Hawthrone 1967	Guinea pig pancreas	Unsaturated fatty acids inhibited the acylation of phosphatidic acid.
Pynadath and Kumar 1963, 1964	Goat mammary tissue	Esterified fatty acids in the order C-16:0 > C-18:1 > C-6 > C-8 > C-4.
Reitz et al. 1968	Rat, bovine, pig, pigeon liver	Number and position of double bond in unsaturated fatty acids important in determining rate of esterification.
Rosenbloom and Elsbach 1969	Toad bladder epithelium	Preferential incorporation of C-18:2 in 2 position, C-16 in 1 and 3 positions.
Sanchez et al. 1969	Rat brain	Fatty acid specificity of acyl transferase not adequate to account for the composition and distribution of fatty acids in triglycerides and phsopholipids of rat brain.
Stitt and Johnston 1966	Rat liver	C-16:0 and C-18:2 were incorporated at differential rates into different lipid classes.

Table 1 Cont.

Investigators	Tissue	Summary of Observations
Vaughan et al. 1964	Rat adipose	C-16:0 esterified greater than C-18:2 for glyceride formation.
Waku and Lands 1968	Human, rat, cow erythrocytes	Found acyl transferase activity in lecithin synthesis varied with species and geometrical isomersim of double bond in fatty acid.

No discrimination between *cis-trans* isomers was exhibited at the 2 position. In human erythrocyte stroma four *cis-trans* isomers were esterified at different relative rates: 18:2 *cc* > 18:2 *tc* > 18:2 *ct* > 18:2 *tt* (Waku and Lands 1968). A species difference between human, rat, and cow erythrocytes was also observed for the specificity of fatty acyl CoA transfer into the 2 position of 1-acyl glycerylphosphatidyl choline. Reitz et al. (1968) have investigated the degree to which different fatty acids are identified by their biosynthetic system. They investigated the importance of the location of the *cis*-ethylenic bonds in influencing the rate at which unsaturated fatty acids were esterified to the 1 and 2 positions of monacyl glycerylphosphatidyl choline. The configuration near carbon atoms 8, 9, and 10 was found to be critical in the metabolism of polyunsaturated fatty acids. Acyl transfer to positions 1 and 2 was relatively fast with

acids containing double bonds near the methyl end and relatively slow when the double bonds were near the carboxyl end of the fatty acid chain. Marked differences in specificity for the Δ^{8-11}, Δ^{6-12}, and Δ^{10-13} isomers indicated that a shift of the double bond by one carbon atom was clearly detected by the enzyme.

Brindley and Hubscher (1966) investigated the rates of esterification of various short and long chain fatty acids by homogenates of cat and guinea pig intestinal mucosa. Different specificities were observed depending upon whether α-GP or monoglyceride was used as the acyl acceptor. The monoglyceride pathway discriminated against fatty acids of 8, 10, and 12 carbons. This is consistent with the direct absorption of short chain fatty acids into the portal system rather than entering the lymph esterified as triglycerides. In a later study with cat intestinal mucosa and rat liver Brindley et al. (1967) found that palmitoleic, oleic, linoleic, and linolenic acids all stimulated the incorporation of palmitate into glycerides and were themselves incorporated. Linoleic acid was especially effective, causing a four-fold stimulation of glyceride synthesis. Linoleate was stimulatory only over a narrow range of concentration, being markedly inhibitory when over 20 μM in the mucosa and 50 μM in the liver. The stimulation of glyceride synthesis by unsaturated fatty acids was not observed when saturated fatty acids replaced unsaturated nor

was it observed when the formation of triglyceride by the monoglyceride pathway was studied (Hubscher et al. 1967). These results indicated a fatty acid specificity for one or more enzymes participating in triglyceride formation by the α-GP pathway. However, Lands and Hart (1964) could not demonstrate a preference for saturated or unsaturated fatty acids in phosphatidic acid formation from α-GP. Since Lands and Hart (1964) did not investigate subsequent triglyceride formation from the phosphatidic acid formed, a specificity of phosphatidate phosphohydrolase (Hubscher et al. 1967) or diglyceride acyl transferase enzymes would have been overlooked.

In summary, investigators have shown that glycerolipid synthesis can be altered by the chain length, degree of unsaturation, position of the double bond and *cis-trans* isomerism of the double bond of the fatty acids presented to the acyl transferase enzymes. Several examples have been presented and discussed. The *in-vivo* significance of these observations is not known. Although specificity was indicated in the examples presented other studies have not demonstrated such pronounced specificities (see Table 1).

D. MILK FAT SYNTHESIS

The review of milk fat synthesis presented here is intended to demonstrate the importance of the uptake and incorporation of long chain fatty acids from blood lipids to milk fat synthesis. Fatty acid synthesis from acetate and

β-hydroxybutyrate will not be discussed in this review although it is realized that they represent an important source of fatty acids for milk fat synthesis.

Origin of Milk Fat

Milk fat is largely (98-99%) composed of triglycerides, the remainder consists of phospholipids (0.2-1.0%) cholesterol and cholesterol esters (0.2-0.4%) and trace amounts of free fatty acids, waxes, and squalene (Hilditch and Williams, 1964). The yield of fat in the milk is influenced by many variables, including nutrition (Kirchgessner et al. 1967).

From quantitative considerations, the synthesis of milk fat is largely the synthesis of triglyceride which is in turn the synthesis of fatty acids and their esterification to glycerol. The physiological locus of milk fat synthesis is the epithelial cells of mammary alveoli. Ruminant milk fats are unique in their content of short chain acids of less than 10 carbon atoms (Kirchgessner et al. 1967).

Fatty acids for milk fat triglyceride synthesis originate from plasma long chain fatty acids and short to medium chain fatty acids (C-4 to C-16) synthesized within the mammary gland (Barry 1966, Linzell 1968, Jones 1969). Fatty acids up to and including C-16 can be synthesized in the mammary gland from acetate and β-hydroxybutyrate taken up from the blood

(Popjak et al. 1951, Kumar et al. 1959, Ganguly 1960, Hibbitt 1966, Annison et al. 1967, Linzell et al. 1967).

Barry (1966) estimated that 20-30% of the carbon of milk fatty acids came from blood acetate. Similar estimates were made by Annison and Linzell (1964). The magnitude of arterial-venous (AV) differences for β-hydroxybutyrate suggested that it could potentially contribute one-half as much carbon to milk fatty acids as acetate (Barry 1966).

Contribution of Blood Fat to Milk Fat

Neutral lipids of the blood have been known to be precursors of milk fat since the late 1930's (Garton 1963, Kirchgessner et al. 1967) but identification of specific fractions and quantitative estimations of blood fat contribution to milk fat were not forthcoming until recently (Jones 1969). Estimations of the quantitative contribution of plasma lipids to milk fat have ranged from 25-82% (Glascock et al. 1956, 1966, Riis et al. 1960, Annison et al. 1967, Barry 1966).

Experiments conducted with the intact goat (Barry et al. 1963, West et al. 1967b), perfused goat udder (Lascelles et al. 1964) and the intact cow (Glascock et al. 1966, Welch et al. 1968) have confirmed that triglycerides of chylomicron and low density lipoproteins ($d < 1.019$) circulating in the blood are taken up by the mammary gland (Barry et al. 1963, Lascelles et al. 1964, Emery et al. 1965, Glascock et al. 1966,

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Welch et al. 1968, Huber et al. 1969). Lipoproteins of density < 1.019 (called variously: chylomicra, very low density lipoproteins, low density lipoproteins, β-lipoproteins) represent a lipid transport agent of high specific activity (Welch et al. 1968). Bovine low density lipoproteins prepared either centrifugally (Evans et al. 1961, Evans and Patton 1962, Emery et al. 1965) or precipitated by dextran sulfate (Glascock et al. 1966) or heparin (Huber et al. 1969) accounted for less than 10% of total blood fat (Evans and Patton 1962, Huber et al. 1969) but accounted for most of the transfer of blood fat to milk fat (Emery et al. 1965, Glascock et al. 1966, Huber et al. 1969).

The major source of long chain fatty acids removed from blood by the mammary gland is the triglyceride of circulating low density lipoproteins. Free fatty acids represent a minor proportion of plasma lipids. Free fatty acids are taken up by the gland (Lauryssens et al. 1961, Annison et al. 1967) but are also released into venous blood resulting in negligible AV differences (Barry et al. 1963, Annison et al. 1967). Cholesterol ester (Riis et al. 1960, Lough et al. 1960, Emery et al. 1965, Varman and Schultz 1968a) and phospholipid (Riis et al. 1960, Lough et al. 1960) AV differences have suggested possible contributions by these lipids to milk fat. However, consideration of data from most AV or radioisotope studies, does not support these lipid

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classes as important milk fat precursors (Linzell 1968, Barry 1966, Barry et al. 1963, Annison et al. 1967, Thomas and Emery 1969).

Mode of Uptake of Chylomicron and Low-density Lipoprotein Triglyceride Fatty Acid

Fatty acid uptake by the mammary gland is believed to involve partial or complete hydrolysis of triglyceride molecules (Patton and McCarthy 1963a, Barry et al. 1963, McBride and Korn 1964d, Annison et al. 1967, West et al. 1967a, 1967b). The most convincing evidence for molecular re-arrangement (McCarthy et al. 1960) is investigations in which ^{14}C-glycerol and ^{3}H-fatty acids were incorporated into chylomicrons and infused intravenously. Substantial shifts in the $^{14}C/^{3}H$ ratio in milk triglyceride relative to that of chylomicron triglyceride were observed in the guinea pig (McBride and Korn 1964d) and the goat (West et al. 1967b).

The hydrolysis of chylomicron and β-lipoprotein triglyceride fatty acid as blood passes through the udder is thought to occur similarly to that of other extra-hepatic tissues. In the goat, release of appreciable quantities (25%) of chylomicron triglyceride fatty acid into mammary venous plasma and the absence of labelled mono-, di-, or triglycerides in mammary lymph (West et al. 1967b) suggested that the enzyme lipoprotein lipase (LPL) acted upon plasma triglycerides liberating free fatty acids into the plasma.

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Lipoprotein Lipase of the Mammary Gland

The first indication that triglyceride fatty acid uptake by the mammary gland might involve LPL was provided by Korn (1962a). He noted the presence of a lipase in cows milk that was similar if not identical to the lipoprotein lipase of heart and adipose tissue. Although LPL was present in milk in relatively high concentrations it appeared not to function there, since it was unable to hydrolyze the triglycerides of cream. Korn deduced that the enzyme probably functioned in mammary tissue and that its appearance in milk reflected cell rupture that occurred during the secretion of milk. McBride and Korn (1963) and Robinson (1963a) subsequently investigated the relationship of guinea pig mammary gland LPL activity to lactation. Virtually no LPL activity was detected in the gland during most of pregnancy. A dramatic increase in enzyme activity occurred immediately prior to parturition and this level of activity remained relatively constant throughout the entire period of lactation. Lipoprotein lipase activity of the mammary gland was one hundred fold greater during lactation than during pregnancy. Suckling and/or continued milk production was a factor in maintaining high lipase activity. No activity could be detected within eighteen hours after cessation of suckling. Fiddler and Falconer (1968) observed increased LPL activity in pseudo-pregnant rabbit mammary tissue following prolactin injections.

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Prolactin released from the pituitary upon suckling (Folley 1961) may be a factor in maintaining mammary LPL activity.

Although direct proof of the existence of LPL in goat or bovine mammary tissue is lacking, mammary venous blood measurements of this enzyme indicate that it may have been released from the mammary gland of goats (Barry et al. 1963, Lascelles et al. 1964). Using live goats, Barry et al. (1963) found that mammary venous blood of live goats contained three times as much LPL activity as did arterial blood. The observation was coincident with a large decrease in concentration of triglyceride fatty acids of chylomicra and low density lipoproteins as blood flowed through the mammary gland. Lascelles et al. (1964) used perfused goat udders and noted an increase in LPL activity of the perfusate after circulation through the gland. This activity was observed whether heparin was present or absent, but the activity was greater when heparin was added.

Although LPL measurements in pregnancy or lactation have not been conducted on the cow, uptake of plasma triglyceride is negligible in the absence of lactation (Varman and Schultz 1968b).

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Fatty Acid Esterification by the Mammary Gland

Esterification of fatty acids by the mammary gland to form triglycerides, phospholipids, and cholesterol esters has been demonstrated with isotopic tracer studies (Patton and McCarthy 1963a, Kinsella 1968a, Kinsella and McCarthy 1968a). Fatty acid esterification by the mammary gland has been investigated by a variety of techniques (Table 2). However, the specific requirements for glyceride synthesis by the ruminant mammary gland are not known (Dimick et al. 1966).

Table 2

Investigations on Fatty Acid Esterification by the Mammary Gland

Species	Technique	Investigator
Goat	Intramammary infusion	Dimick et al. (1966), Patton et al. (1966a), Patton and McCarthy (1963a)
Goat	Tissue homogenates	Pynadath and Kumar (1963, 1964)
Cow	Intramammary infusion	Al-Shabibi et al. (1969)
Cow	Tissue slices	Patton et al. (1966a)
Cow	Dispersed cell cultures	Kinsella (1968a, b), Kinsella and McCarthy (1968a, b)
Guinea pig	Tissue homogenates	McBride and Korn (1964a, b), Kuhn (1967a, b)
Rat	Tissue homogenates	Dils and Clark (1962)

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Glyceride synthesis by the mammary gland has reveiced little detailed study at the enzymic level. Studies conducted with rat, guinea pig, and goat tissue (Table 2) suggest that the phosphatidic acid pathway of glyceride synthesis occurs, although certain observations to be discussed later suggest possible alterations.

Dils and Clark (1962) first showed that fatty acid esterification by rat mammary gland homogenates required ATP, Mg^{2+} and CoA. A strong requirement for glycerol-3-phosphate (α-GP) that could not be replaced by glycerol or monoglyceride was observed. Pynadath and Kumar (1963, 1964) found that goat mammary tissue exhibited similar requirements. Both diglyceride and α-GP could serve as acyl acceptor. Little or no acylation of monoglyceride was observed.

McBride and Korn (1964a, 1964b) observed similar requirements with guinea pig mammary tissue. They found evidence that several acyl acceptors could substitute for α-GP. Glycerol as well as glucose was effective in generating α-GP in this system, demonstrating for the first time the existence of a glycerokinase in mammary tissue. Ninety-six percent of the glycerokinase activity was found in the soluble portion of the cell, whereas most of the glyceride synthesizing activity was found in the particulate portion. Although acylation of monoglyceride was observed, phosphatidic acid

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was much more active in stimulating palmitate incorporation. Several peculiarities were observed with this system. Triglyceride was as effective as diglyceride in acting as an acyl acceptor. The incorporation of ^{14}C-palmitate using triglyceride as acyl acceptor was ATP and CoA dependent. The authors suggested that perhaps lipase hydrolysis of triglycerides to diglycerides, which in turn acted as acyl acceptor, might explain these results. Ethanol was also esterified to palmitate by this system. The reaction was enzymatic, requiring ATP, CoA and homogenate. Patton and McCarthy (1966) have also noted the formation of ethyl palmitate by fresh goat milk. The biologic significance of this reaction is unknown, but illustrates the importance of omitting ethanol from any portion of an assay where fatty acid esterification is measured.

Kinsella (1968a, 1968b) and Kinsella and McCarthy (1968a, 1968b) applied dispersed bovine alveolar cell culture techniques to studying bovine mammary lipid metabolism. These studies demonstrated the ability of bovine mammary tissue to utilize glycerol for milk fat synthesis. Small quantities of phosphatidic acid were detected when cell lipids were separated. When isotopically labelled fatty acids were added to the cell culture specific activities of the di- and triglycerides indicated a precursor product relationship. These findings all indicated that the phosphatidic

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acid pathway was operating. However, Patton et al. (1966a) were unable to demonstrate incorporation of labelled fatty acid into phosphatidic acid in cow and goat mammary tissue and concluded that the monoglyceride pathway predominated.

Kuhn (1967a, 1967b) has provided the most convincing evidence for the operation of the phosphatidic acid pathway in guinea pig mammary tissue. The presence of three of the four enzymes of the phosphatidic acid pathway (fatty acid thiokinase, acyl transferase, and phosphatidate phosphohydrolase) were demonstrated. Accompanying a large increase in triglyceride synthesis at parturition was a 37 fold increase in acyl transferase activity and a 2-3 fold increase in the concentration of glycerol-3-phosphate and free fatty acids. Pynadath and Kumar (1964) found that lactating tissue was four times more active in glyceride synthesis than non-lactating.

Mammary gland acyl transferase may be a rate limiting enzyme in milk fat synthesis. Since fatty acyl CoA may enter any one of several different pathways in lipid metabolism, the esterification of glycerol-3-phosphate would be a suitable point at which fat synthesis might be regulated. Glycerol-3-phosphate may be limiting, as has been suggested for other tissues (Tzur et al. 1964, Howard and Lowenstein 1965). Kuhn found the Km of guinea pig transferase for glycerol-3-phosphate to be 2.7 mM, well above the 0.089 mM concentration of

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glycerol-3-phosphate found in the tissue. Baldwin et al. (1969) has found similar concentrations of glycerol-3-phosphate (0.154 mM) in the mammary tissue of cows. Kuhn (1967b) speculated that the concentration of glycerol-3-phosphate may act as a fine control coordinating triglyceride synthesis with carbohydrate degradation. Accumulation of diglyceride in *in-vitro* assays (McBride and Korn 1964b, Kuhn 1967a, Pynadath and Kumar 1964) suggested that specifically the third acylation may be limiting. However, this may merely reflect lipolysis (Clark and Hubscher 1961) or unfavorable assay conditions. Contrary to Pynadath and Kumar (1964), Kuhn (1967a) found endogenous generation of palmityl-CoA permitted greater conversion of phosphatidate to glyceride than when synthetic palmityl-CoA was added. This observation plus work done on fatty acid activation in other tissues (Daniel and Rubinstein 1968) suggested that formation of acyl-CoA probably is not limiting. The inhibitory nature of CoA derivatives at certain concentrations (Daniel and Rubinstein 1968, Kuhn 1968a) suggested that long chain acyl CoA molecules may have a role in regulating the activity of the transferase.

The source of the glycerol moiety of the triglyceride molecule has been the subject of controversy (Folley 1961). Milk fat glycerol can come from three sources: blood glucose

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(Popjak et al. 1952, Luick 1961, Luick and Kleiber 1961, Hardwick et al. 1963, Annison and Linzell 1964), plasma lipoprotein triglycerides (Barry 1964, West et al. 1967a), and free plasma glycerol (Barry et al. 1963, Barry 1964, Linzel 1968). Although the relative contribution of each source is uncertain, 20-70% of milk fat glycerol can come from blood glucose, 50% from plasma triglyceride glycerol, and possible 10% from free plasma glycerol. From a consideration of quantitative estimates of glycerol origin from the literature, Dimick et al. (1966) has noted that a large proportion of milk fat glycerol is unaccounted for. Dimick et al. (1965) have noted a preferential occurrence of palmitic acid at the two position in high molecular weight milk fat triglycerides. These investigators noted that upon infusion of ^{14}C-palmitate into the udder, the specific activity of palmitic acid in the 2-monoglycerides was considerably lower than in the corresponding triglycerides. These data suggested that a 2-monoglyceride derived by partial hydrolysis of blood triglycerides may be contributing additional carbons for glyceride synthesis in the gland.

Adipose tissue lacks the enzyme glycerokinase, necessary for utilization of glycerol in fat synthesis, and some workers (Dils and Clark 1962, Pynadath and Kumar 1964) have been unable to demonstrate its presence in mammary tissue. However, evidence now exists for glycerokinase activity in mammary tissue

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of the rat (Carlson et al. 1964, Kinsella 1968b), guinea pig (McBride and Korn 1964a) and the bovine (Kinsella 1968b).

Ruminant milk fats are characterized by a high proportion of short chain fatty acids which account for the large quantities of triglycerides with 26-44 acyl carbons (Breckenridge and Kuksis 1967). These triglycerides account for approximately 50% of milk fat. Ruminant milk fat consists predominantly of two populations of triglycerides, one with 48-54 acyl carbons and the other with 36-40 (Glass et al. 1969). In an analytical study of milk fats from 15 species of ruminants and 40 species of non-ruminants Glass et al. (1969) found ruminant milk fat separated into two distinct triglyceride spots upon thin layer chromatography, whereas non-ruminant milk fat exhibited only one spot. No butyrate was found in non-ruminant triglycerides whereas appreciable butyrate and caproate were found in the slower moving triglyceride spot of ruminant milk fat that corresponded to triglycerides of 36-40 acyl carbons (Glass et al. 1967, 1969). Furthermore, analytical data indicates that 95% of the C-4 to C-8 fatty acids of milk fat are esterified to the 3 position of the glycerol molecule (Breckenridge and Kuksis 1968). The general pattern of fatty acid distribution in milk fat suggests specific placement. Short chain and 18-carbon fatty acids predominate in the external positions of the glycerol molecule while medium chain fatty acids are concentrated on the internal carbon (De Man 1968,

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Jensen et al. 1961, Kumar et al. 1960). Dimick and Patton (1965) analyzed milk fat and could find no more than one mole of butyrate per mole of triglyceride. Mammary tissue diglycerides contain a low proportion of short chain fatty acids, (particularly butyrate) compared with tissue and milk fat triglycerides (Patton and McCarthy 1963b). Breckenridge and Kuksis (1968) have stated that any interpretation of the mechanism of biosynthesis of milk fat will have to account for the specific placement of the short chain fatty acids in milk fat triglycerides. Analytical data from studies on milk fat composition is consistent with the hypothesis (Dimick and Patton 1965, Breckenridge and Kuksis 1968) that short chain fatty acids, in particular butyrate, are esterified during the final step in biosynthesis of milk fat triglycerides. Although this hypothesis remains to be proven there is indirect evidence to support this theory. Pynadath and Kumar (1964) could not demonstrate butyrate esterification by goat mammary tissue. Their system (using palmitate and α-GP) formed only 25% triglyceride and 70% diglyceride. Patton and McCarthy (1963b) have postulated the existence of two separate fatty acid pools in alveoli, one at the base of the cell derived from plasma triglycerides and another pool of intermediate and short chain acids in the upper portion of the cell arising from *de novo* synthesis from acetate. Tracer studies in goats (Annison et al. 1967, West et al. 1967a) have shown that

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fatty acids synthesized within the secretory cells are not in equilibrium with the long chain fatty acids in the blood supplying the mammary gland. Wood (1966) has suggested that the pathway for the incorporation of short chain fatty acids into milk fat triglycerides may differ from that of long chain acids. The mechanism of incorporation of short chain fatty acids into milk fat is unknown and appears to be unique to the ruminant mammary gland.

Another peculiarity of ruminant milk fat is the content and molecular positioning of palmitic acid. Dimick et al. (1965) have observed from analyses of different triglyceride classes of cow and goat milk fat that the over-all concentration of palmitic acid is relatively constant and independent of the molecular weight of the triglyceride fraction. These observations led the authors to suggest that palmitic acid acts as a pivoting acid (i.e. is esterified first) about which the other fatty acids orient during triglycerides synthesis. Although palmitate was distributed randomly over total milk fat it showed a definite tendency to be preferentially esterified in the 2 position in the high molecular weight triglycerides. As molecular weight decreased, palmitate shifted to random distribution and was completely reversed in low molecular weight triglycerides, where it was preferentially esterified in the 1 and 3 positions of glycerol. Since palmitate is the only major acid of milk fat supplied to the gland by

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both circulating lipids and acetate condensation (Glascock 1958, Popjak et al. 1951), Dimick et al. (1965) suggested that the preferential esterification of palmitate in the two position may be attributed to a 2-monoglyceride or 1, 2 diglyceride derived by partial hydrolysis of blood lipids. In connection with the formation of 2-monoglyceride, lipoprotein lipase has been reported to preferentially cleave acyl groups esterified at the α-position and as a result ß-monoglyceride accumulates (Carlson and Wadstrom 1957). If ß-monoglycerides are taken up by the mammary gland as suggested by Dimick et al. (1965) positional specificity of LPL may provide an explanation of their origin. Palmitate in the terminal positions of the low molecular weight triglycerides may be derived from classical acetate condensation with a mixing of the two sources giving the random distribution of the intermediate triglyceride classes.

For unknown reasons the mammary gland of the bovine Lauryssens et al. 1961) and goat (West et al. 1967a, nnison et al. 1967) desaturates stearic acid to oleic acid. he mammary gland takes up more stearic than oleic acid from lood (Barry et al. 1963) but milk contains 3-4 times more leic than stearic. McCarthy et al. (1965) have found an nzyme in goat milk capable of converting stearic to oleic. insella (1968a) found that dispersed mammary alveolar cells esaturated 37% of the added unesterified stearic acid to

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oleic. Following intravenous injections of acetate-1-^{14}C, Gerson et al. (1966, 1968) found higher specific activity in oleic acid of milk fat than stearic indicating that oleic may be synthesized from sources other than stearic.

Patton et al. (1966b) have suggested that mammary gland lecithin may be an acceptor for short chain acyl groups and as such function as an intermediate in the synthesis of triglycerides of short and medium chain length. This postulate was based upon the high specific activity of lecithin found in mammary tissue and milk when ^{14}C-fatty acids were incubated with tissue (Patton et al. 1966a) or milk (Patton et al. 1965) or infused into the teat canal (Patton et al. 1966a, Al-Shabibi et al. 1969). If indeed lecithin is serving as a precursor of neutral lipids, a relationship between the fatty acid composition of milk fat lecithin and short chain triglycerides would be expected. However, Kuksis and Breckenridge (1968) could not find a good relationship between these two classes. Kinsella (1968b) did not find lecithin to be particularly active in milk fat synthesis although it incorporated more ^{14}C-glycerol than any other class of phospholipid. Nevertheless the work of Lands (see discussion of glyceride synthetase specificity) on acyl glycerylphosphatidyl choline provides credence to Patton's et al. (1966b) proposal.

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etate (Clarenburg and Chaikoff 1966). Less than one-half
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erides than the free fatty acids in the medium. This
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e Kinsella (1968a) demonstrated that only 5% of the
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E. NUTRITIONAL FACTORS INFLUENCING MILK FAT SECRETION

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Since the daily intake of dietary fat by the cow is of the same order of magnitude as the daily output of fat in the milk (Moore and Steele 1968) the possibility that the level of dietary fat might exert some influence on milk fat production as been extensively investigated over the last 50 years. olyunsaturated fatty acids of the ruminant diet can influence he composition of blood and milk lipids, depending upon the mounts that escape ruminal hydrogenation (Hilditch and illiams 1964). Under dietary conditions where ruminal ydrogenation capacity is not grossly exceeded, di- and iunsaturated C-18 fatty acids escaping the rumen are sociated mainly with the phospholipid portion of intestinally rmed chylomicrons (Leat and Hall 1968, Wadsworth 1968). ospholipid fatty acids have not been found to make a antitative contribution to milk fat (Linzell 1968, Annison al. 1967). The remaining di- and triunsaturated fatty ds that are incorporated into chylomicron triglycerides normally present in minor quantities (Leat and Hall 1968, sworth 1968), and as such probably do not make a very nificant contribution to milk fat. However, when the acity of the rumen to saturate dietary fatty acids is eeded, or when the rumen is by-passed by abomasal infusions, ss unsaturated fatty acids may be taken up as chylomicron

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iglycerides (Moore et al. 1969) in sufficient quantities to low their increased transfer to milk fat. Chylomicron iglycerides appear to be a more likely candidate for transfer polyunsaturated fatty acids to the mammary gland than oprotein triglyceride synthesized in the liver. Leat and l (1968) found that the content of di- and triunsaturated ty acids was greater in triglycerides derived from the estine than those found in the plasma. Whether this is to the specificity of liver enzyme systems responsible glyceride synthesis as suggested by Moore et al. (1968) ot known. Since only one-third of chylomicrons of gut in are believed to reach the peripheral circulation inson 1963b), large increases in a component fatty acid d seem necessary to produce a measureable alteration in fat composition by this route.

The literature concerning the effects of supplemental ry fat on milk yield, milk composition, and ruminal ntation is extensive and contradictory. Part of the tion observed in response to dietary fat may have been o different experimental conditions. Gibson and Huffman) were among the first to recognize the composition of asal ration as an important factor in determining the of the addition of dietary fat on milk and fat ion. The following considerations have been implicated ation to the responses observed upon oil administration:

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2) Quantity of roughage in the basal diet (Steele and ›ore 1968a, 1968b, Brown et al. 1962),

3) Mode (i.e. *per os*, abomasal, intravenous) of ministration (Tove and Mochrie 1963),

4) Length of time administered (Steele and Moore 1968b),

5) Quantity and/or frequency of the oil feeding (Moore al. 1945), and

6) Degree of unsaturation of the oils administered ›Cay et al. 1938, Steele and Moore 1968a).

Increases in yield of milk fat have been reported when diet of the cow was supplemented with fats or oils low in yunsaturated acids, i.e., tallow, butter, palm oil, coconut (McCandlish and Weaver 1922, Garner and Sanders 1938, en and Fitch 1941, Peters et al. 1961, Brown et al. 1962, ele and Moore 1968a). Increased yields of milk fat have been observed when vegetable oils rich in polyunsaturated y acids have been fed for a short period of time (Nevens l. 1926, Allen 1934, Garner and Sanders 1938, Davis and and 1946, Steele and Moore 1968a, 1968b). Conversely, investigators have observed a reduced yield of milk fat vegetable oils rich in polyunsaturated fatty acids were rporated into the ration of cows over longer time periods ner and Sanders 1938, Allen and Fitch 1941, Parry et al. Steele and Moore 1968a, 1968b, Varman et al. 1968,

Adams et al. 1969
of milk fat have
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(Petersen 1932, (
Reitz and Davis 1
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ms et al. 1969, Haenlein et al. 1968). Decreased yields
milk fat have also been observed when highly unsaturated
s of marine origin have been incorporated into the diet
tersen 1932, Garner and Sanders 1938, Shaw and Ensor 1959,
z and Davis 1964, Varman et al. 1968, Haenlein et al.
9). Cod liver oil has been shown to be especially effective
ecreasing milk fat yield. The fat depressing properties
od liver oil appear to be associated with its high content
-20 and C-22 unsaturated fatty acids. McCay et al. (1938)
nstrated that the milk fat depressing effect of cod liver
was destroyed if the unsaturated fatty acids were hydrogenated
to feeding. Moore et al. (1945) found that milk fat
was depressed when 5-8 ounces of cod liver oil were
once a day but was unaltered when the same amount was
in several smaller doses during the day. The possibility
e unsaturated C-20 and C-22 fatty acids of cod liver oil
tly interfering with milk fat synthesis at the mammary
has been suggested (Hilditch and Williams 1964, Beitz
avis 1964, Storry et al. 1969a). The most convincing
nce for an extra-ruminal effect by cod liver oil has
rovided by Storry et al. (1969b). Intravenous infusions
liver oil emulsions decreased the yield of milk fat
rly to *per os* administration indicating that the decreased
at secretion was accomplished in some manner not mediated
ifts in rumen VFA proportions. Storry et al. (1969b)

speculated that
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rations (Steele
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Although fe
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speculated that unsaturated C-20 and C-22 fatty acids escaped
ydrogenation in the rumen and acted directly on the mammary
gland, possibly by inhibiting the enzyme lipoprotein lipase.

The results of feeding oils or fats containing C-18
nsaturated fatty acids are less clear. Shaw and Ensor (1959)
ound that oleic and linoleic acids were as effective as cod
iver oil in lowering milk fat yields when administered orally.
arman et al. (1968) found safflower oil (ca 75% C-18:2) was
s effective *per os* as cod liver oil in decreasing milk fat
st. Haenlein et al. (1968) found that safflower oil,
d liver oil, and a pelleted high grain ration
creased milk fat yield 13, 21, and 30% respectively. Parry
al. (1964) also reported lower milk fat yields when safflower
l was fed as part of the concentrate mix. Cottonseed oil
a 50% C-18:2) has been reported to decrease milk fat yield
en fed over long time periods (28 days) (Steele and Moore
8a, 1968b) and in conjunction with restricted roughage
ions (Steele and Moore 1968a, 1968b, Brown et al. 1962).
ms et al. (1969) found that a mixture of 50% wheat germ
and 50% cottonseed oil fed at 10% of the concentration
ture lowered milk fat yield.

Although feeding experiments with cottonseed oil have
lted in decreased milk fat yields, contrary results have
noted in infusion studies. Tove and Mochrie (1963)

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infused up to 900 grams of cottonseed oil emulsion intravenously without noting any significant effect on milk fat yield. Storry and Rook (1965) observed an increased yield of milk fat when 700-1000 g/day of cottonseed oil emulsion was intravenously infused for 2-3 days. Storry et al. (1969b) noted an increased yield of milk fat when 1000 grams of soybean oil emulsion (54.2% C-18:2) was infused intravenously. These infusion experiments were of relatively short duration compared to the feeding experiments cited.

Results from feeding saturated fats such as tallow, coconut oil, and palm oil indicated either no effect or increased milk fat yield (Brown et al. 1962, Steele and Moore 1968a, Adams et al. 1969, Storry et al. 1967, Storry et al. 1968). These same studies demonstrated that feeding saturated fats affects milk fat yield differently than feeding unsaturated fats, especially in long term studies.

Several attempts have been made to differentiate between the effects of various individual fatty acids of the oil or fat on milk fat synthesis. Previously mentioned work by Kay and Ensor (1959) demonstrated that either oleic or linoleic acid decreased milk fat yield within 63 hours of feeding. Linoleic was much more effective than oleic in this respect. When individual fatty acids were included at 5-10% of the concentrate mix, lauric and oleic decreased milk fat yield,

myristic had no

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ristic had no effect, and palmitic and palmitic and stearic
creased milk fat yield (Steele and Moore 1968d, Steele and
ore 1968c).

In summary, polyunsaturated fatty acids are effective in
reasing milk fat yield. Cod liver oil high in C-20 and
2 polyunsaturates has lowered milk fat yield either when
or intravenously infused. Feeding vegetable oils such as
tonseed oil or safflower oil high in C-18 unsaturates
ers milk fat yield. Limited evidence suggests oleic and
oleic acids also lower milk fat yield. In contrast,
ravenous infusions of cottonseed oil or soybean oil
sions under similar conditions to cod liver oil infusions
not been shown to lower milk fat yield. Milk fat
ession caused by feeding polyunsaturated oils may be
ted to the milk fat depression caused by feeding restricted
hage-high grain rations. Both dietary treatments alter
composition of long chain fatty acid precursors of milk
found in the blood.

icted Roughage-High Grain Feeding

Restricting the fibrous portion of the diet fed to
ting dairy cows frequently results in decreased yields
lk fat. Restriction of the fibrous portion of the ration
e accomplished by grinding and pelleting the ration or
icting the roughage intake and concurrently increasing
ncentrate portion of the ration.

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The percent and yield of fat in the milk usually commence to decline within a matter of days following the feeding of a restricted roughage-high grain ration. Milk yield is usually not affected although slight increases, probably due to a higher plane of nutrition, have been demonstrated. Milk lactose remains constant although milk protein may increase slightly (Armstrong 1968).

E. B. Powell, a former nutritionist for the Ralston urina Feed Manufacturing Company, is credited with the riginal observation (Powell 1938) that depression of fat ercentage in milk followed the feeding of diets high in rain and low in roughage. Powell (1939) demonstrated a % variation in the fat content of milk by regulating the ysical characteristics and total intake of the roughage rtion of the ration. Fat production was brought back to rmal by dietary means after three lactations of depression dicating that the metabolic change was adaptive, rather than rmanent. Powell's origional observations have stimulated estigation of the causes and prevention of nutritionally cited milk fat depression. Van Soest (1963) reviewed the erature on milk fat depression and summarized the more sible theories that might explain the cause of depressed k fat of cows fed high grain-restricted roughage diets. e theories observed were: 1) deficiency of acetate uced by the rumen; 2) deficiency of ß-hydroxybutyrate

(BHBA) available

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Brown et al. 196

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3) The fee

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BHBA) available to the mammary gland and 3) glucogenic
ppression of the fat mobilization required for milk fat
ynthesis. These theories will be discussed with special
mphasis upon the latter of the three since it bears more
on the nature of the research presented in this thesis.

The first and most popular theory suggests that the
crease in fat test accompanying high grain feeding is caused
a reduction in the amount of acetate produced by the rumen
croorganisms (Tyznik and Allen 1951, Balch et al. 1955,
own et al. 1962, Rook 1959). This theory is supported by
e following observations:

1) Acetate taken up from the blood (McClymont 1951)
used by the mammary gland for fatty acid synthesis
pjak 1952). Fatty acid synthesis from acetate usually
ounts for ca 40-50% of total milk fat (Linzell 1968).

2) The molar percent acetate in the rumen of cows fed
tricted roughage-high grain rations decreases (Balch et al.
5).

3) The feeding or intraruminal infusion of acetate
lly results in an increase in the fat percent of the
(Tyznik and Allen 1951, Van Soest and Allen 1959, Balch
Rowland 1959, Rook and Balch 1961).

However, acetate administration does not always result
ncreased milk fat yield. Stoddard et al. (1949) were

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able to completely correct milk fat test to normal levels
adding acetic acid to the rumen of cows fed a high grain
ion. Although Balch and Rowland (1959) were able to
rease the fat test of cows producing low fat milk by
ding or infusing acetate, they noted variations between
s in response to this treatment. One of the cows exhibiting
k fat depression in the study of Balch and Rowland (1959)
not respond to acetate treatment. Jorgensen et al.
55) were unable to correct milk fat depression by intra-
nal acetate infusion even though blood acetate was
eased five-fold.

A recent investigation by Davis (1967) has cast further
t on the acetate deficiency theory. By an isotope
tion technique Davis has estimated ruminal acetate
uction on normal and restricted roughage-high grain rations.
ite production values were 29.3 and 28.1 moles/24 hours
ctively, indicating that acetate production was not
ssed in the rumen. The constant rate of decline in the
fic activity of rumen acetate with time, regardless of
indicated that substrate for acetate production was
imiting and that acetate production was constant.
ugh Davis (1967) allowed for some ruminal acetate
rsion to butyrate which gave corrected acetate production
of 25.1 and 21.8 moles/24 hours respectively for normal
stricted roughage-high grain rations, no significance

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attached to the differences in these values. However, the acetate supply to the udder is critical, and if ess butyrate is not utilized as well for milk fat synthesis, conversion of acetate to butyrate in the rumen of cows fed ricted roughage-high grain rations may be a significant or. Although the differences in acetate production s (1967) observed were small they were probably conservative e the control cows in this experiment were receiving tively high quantities (10.9 Kg) of concentrate mixture. magnitude of an acetate deficiency at the mammary gland ed by acetate conversion to butyrate would seem too small ccount for the milk fat depression observed.

As Davis (1967) noted, the lack of a decreased acetate action by the rumen does not rule out an acetate shortage he mammary gland. Acetate utilization could be enhanced ssues such as adipose which would divert acetate away the mammary gland. Some studies on blood acetate under onditions of milk fat depression support this concept an and Schultz 1968a, Huber et al. 1969) while studies hers do not (McClymont 1951, Van Soest and Allen 1959).

-hydroxybutyrate (BHBA) derived from rumen epithelium ver metabolism of butyric acid is taken up from the by the mammary gland (Linzell 1968) and is an essential sor of the short-chain fatty acids of milk fat (Shaw odt 1941). Although the molar percent butyrate in the

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ughage-high grain rations are fed, decreased blood con-
ntrations and arteriovenous differences in ketone bodies
ve been associated with milk fat depression (Van Soest and
en 1959, Van Soest 1963, Huber et al. 1969). Other
estigators have not observed significant decreases in
od ketones with milk fat depression (Varman and Schultz
8a).

Palmquist et al. (1969) have recently determined the
y rate of BHBA into the mammary gland from blood in
fat depressed and normal cows. Although total fat
uction was decreased in the cows fed restricted roughage-
grain rations, the specific activity (dpm/g fat) was
same for the control and restricted roughage-high grain
cows following intramammary butyrate-1, 3-^{14}C infusions.
fed restricted roughage rations incorporated less BHBA
2 units than cows on normal rations. Lower specific
ity (dpm/g fat) for the restricted roughage-high grain
was observed when acetate-1-^{14}C was infused. Although
incorporation into milk fat as a four carbon unit did
pear to be affected by rations in this study, acetate
ation was slightly depressed under the conditions of
at depression. Palmquist et al. (1969) have estimated
HBA could contribute only 8% of the total milk fatty

acid carbon. T
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id carbon. They concluded that a deficiency of BHBA would t likely cause the decrease (50%) in milk fat observed when ws were fed restricted roughage-high grain rations.

McClymont and Vallance (1962) have proposed that the cogenic nature (i.e., increased ruminal propionate duction) of restricted roughage-high grain rations may oke hormonal responses that suppress mobilization of fat m adipose tissue. This theory is the most difficult of three theories of milk fat depression to test. Several ervations lend support to this concept:

1) Glucose and propionate infusions have been shown to ease milk fat (Vallance and McClymont 1959, Storry and 1965b, Fisher and Elliot 1966, Fisher et al. 1967).

2) Infused glucose can cause increased secretion of lin (Folley and Greenbaum 1960, Tepperman and Tepperman).

3) Insulin can decrease yield of milk fat (Gowen and 1931, Rook et al. 1965) by promoting increased lipid e by adipose tissue. This may be accomplished by ased adipose tissue LPL activity (Wing et al. 1967, la and Pykalisto 1968) and fatty acid synthesis horn and Benjamin 1965) and by inhibiting the mobilization tty acids from adipose tissue.

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The conclusion from this hypothesis is that mobilization f long chain fatty acids from adipose tissue could be creased by restricted roughage-high grain rations. In this se arterial concentrations of plasma triglycerides would crease, causing reduced mammary uptake of triglyceride ng chain fatty acids. This has been observed in some stances (Storry and Rook 1965a) but not in others (Varman d Schultz 1968a, Huber et al. 1969).

The glucogenic theory has recently been strengthened by zymic studies in mammary and adipose tissues. Opstvedt al. (1967) and Baldwin et al. (1969) have observed a three four fold increase in the activities of several enzymes ociated with fatty acid synthesis and esterification in adipose tissue of lactating cows fed restricted roughage- h grain rations. Mammary enzymes were relatively unaffected the dietary treatment. A two-fold increase in the level of ? was observed in adipose tissue of cows exhibiting milk depression (Baldwin et al. 1969). This suggests that bolic conditions in adipose tissue increased fatty acid rification. Opstvedt et al. (1967) proposed that the of fatty acid esterification in adipose tissue was eased while fatty acid mobilization was decreased lting in a decreased availability of milk fat precursors he mammary gland.

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In summary
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The role of plasma triglyceride long chain fatty acids milk fat depression was further emphasized by the servations of Opstvedt and Ronning (1967) who quantitatively aluated the magnitude of change in the individual fatty ids in milk fat during milk fat depression. Reduced retion of fatty acids with 16 carbons or more accounted 74% of the reduced fat output in the milk fat depression erved.

In summary, nutritionally elicited milk fat depression the cow has been related to possible decreased acetate long chain fatty acid availability to the mammary gland to adaptive lipogenesis occurring in adipose tissue. ough proof of increased deposition of fat in adipose ue has been found, a deficiency of long chain fatty acids he mammary gland has not been demonstrated.

A. PROCEDURE
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CHAPTER III

METHODS AND MATERIALS

PROCEDURE FOR ASSAYING LIPOPROTEIN LIPASE FROM TISSUE HOMOGENATES OF BOVINE MAMMARY GLAND

The procedures used for this assay were a modification the methods of Korn (1959) and McBride and Korn (1963).

1. Preparation of Tissue for Assay

Mammary tissue from lactating cows was procured from a l abattoir. Whenever possible cows from the Michigan e University dairy herd with a known lactational history used. The tissue was removed within five minutes of ghtering, rinsed in ice cold (± 4°C) 0.15 M KCl, blotted heese cloth and either frozen immediately on Dry Ice or ed directly. Tissues to be assayed fresh were transported e laboratory in ice cold (± 4°C) 0.15 M KCl and assayed n 45 minutes of slaughter. Thin slices of tissue were ed on a direct reading balance sensitive to the nearest of a gram. The tissue was first disrupted in eight s of cold (± 4°C) M KCl with an Omni-Mixer (Ivan Sorvall, Norwalk, Conn.) and then homogenized with three passes

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f a teflon pestle in a Thomas (A. H. Thomas Co., Philadelphia, a.) glass homogenizer. A Powerstat (Superior Electric Co., ristol, Conn.) was used to adjust homogenization speeds to pproximately 1000 revolutions per minute. The homogenate as centrifuged 800 x g for 10 minutes in a refrigerated entrifuge at 0° centigrade. The 800 x g supernatant was ltered through glass wool to remove cream and cellular bris. The resulting filtrate is the fraction referred to homogenate. In some instances the homogenate was centrifuged rther (White et al. 1964). The material sedimenting after ntrifuging the 800 x g supernatant for 20 minutes at ,000 x g is referred to as "mitochondria." The material limenting after centrifuging the 12,000 x g supernatant for minutes at 100,000 rpm is termed "microsomes." The ction of the 800 x g supernatant sedimenting after trifuging at 100,000 x g for one hour is termed "particulate". some instances the 800 x g supernatant was further centrifuged 80,000 x g (Pynadath and Kumar 1964) for 45 minutes to ment the "particulate" fraction of the cell. The 80,000 x ellet was resuspended in buffer and centrifuged 12,000 x g 20 minutes to sediment the "mitochondrial" fraction. The 00 x g supernatant is referred to as the "microsomal" tion. High speed centrifugations were done at 0°C in a igerated preparative ultracentrifuge.

2. Prepa

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Ediol:6 parts

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2. Preparation of Substrate

The substrate was prepared from a commercial coconut oil
ulsion known as Ediol.[1] Pure Ediol was diluted 1.0 part
iol:6 parts water resulting in a ca 8.0% triglyceride
ncentration. Diluted Ediol will be referred to subsequently
Ediol. Ediol was "activated" by incubating with an equal
lume of fresh cow serum in a glass stoppered flask for
irty minutes at 37°C in a Dubnoff Metabolic Shaking
cubator (Precision Scientific Co., Chicago, Ill.,). This
the mixture referred to as "activated" Ediol or substrate.

Triglyceride concentration in "activated" Ediol was
culated after making the following assumptions: 1) Ediol
50% coconut oil; 2) Coconut oil is 100% triglyceride;
Average molecular weight of coconut oil triglyceride is
g/mole. One ml of activated substrate would then contain
54.3 µmoles of triglyceride.

3. Incubation Mixture

The incubation mixture consisted of 1.0 ml of 10% bovine
m albumin (BSA Fraction V Sigma Chemical Co., St. Louis,
adjusted to pH 8.5 with concentrated ammonium hydroxide,
able amounts of substrate (0.0 - 0.8 ml) and variable
nts of homogenate (0.0 - 0.3 ml). The mixture was made

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[1] Substrate-CB (Ediol) stable 50% emulsion of coconut oil, A
ade, CalBiochem, Los Angeles, Calif. Composition: Coconut
50%, sucrose 12%, glyceryl monostearate 1.5%, poly-
thylene sorbital monostearate 2.0%.

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In-Vitro Assay

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to a total volume of 2.0 ml with 0.15 M KCl and incubated
glass stoppered 25 ml flasks at 37°C for 30 minutes in a
abolic shaker (50 oscillations/minute). The incubation
ture used in standard assays for lipoprotein lipase
ivity is shown in Table 3.

Table 3

Vitro Assay System for Bovine Mammary Lipoprotein Lipase[1]

Component	Quantity
(Fraction V, 10%)	1.00 ml
um	0.25 ml
ol (8.0% triglyceride)	0.25 ml
x g supernatant	0.10 ml
(0.15 M)	0.40 ml

cubate 1/2 hour at 37°C in a 2.0 ml assay volume,
8.3. Serum and Ediol components pre-incubated
gether (1:1) at 37°C for 1/2 hour prior to assay.

4. Termination of Reaction

The reaction was terminated by the addition of 5.0 ml of
xture of heptane:isopropanol:1.0 N sulfuric acid (10:40:1)
tly into the incubation flask.

5. Extraction of Free Fatty Acids

Free fatty acids (FFA) were extracted by a modification
e method of Dole and Meinertz (1960). The terminated
ion mixture was allowed to stand at room temperature for

five minutes.
were added, th
glass test tub
two phases by
A 2.0 ml aliqu
was pipetted w

6. Titra

One ml of
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ive minutes. Two ml of distilled water and 3 ml of heptane
ere added, the contents shaken and transferred to a 15 ml
lass test tube. The mixture was allowed to separate into
o phases by standing at room temperature for five minutes.
2.0 ml aliquot of the upper heptane phase (containing FFA)
s pipetted without delay into a 5.0 ml glass vial.

6. Titration of Free Fatty Acids

One ml of indicator solution [9 parts redistilled
anol + 1 part 0.1% Nile Blue A (Allied Chemical Corp.,
., N.Y.) with the acidity adjusted so that 1.0 ml of the
icator solution required 10-15 µl of 0.02 NaOH for color
nge] was added to each vial. The contents were then
rated with 0.02 N NaOH (standardized against potassium
d phthalate). The titrant was delivered from a Manostat
tal reading pipette (Greiner Scientific Co., N.Y., N.Y.)
rate to 0.1 µl. The contents were titrated to a red-
et end point with continual bubbling of nitrogen through
two phase system. The nitrogen was bubbled through 0.1 M
phate buffer prior to delivery into the titrating flask
precaution against acidic contaminants. Known quantities
re palmitic acid were also extracted and titrated by
rocedure just described. Corrections for extraction
iency (80-90%) were made when appropriate.

7. Calcu

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B. PROCEDURE
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7. Calculation and Expression of Results

Appropriate substrate and homogenate blanks were run
ith each assay. Free fatty acid contributions from these
ources were subtracted from each estimate of enzyme activity
o reduce error. A sample calculation is shown below:

a) µl titrant attributable to enzyme activity = [µl
itrant total] - [µl titrant for enzyme blank]

b) µeq. FFA liberated = (a) x normality of titrant

c) µeq. FFA liberated/hr/g tissue = (b) x 2 (aliquot
orrection) x 2 (time correction) ÷ [% extraction efficiency
g tissue or mg. tissue protein used in the assay]

In some instances kinetic data (Km, Vmax) were derived
rom 1/V vs 1/S plots according to the method of Lineweaver
nd Burk (1934). Enzyme activity is expressed as µeq. FFA
berated/hour/gram tissue or µeq. FFA liberated/hour/mg.
tractable protein. Lipolytic activity toward "activated"
iol by mammary tissue is referred to as LPL activity.
though it is realized that a portion of this activity is
obably due to lipase(s) other than lipoprotein lipase.

PROCEDURE FOR ASSAYING GLYCERIDE SYNTHETASE FROM TISSUE HOMOGENATES OF BOVINE MAMMARY GLAND

The procedures used for this assay were a modification
those given by McBride and Korn (1964b).

1. Prepa

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2. Prepa

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Sonicatio

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accurately by

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Illinois)
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Illinois)
Linoleic aci
Illinois)
Linolenic ac
Illinois)
Sodium n-But
Des Plaines,
Potassium-ß-
From C. L. D
Science, Uni

1. Preparation of Tissue for Assay

The tissue preparation was as previously described for lipoprotein lipase (see A, 1).

2. Preparation of Substrate

Approximately 2 µc of fatty acyl-1-^{14}C [1] was added to 1000 µm of its unlabelled analog. Two ml of 0.1 N NaOH was added to convert the acid to its sodium salt. The mixture was emulsified in an ultrasonic cleaner for five minutes. Three ml of 0.1 M phosphate buffer pH 7.5 was added to bring the final volume to five ml. Aliquots of the substrate were counted to establish its specificity activity. Quenching was determined by internal standardization with ^{14}C-Benzoic acid. A typical substrate contained 0.02 µmoles fatty acid/ml, 1000 dpm/µl, or 50,000 dpm/µmole fatty acid.

Sonication of the substrate in an ultrasonic cleaner for five minutes gave an emulsion that could be transferred accurately by a microliter syringe with good repeatability.

---

[1] Palmitic acid-1-^{14}C, 56.2 mc/mM (Nuclear-Chicago, Des Plaines, Illinois)
Stearic acid-1-^{14}C, 48.4 mc/mM (Nuclear-Chicago, Des Plaines, Illinois)
Oleic acid-1-^{14}C, 43.2 mc/mM (Nuclear-Chicago, Des Plaines, Illinois)
Linoleic acid-1-^{14}C, 59.2 mc/mM (Nuclear-Chicago, Des Plaines, Illinois)
Linolenic acid-1-^{14}C, 41.5 mc/mM (Amersham/Searle, Des Plaines, Illinois)
Sodium n-Butyrate-1-^{14}C, 15.0 mc/mM (Nuclear-Chicago, Des Plaines, Illinois)
Potassium-ß-hydroxybutyric acid-3, 4, -^{14}C 15.0 mc/mM (Gift from C. L. Davis and D. S. Sachan, Department of Dairy Science, University of Illinois)

3. Incu

The comp

assays for gl

In-Vitro Assa

Compo
ATP
CoA
D, L-a-GP
$MgCl_2$
NaF
DTT
BSA
Na-palmitate-
800 x g superr
Phosphate buf

[1] Incubate 1 h

The incu

concentrations

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Mo.), Dithio
Cleveland, O
(Sigma Chemi

3. Incubation Mixture

The composition of the incubation mixture used in standard assays for glyceride synthesis is shown in Table 4.

Table 4

In-Vitro Assay System for Bovine Mammary Glyceride Synthesis[1]

Component	Concentration
ATP	10.5 mM
CoA	0.4 mM
, L-α-GP	20.0 mM
$MgCl_2$	2.0 mM
NaF	50.0 mM
DTT	4.0 mM
BSA	5.0 mg
a-palmitate-1-^{14}C	0.2 mM
00 x g supernatant	0.2 ml
hosphate buffer, pH 7.5	90.0 mM

Incubate 1 hour at 37°C in a 2.0 ml assay volume at pH 7.2

The incubation mixture contained the cofactors[1] in the concentrations shown in Table 4, variable (µl) amounts of substrate and variable (0.0 - 0.4 ml) amounts of homogenate

[1] Adenosene tri-phosphate, disodium salt (ATP) (Sigma Chemical Co., St. Louis, Mo.), Coenzyme A, free acid (CoA) (Sigma Chemical Co., St. Louis, Mo.), Magnesium chloride, hexahydrate ($MgCl_2$) (Baker Chemical Co., Phillipsburg, New Jersey), Sodium Fluoride (NaF) (Baker Chemical Co., Phillipsburg, New Jersey), D, L-α-glycerol-3-phosphate disodium salt (D, L-α-GP) (Sigma Chemical Co., St. Louis, Mo.), Dithiothreitol (DTT) (Nutritional Biochemicals Corp., Cleveland, Ohio), Bovine Serum Albumin, fraction V (BSA) (Sigma Chemical Co., St. Louis, Mo.).

in a final v
buffer. The
flasks at 37
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4. Ter

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washed twice
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5. Det

Ten ml
ml xylene, 4
(PPO), 160 g.
(α-NPO)] was
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Contamir
palmitate-1-

in a final volume of 2.0 ml with 0.1 M sodium phosphate buffer. The reactants were incubated in 25 ml glass stoppered flasks at 37°C for one hour in a metabolic shaker (50 oscillations/minute).

4. Termination of Reaction

The reaction was terminated by adding with 8.0 ml heptane:isopropanol (1:1) and 6.0 ml water:1.0 N sodium hydroxide (30:1). The mixture was transferred directly into a 15 ml glass test tube and allowed to separate into two layers. The heptane layer, containing neutral lipids, was washed twice with fresh 6 ml aliquots of water:1.0 N sodium hydroxide (30:1). This served to remove FFA as their sodium salts in the aqueous phase. A 2 ml aliquot of the heptane phase was transferred to a scintillation vial.

5. Determining Specific Activity of Product

Ten ml of scintillation fluid [770 ml paradioxane, 770 ml xylene, 460 ml absolute ethanol, 10g. 2, 5 diphenyloxazole (PO), 160 g. napthalene, 100 mg α-Naphthylphenyloxazole (α-NPO)] was added directly to the counting vial. Samples were counted in a Nuclear-Chicago model 720 liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Illinois) for two - ten minute counts.

Contamination of the heptane layer by nonesterified palmitate-1-^{14}C was determined. Following enzyme blank

incubations, o
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a) µm fa
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b) µm fa
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Enzyme ac
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ncubations, only approximately 100 cpm (0.002 µmoles) tributable to palmitate-1-^{14}C was found in the heptane yer after two washes with water:1.0 N NaOH (30:1). This ount of FFA contamination was constant regardless of the ecific activity of the substrate or the fatty acid-1-^{14}C sted. Error from nonesterified substrate fatty acid-1-^{14}C ntamination of heptane layer was estimated to be ca 2.0% 002 µmole contamination ÷ 0.100 µmole typical esterification).

Quenching was determined by adding a known amount of tope (^{14}C-benzoic acid) to the samples and recounting. nting efficiency through out the studies was approximately ercent. No quenching by products synthesized in the ceride synthetase system was observed.

6. Calculation of Data and Expression of Results

a) µm fatty acid esterified/hour/aliquot counted = in sample] - [DPM in blank] ÷ DPM/µm fatty acid in trate

b) µm fatty acid esterified/hour/gram tissue (or mg. ue protein) = (a) x 2 (aliquot correction) ÷ gram tissue ssay (or mg. tissue protein per assay).

Enzyme activity was expressed as µmole fatty acid ified/hour/gram tissue or µmole fatty acid esterified/ mg extractable protein. In some instances kinetic date Vmax) were derived from 1/V vs 1/S plots according to ethod of Lineweaver and Burk (1934).

C. ANALYT

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C. ANALYTICAL LIPID TECHNIQUES

1. Extraction Procedure

The following samples were all extracted similarly: ammary gland tissue homogenates, serum, rumen fluid, cream, nd glyceride synthetase reaction products. One volume of ample was extracted in a Teflon stoppered 250 ml separatory unnel two times with 10 volumes of chloroform:methanol 2:1). Chloroform:methanol extracts were collected in a ound bottom 250 ml rotary evaporation flask. The samples ere evaporated under reduced pressure at 40°C in a rotary aporator. The samples were immediately resuspended in xane:ethyl ether (1:1) and transferred to a clean separatory nnel containing 100 ml distilled water. The rotary evapora- on flask was rinsed three times with 5 ml of hexane:ethyl her. Each rinsing was transferred to the separatory funnel. e hexane:ether:water mixture was shaken and allowed to arate in two layers. The aqueous layer was discarded. e hexane layer was passed through anhydrous sodium sulfate collected in a Teflon lines screw cap 15 ml test tube. The ane was evaporated to dryness under a stream of nitrogen in 0°C sand bath. This lipid extract was then methylated ectly or separated by thin layer chromatography.

2. Thin Layer Chromatography (TLC)

One tenth ml of hexane:ethyl ether (1:1) was added to lipid extract described in (1) above. The extract was

applied with a 50 µl syringe to an Eastman 6061 chromatogram sheet precoated with silica gel G (Eastman Kodak, Rochester, N.Y.). The sample was developed in an Eastman Chromatogram Developing Apparatus (Eastman Kodak, Rochester, N.Y.). Neutral lipids were separated by developing the chromatogram sheet with hexand:ethyl ether:acetic acid (80:20:1). Polar lipids were separated by developing the chromatogram sheet with chloroform:methanol:ammonium hydroxide (75:25:4). The chromatogram sheet was sprayed with appropriate reagents listed in Table 5 to develop the lipid spots for visual observation. The color spray reagents listed in Table 5 were prepared as described by Randerath (1966).

Table 5

Color Spray Reagents for Detecting Lipid Classes on Chromatogram Sheets

Reagent	Lipid Class Detected
', 7' Dichlorofluorescein	All lipids
romothymol Blue	All lipids especially monoglycerides
ılfuric Acid:Acetic Acid	Cholesterol and cholesterol esters
ılybdenum Blue	Phospholipids
nier - Macheboeuf	Phosphatidyl choline
nhydrin reagent	Amino-lipids

An authentic neutral lipid standard containing monoglyceride, 1, 2- and 1, 3-diglycerides, free fatty acids and triglyceride was co-chromatographed with all netural lipid separations. A phospholipid extract of egg yolk and phosphatidic acid separated from egg yolk phospholipids was used as a standard in identification of polar lipids.[1] Phospholipid identification was further facilitated by the use of spray reagents that yielded color responses characteristic of the lipid class being identified.

Once the lipid classes were located on the chromatogram sheet their spots were either cut out and scraped into a counting vial for liquid scintillation counting or into a Teflon lined screw cap tube for methylation. When assaying for radioactivity, spots of equal size from a non-radioactive portion of the chromatogram sheet were scraped into a separate vial and used to allow estimates of quenching. Scintillation fluid was prepared according to Randerath (1966) [10.5 g 2, diphenyloxazole (PPO), 0.45 g p-bis-2-(4-methyl-5-henyloxazolyl-benzene) (POPOP), and 150 g naphthalene were ade up to 1500 ml with analytical grade paradioxane. The olution was then diluted to 1800 ml with distilled water.] en ml of this scintillation fluid was added to the scrapings the vial and counted in a liquid scintillation counter.

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[1] This standard was provided by the courtesy of L. Goodman and L. Dugan, Department of Food Sciences, Michigan State University.

3. Methylation of Lipids

Lipids were methylated by one of two methods, prior to gas liquid chromatography. Serum and cream samples from the cow milk fat depression experiment (to be described later) were methylated by the method of Dugan et al. (1966). When the fatty acid composition of free fatty acids became of interest a modification of the Boron trichloride (BCl_3) method was used. The method of Metcalfe et al. (1966) was modified as follows: The lipid extract from part (1) or TLC scrapings from part (2) were dissolved in 1 ml of benzene in a 15 ml Teflon lined screw cap tube. One ml of BCl_3-methanol reagent (10% methanol) was added. The tube was sealed, mixed well, and placed in a heating block at 100°C for 60 minutes.

At the end of 60 minutes the tubes were allowed to cool. The reaction was terminated by the addition of 1.0 ml distilled water. The contents of the tube were transferred to a 250 ml separatory funnel containing 100 ml 15% NaCl. The tube was rinsed three times with 2.0 ml pentane per rinse. All rinses were transferred into the separatory funnel. The funnel was shaken and allowed to separate into two layers. The lower aqueous layer was drawn off and discarded. The pentane layer was washed twice with 100 ml of 15% NaCl and once with distilled water. After the final rinse the pentane layer was passed through anhydrous sodium sulfate. The funnel and the sodium sulfate were rinsed twice with one ml pentane per rinse.

Sixty minutes was found ideal for good methylation efficiency. At 60 minutes known quantities of tripalmitin, cholesterol stearate, palmitate, and linoleate were methylated at 56%, 100%, 100%, and 98% efficiency respectively. Known quantities of palmitic acid and tripalmitin were applied to a chromatogram sheet, scraped and methylated. These lipids were recovered and methylated at 47-50% efficiency.

4. Gas Liquid Chromatography (GLC)

The methyl esters dissolved in pentane were evaporated o dryness under a stream of nitrogen in a 40°C sand bath. he methyl esters were resuspended in a volume of carbon lsulfide appropriate to achieve good recorder response when aliquot of the solution was injected into the chromatograph. nce all samples analyzed contained only trace quantities heptadecanoic acid (C-17), methyl esters of this acid re added to each sample as an internal standard. The mples were chromatographed isothermally on a Aerograph Hy- model 600 gas chromatograph (Varian Aerograph Co., Walnut ek, Calif.) equipped with a hydrogen flame detector and ached to a Sargent model SRL recorder (E. H. Sargent Co., cago, Ill.). The column was purchased from Applied Sciences oratories and had the following specifications: 15% Eff-1BP (Diethylene Glycol Succinate) on Gas Chrom P 80/100 , stainless steel 7 ft. x 1/8 in. O.D. Oven temperature 180°C. Nitrogen was used as the carrier gas at a flow of 70 ml/minute.

Detector response was measured with known quantities of
17 and found to be linear over the concentration range
sayed. Estimates of weight percent component fatty acids
samples was found to be identical whether peak height or
ak area was taken as a measure of recorder response. Peak
ight was routinely taken as a measure of recorder response.

A standard was run every four hours of chromatograph
erating time. Weight percent fatty acids in samples was
lculated as outlined in F and M Methods Bulletin No. 117.

Most of the solvents used were of highest purity
mmercially available, all were reagent grade or higher.
lvents were checked for contaminants by blank extractions,
thylation, and subsequent chromatographic separation. No
aces above background were noted when reagent blanks were
racted and chromatographed.

OTHER PROCEDURES

Protein was determined by the method of Lowry (1951).
roxyproline was determined by the method of Firschein and
ll (1966). Milk samples were tested for butterfat by the
ock method. Blood samples were drawn in vacuum tubes and
wed to clot for twelve hours at 10°C. Serum was prepared
entrifuging clotted blood at 1000 x g for 20 minutes.
serum was drawn off, gassed with nitrogen, sealed and
ed at -10°C until analyzed for fatty acids.

Milk samples were allowed to stand overnight at 10°C. One gram of the cream layer was removed; gassed with nitrogen, sealed and stored at -10°C until analyzed for fatty acids.

. SURGICAL PROCEDURES

Mammary tissue samples were obtained from twenty-two surgical biopsies of eleven cows. The surgery was conducted the Michigan State University Large Animal Veterinary linic. Immediately prior to biopsy the cows received 100 its of oxytocin intravenously. The residual milk was moved by hand milking. The udder was clipped and scrubbed th an iodine soap solution. The local anesthetic (xylocaine, cc, Astra Pharmaceutical Products, Inc., Worchester, Mass.) administered subcutaneously on the udder as a line block inches above the surgical field. The surgical field was hed again with iodine and rinsed with alcohol. Three to e grams of mammary tissue was cut out and rinsed in ice d (± 4°C) 0.15 M KCl. The tissue was blotted on cheese th and frozen immediately on Dry Ice. After bleeding was rolled an absorbable hemostat (oxycel, Parke, Davis and Detroit, Mich.) was placed in the wound. The inner ule and overlying connective tissues were sutured with bsorbable surgical gut. The skin was sutured with a absorbable suture. The wound was sprayed locally with nitrofurazone. Biopsied quarters were injected through eat canal with 25 cc of Darbiotic (S. E. Massengill Co.,

ristol, Tenn.) to prevent mastitis. The animals were given
) cc of Procaine Penicillin G (300,000 units/cc) intra-
uscularly immediately following biopsy, and 40 cc/day for
hree days post biopsy. Biopsied quarters were milked out
y hand at subsequent milkings until clot formation ceased.
hereafter machine milking was used. Sutures were removed
-3 weeks after biopsy. Adipose and liver biopsies were
aken simultaneously as described by Benson (1969).

. STATISTICAL DESIGN AND METHOD OF ANALYSIS

The basic experimental design employed in feeding
xperiments was that of the latin square as described by
teel and Torrie (1960). Three replicates of a 3 x 3
experiment I) and one replicate of a 2 x 2 (experiment II)
atin square were employed, involving a total of eleven
imals. An example of one replicate of a three 3 x 3 latin
uare is shown in Table 6.

Table 6

Experimental Design, Experiment I[1]

is	Period I	II	III
	MgO	N	RR-HG
	N	RR-HG	MgO
	RR-HG	MgO	N

reatment designations: RR-HG = Restricted Roughage-
igh Grain; MgO = Restricted Roughage-High Grain +
agnesium Oxide; N = Normal.

Each period was of approximately 30 days duration. At
end of each period mammary tissue was obtained by surgical
psy or slaughter. Adipose and liver tissue samples were
o obtained (Benson 1969). The energy requirement for each
mal was calculated from a feeding standard (Moe et al. 1963)
ediately prior to ration change. This requirement represented
inimum value. No animal received less energy than its
culated requirement. Grain feeding was increased and
hage feeding was decreased to achieve milk fat depression
HG treatment). The MgO treatment was identical to the
G treatment except that 1.0% MgO was included in the grain
ure. Typical rations are shown in Table 7.

Table 7

Typical Rations Fed, Experiment I

ion onent	Normal	Restricted Roughage - High Grain	Magnesium Oxide
		Kg	
	15.9	1.4	1.4
Silage	4.5	4.5	4.5
	5.0	15.0	15.0
	12.5	12.8	12.8

he composition of the grain mixture fed is shown in
8.

Table 8

Experimental Grain Ration

Components	Kg
Corn	613.6
Soybean Oil Meal	181.8
Molasses	68.2
Dicalcium Phosphate	11.4
Trace Mineralized Salt	9.1
Vit. A, IU/Kg	454.5
Vit. D, IU/Kg	45.5

One cow (330) sustained a lesion in the large intestine
ring surgical biopsy for abdominal adipose tissue and died
lor to completion of the third treatment (N). Hence values
orted for RR + MgO rations are averages of 9 determinations
le values for N rations are averages of 8 determinations.
to the missing data, the results of the nine cow 3 x 3
in square experiment were analyzed by the method of least
ares. Two cows were assigned to a 2 x 2 latin square
ign feeding experiment to confirm the data of the previous
3 experiment and allow certain analyses to be conducted
issue samples that were not measured in the previous
riment. The design of this experiment is shown in
e 9.

Table 9

Experimental Design, Experiment II[1]

Cow	Period I	Period II
444	N	RR-HG
445	RR-HG	N

[1] Treatment designations: RR-HG = Restricted Roughage-High Grain; N = Normal.

The rations for this experiment differed from the previous
eriment in these respects: 1) No corn silage was fed;
No hay was included in the RR-HG treatment; 3) MgO was not
d.

The grain mixture was the same as that shown in Table 8.
ical rations fed are shown in Table 10.

Table 10

Typical Rations Fed, Experiment II

on Component	Normal	RR-HG
Hay	11.4	0.0
Grain	7.3	14.5
TDN	11.2	10.9

Enzyme velocities were determined at fixed substrate
ntrations existing in the range of substrate saturation

enzyme. All three tissue samples from any one cow were
sayed simultaneously. Lipoprotein lipase and glyceride
nthetase assays were conducted on the same tissue homogenate.
ch assay was conducted simultaneously in triplicate and the
an value reported.

CHAPTER IV

RESULTS AND DISCUSSION

To be valid, an enzyme assay must satisfy at least ree conditions: 1) activity must be proportional to the ount of enzyme added, 2) activity must be constant during e time period of the assay, and 3) the assay (fixed bstrate assay) must be conducted at saturated substrate ncentrations (Reiner 1959). The importance of measuring tial velocity is emphasized by Dixon and Webb (1964). y at the initial point in an enzyme assay where unknown iables (i.e., pH change, substrate disappearance, cofactor ltation, end product inhibition) have not had time to ome operative are assay conditions accurately known. n and Webb (1964) list the chief factors affecting initial city as enzyme concentration, substrate concentration, pH, ence or absence of activators or inhibitors, and temperature. n and Webb (1964) state that the effect of a variable e tested on the initial velocity of an enzyme should be rmined by varying only one factor at a time and holding thers constant.

The above criteria were adhered to in the determination
nzyme activity reported in these studies.

CHARACTERIZATION OF LIPOPROTEIN LIPASE (LPL)

Studies were conducted to devise a method of assaying and
acterizing some of the properties of LPL in bovine mammary
ue prior to investigating its role in milk fat depression.

1. Evaluation of Analytical Capabilities of the Assay System

Since an estimate of enzyme activity is only as accurate
he method detecting that activity, an evaluation was made
ome of the variables of the Dole extraction procedure.
n quantities of palmitic acid were subjected to a modifica-
of the Dole procedure to ascertain the analytical
bilities of the system. The modified Dole procedure
cted 93.4% of the added palmitic acid and gave a linear
onse from 0.2 - 1.4 µmoles of palmitic acid standard.
and Meinertz (1960) stressed the importance of pH of
aqueous phase and length of standing time during the
ction procedure. Studies were conducted to ascertain
mportance of these variables in this system. The
ts are given in Table 11.

These results indicate that 1) the pH of the aqueous
need not be adjusted, 2) standing time is not critical,
3) one-half the volume of extractants recommended by

Table 11

Evaluation of Variables in Dole Procedure[1]

l r	Adjusted Aqueous Phase pH	Standing Time (Minutes)	Volume of Extractants Recommended by Dole %	µeq. FFA/hr./ g. tissue[2]
	1.5	5	50	127.0
	2.0	5	50	132.0
	3.3	5	50	129.0
	3.3	15	50	129.0
	3.3	30	50	127.0
	3.3	60	50	128.0
	3.3	5	100	127.0

trials conducted simultaneously with the same tissue
rce. All determinations conducted in triplicate, and
rage values are given. Incubation conditions were as
cribed in Table 3. Similar results were obtained in
er studies wherein each variable was investigated
arately.

n value ± standard error of mean = 128.4 ± .7.

and Meinertz (1960) can be satisfactorily used for this

Dole and Meinertz (1960) also emphasized that the
extraction procedure may not be adequate for the study
sue lipids due to contamination of the heptane layer
rganic acids and acidic phospholipids from the aqueous

With mammary tissue, the double extraction procedure
to be no more accurate than the single extraction
ure. Therefore, the single extraction procedure was
outinely throughout this study.

An estimate of repeatability of assay can be obtained
n the results of Table 11. The assay was very repeatable
e the titration end point was mastered. Typical values
quadruplicate incubations under identical conditions from
same homogenate were 111, 116, 117, 119 µeq./hr./g. tissue
5.8 ± 1.7). Typical values for quadruplicate incubations
er identical conditions from four separate homogenates of
same tissue were 143, 138, 129, 146 µeq./hr./g. tissue
9.0 ± 3.7). Most of the values reported in LPL characteriza-
n studies are the average of at least two identical
ltaneous incubations. Tissue from a total of four cows
used for the majority of the characterization studies.
e results were further supported by substrate saturation
tic data of eleven cows from the feeding study described
aterials and methods.

2. Cofactor Requirements

a) Cation and free fatty acid (FFA) acceptor

According to Korn (1959) LPL from tissues requires a
acid acceptor and a divalent cation or ammonium ion.
ystem was found to be stimulated by FFA acceptor, bovine
albumin (Figure 1) but was inhibited at all concentrations
$H_4)_2SO_4$ or $CaCl_2$ (Figure 2). These results do not
de information on the requirement of NH_4^+ for this system
the pH of the BSA in the incubation mixture was adjusted
NH_4OH. On the basis of the results just discussed cation

was omitted from the incubation mixture. The concentration was selected to be 100 mg/2.0 ml of incubation mixture. ris and Krebs-Ringer phosphate buffers were tried but t enhance FFA release when compared to BSA in 0.15 M KCl.

b) Activation of substrate

nen artificial triglyceride emulsions (Ediol) are used ubstrate in LPL assays, they must be first "activated" ubation with serum (Korn 1959). The ratio of serum to was found to be critical in activating the substrate e 3). All determinations were conducted at equal trations of Ediol (2.0 mg triglyceride). The optimum of serum:Ediol was found to be one part serum to one Ediol. This corresponded to 0.125 ml serum/mg of ceride. This value is in general agreement with (Robinson 1963b) who found 0.8 ml serum/mg of eride to optimum for Ediol activation.

activated Ediol (0% serum) was hydrolyzed only 17% as vely as activated Ediol (50% serum, Figure 3). This y may represent that portion of the total activity table to a lipase other than lipoprotein lipase. tively a portion of the 17% activity at 0% serum may butable to partial activation of substrate by te proteins. The decrease in effectiveness of high

---

ferences to Ediol will be to Ediol diluted one part six parts distilled water (8% triglyceride).
ated" Ediol or substrate refers to Ediol pre-incubated n equal quantity of serum one-half hour at 37°C.

;rations of serum to activate ediol may be due to the
;e of an inhibitor in the serum (Robinson 1963b).

c) pH optimum

lpoprotein lipase has been reported to function best
aline pH (Robinson 1963b). In these studies LPL
ty was found to be dependent upon pH. Optimum activity
nieved between pH 8.2-8.5 (Figure 4). In all further
a pH of 8.3 was used. The pH of the incubation
e decreased to 8.1 after one hour of incubation. The
imum determined here is in agreement with Korn (1959)
und a pH optimum of 8.5 for adipose lipoprotein lipase.

d) Activation by heparin

eparin has been demonstrated to be a cofactor for LPL
y (Korn 1957).

e addition of heparin to the incubation medium for
sed 3.0-25.0% increased in lipolytic activity. Two
nt sources of heparin were tried with identical results.
stimulation of LPL activity for several different
tissue sources and several levels of heparin are
n Table 12.

two out of the three tissues tested, heparin stimulated
vity. The third tissue was inhibited by heparin at a
ation demonstrated to be stimulatory for the other two

Table 12

Heparin Stimulation of Bovine Mammary LPL[1]

ssue Condition	Heparin added units/ml incubation mixture	Stimulation[2] %
Frozen	0.07	12
Fresh	0.50	24
Fresh	3.00	26
Fresh	5.00	25
Frozen	0.50	3
Frozen	1.00	1
Frozen	3.00	-5

y conditions were those described in Table 3, except rin was added as indicated.

ulation = Percent increase in FFA release above non-rin control.

s. Tissue of animal 1120 was analyzed frozen while s of 812 (with one exception) and 773 were analyzed Freezing may destroy the ability of the tissue to i to heparin. This conclusion cannot be drawn from udy since the tissue of 1120 was not tested for heparin ition prior to freezing. There is also some evidence ie failure of tissue of 1120 to respond to heparin may ndividual tissue difference since that of 812 (frozen) mulated 12% by 0.07 units/ml heparin. Figure 5 ates the effect of heparin on kinetics of FFA release ary tissue homogenates from cow 812.

In another experiment tissue was homogenized in buffer
aining heparin (0.7 units/ml) and compared to the same
ue homogenized in the absence of heparin. A 11.7%
ulation in activity was observed indicating that the
rity of the enzyme was liberated upon homogenization of
issue in the absence of heparin.

The response of bovine mammary tissue to heparin was
mediate to that observed for guinea pig mammary tissue
ported by Robinson (1963a) and McBride and Korn (1963).
son used acetone-ether powders of the tissue while
de and Korn used tissue homogenates. Method of tissue
ration may possibly explain differences in response to
in. Heparin stimulation of bovine mammary tissue (3-25%)
ess than that (50-60%) reported for rat heart and adipose
(Gartner and Vahouny 1966, Ho et al. 1967).

Due to the small amount of stimulation and variable
se to heparin observed in these studies, heparin was
d from the assay system for bovine mammary LPL.

) Inhibition by sodium chloride

odium chloride has been reported to be a potent
tor of LPL at concentrations of 0.35-1.0 M (Korn 1959).
chloride present at 1.0 M in the assay system used in
esent studies caused a 90% inhibition of lipolytic
y (Figure 5). Sodium chloride present at 0.25 M

t shown) caused a 64% inhibition of FFA release. The
h degree of inhibition by 1.0 M NaCl suggested that the
ority of the observed lipolytic activity was attributable
lipoprotein lipase. Monoglyceride lipase activity is
slightly inhibited by 1.0 M NaCl (Biale and Shafrir 1969,
en et al. 1969). Increasing the concentration of
trate while keeping NaCl concentration constant did not
rse inhibition. These results are in agreement with the
ept (Patten and Hollenberg 1969) that NaCl inhibits LPL
nteracting directly with enzyme but not with the substrate.

3. Kinetics of Lipoprotein Lipase

Kinetic data was obtained by measuring the velocity of
ion in response to variable concentrations of homogenate,
rate, and length of incubation period. During a one
incubation period response to variable amounts of tissue
enate was linear to 4.0 mg tissue/ml of incubation
n (Figure 6). The reaction was linear during 30 to 60
es of incubation time (Figure 7). The departures from
rity observed with homogenate concentration and time
ize the importance of selecting a value for these
les that will allow a true estimate of initial velocity.
minutes incubation time and 2-5 mg tissue/ml of
tion medium were selected for use in routine assays.

Saturation kinetics were exhibited in response to reasing levels of substrate (Figure 8). An apparent Km Vmax were determined by Lineweaver Burk transformation he data shown in Figure 8, and plotting as shown in re 9. Although a Km for an impure enzyme(s) using a ly defined complex substrate is of limited value, the ulations were made to allow comparisons between values rted in the literature (using a similar substrate) and es obtained with this system. A Km of 2.3 mM triglyceride a Vmax of 532 µeq. FFA/hr. g. tissue were obtained. This alue is comparable with 6.1 mM Km triglyceride found by (1962b) for LPL of chicken fat. McBride and Korn (1963) ned velocities of 600-900 µeq. FFA/hr./g. of guinea pig ry tissue. Such values are in the same range as those mined for bovine mammary tissue in this investigation.

4. Subcellular Localization of Lipoprotein Lipase Activity

Mammary tissue was homogenized and separated into the llular fractions shown in Table 13 as outlined in ials and methods. Each sedimenting fraction was re-ded in a volume of buffer equal to that from which it rived. Lipolytic activity towards "activated" Ediol sted on each fraction (Table 13).

he 80,000 x g supernatant corresponds to the soluble on of the cell, the 80,000 x g pellet to the particulate on, the 12,000 x g supernatant to the "microsomal"

Table 13

Subcellular Localization of Bovine Mammary Lipoprotein Lipase Activity[1]

Fraction	Total Activity[2]	Protein[3]	Specific Activity[4]
g Supernatant	33.3	14.2	2.35
,000 x g Supernatant	6.4	9.8	0.65
,000 x g Pellet	25.1	5.1	4.92
ended 80,000 x g Pellet:			
000 x g Supernatant	9.0	1.8	5.00
000 x g Pellet	13.9	2.7	5.15

lar results were obtained in two other experiments that mented the particulate fraction at 100,000 x g.

l Activity = µeq. FFA released/hr./ml fraction assayed

ein = mg. extractable protein/ml fraction assayed

ific activity = µeq. FFA released/hr./mg protein

on and the 12,000 x g pellet to the "mitochondrial" on. It should be emphasized that these fractions are y not pure since their identity was not rigorously shed. The majority of the 800 x g supernatant y was found in the particulate fraction. When the were expressed on a extractable protein basis the late activity was distributed about equally between tochondrial" and "microsomal" fractions. In three e determinations (not shown) 95-100% of the total ite activity (prior to centrifuging 800 x g) was in x g supernatant. These results are similar to those

ollet and Auditore (1967) and Gartner and Vahouny (1966) found 75 and 73% of the lipolytic activity associated with particulate fraction of rat adipose and rat heart tissue ectively. Gartner and Vahouny (1966) found most of the iculate activity was in the fraction corresponding to osomes, while Hollet and Auditore (1967) found more of particulate activity in the fraction corresponding to hondria. The 20% lipolytic activity attributable to the 0 x g supernatant in this study may represent the activity lipase other than lipoprotein lipase. Gorin and Shafrir) found most of the monoglyceride lipase activity of pididymal fat pad cells to be located in the soluble on of the cell. Data shown in Figure 3 also indicate FFA were liberated from non-activated Ediol at 17% of ctivity of activated Ediol, suggesting the possible nce of a lipase other than lipoprotein lipase.

5. Lipoprotein Lipase of Cows Milk

Gorn (1962) reported the presence of a lipase in cows hat had many of the properties of lipoprotein lipase. ination of mammary tissue by variable amounts of milk potentially cause variation in assessment of tissue activity. Lipolytic activity of cows milk was igated prior to assessing the potential contribution lipolytic activity to tissue lipolytic activity.

resh milk from selected cows of the University Dairy
as centrifuged 800 x g for ten minutes to facilitate
ion of a cream layer at the top of the centrifuge tube.
lk was then filtered through glass wool to remove
. The resulting skim milk was diluted by mixing one
kim milk with nine parts cold (± 4°C) 0.15 M KCl.
s of the diluted skim milk were analyzed for lipolytic
y. The assay system was similar to that previously
ed for the assay of LPL of mammary tissue, except
3.0 ml incubation volume was employed instead of a
volume.

tty acid release was tested and found linear from
0.7 ml of diluted skim milk and for 30 minutes
ion time. Some of the characteristics of the lipolytic
e of skim milk dependent upon activation of Ediol are
n Figure 10. Lipolytic activity in the presence of
"non-activated" Ediol or "activated" Ediol plus NaCl
ed that 14-15% of the lipolytic activity of skim milk
"activated" Ediol was the result of lipases other than
tein lipase. Further characteristics of the lipolytic
of cows milk are listed in Table 14.

Table 14

Lipolytic Activity of Cow's Milk Toward Endogenous and Exogenous Triglyceride[1]

Milk	Whole Milk	Serum	"Activated" Ediol[2]	FFA Release
	ml	ml	ml	µeq. /hr./ml milk
5	0	0.5	0	0
5	0	0	1.0	66
	0.5	0	1.0	62

e results were obtained using fresh milk from one cow. lar results were obtained when one day old refrigerated from several different cows was used.

ivated" Ediol contains 0.5 ml serum + 0.5 ml Ediol.

lthough LPL (or a lipase with similar characteristics to as present in milk it did not hydrolyze the triglycerides n milk or whole milk in the presence of serum (Table 14). 1962a) found similar results using cream plus serum as ate for milk lipoprotein lipase. The triglycerides of oil (Ediol) were hydrolyzed in the presence of serum this study and the study of Korn (1962a). These indicate that LPL is present in milk but does not n at that locus. The appearance of LPL in milk may result of cell rupture during fat secretion (Korn

Heparin did not stimulate milk lipolytic activity ctivated" Ediol was used as substrate (Table 15).

Table 15

Lipolytic Activity in the Presence of Heparin[1]

ubstrate[2]	Heparin	FFA Release
	(units/ml)	(μeq. FFA/hr./ml milk)
(Activated)	0.0	229
(Activated)	0.3	228
(Activated)	1.6	228
(Non-activated)	0.0	40
(Non-activated)	0.3	35
(Non-activated)	1.6	13

ar results were obtained in a previous study where one concentration of heparin (1.6 units/ml) was yed.

ated Ediol = Serum + Ediol; Non-activated Ediol =

at similar concentrations inhibited lipolytic activity cubated with "non-activated" Ediol. If the addition rin to "activated" Ediol inhibited the non-LPL activity / slight stimulatory effect of heparin on LPL activity e been masked.

apparent Km value was calculated by the method of er and Burk for milk LPL from the upper substrate on curve shown in Figure 10. Values were based on lipolytic activity using "activated" Ediol as well his activity minus lipolytic activity using "non- i" Ediol. A Km of 1.0 mM triglyceride was obtained

time, indicating that the presence of other lipases did
nfluence the affinity of LPL for its substrate at high
rate concentrations. The 1.0 mM Km for milk LPL is
than but similar to the 2.3 mM Km previously calculated
ammary tissue.

Since milk possesses LPL activity it appeared possible
this activity might be related to milk fat test. If a
relationship did exist, then measurement of milk LPL
ity might provide a convenient method of estimating
e LPL activity. Milk samples were collected from eleven
ting Holstein cows and analyzed for LPL activity. The
ts are shown in Appendix Table 1. Lipoprotein lipase
ity was not positively related to fat test either on a
milk basis ($r = -0.3$) or total daily milk production
($r = -0.6$).

An estimation of the contribution made by milk to tissue
easurements was made. The following assumptions were
1) the average weight of an udder was 20.0 kg;
s quantity of tissue might contain 10.0 kg of milk;
olytic activity of milk equalled 200 µeq./hr./ml milk;
olytic activity of tissue equalled 600 µeq./hr./g
. The udder plus the milk would weigh 30 kg, and every
this tissue sampled would contain 0.33 g milk.
ic activity of 0.33 ml of milk would be 0.33 ml x 200

q./hr./ml = 66.00 μeq./hr. Dividing this figure by the
tal activity of a gram of tissue, 66 ÷ 600, indicated that
proximately 10% of the total lipolytic activity of a gram
tissue could be attributed to milk. The actual contribution
milk would probably be much less since the assumed quantity
milk would probably not be present when tissues were sampled.
o a portion of the milk present would be in ducts and
terns rather than the tissue proper. Oxytocin injections
or to tissue sampling would further remove a large portion
the milk present in the lumen of alveoli cells.

In summary milk possesses a lipase with properties
llar to tissue lipoprotein lipase. This lipase accounts
greater than 80% of the lipolytic activity of milk when
ed on serum activated Ediol. Any contribution of milk
to tissue LPL activity would probably be less than 10%.

6. Other Factors Influencing Lipoprotein Lipase Determinations

Since the assay of activity in frozen tissues would be
derably more convenient than assaying fresh tissue, the
t of freezing upon LPL activity was investigated. The
ts are shown in Table 16.

The results indicated that tissue samples could be frozen
tored at -10°C until assayed. The maximum storage period
e loss of LPL activity was not determined. Samples stored
ive months still retain high levels of LPL activity.

Table 16

ipoprotein Lipase Determinations on Fresh and Frozen Tissue[1]

w	Fresh	Frozen	% Change[2]
	- - µeq. FFA/hr./g. tissue - -		
	115.5	106.9	- 7.4
	136.0	141.5	+ 4.0

Slaughter tissue was obtained from two cows. A sample was removed and assayed immediately, the rest of the tissue was frozen and assayed 3 weeks later. Assay conditions were those of Table 3.

% Change = (Activity fresh - Activity frozen) ÷ Activity fresh.

The biopsy technique was sometimes used in securing sue samples. To determine if the quarter of the udder pled influenced estimates of LPL activity, slaughter sue samples were obtained from all four quarters of an er and assayed for LPL activity (Table 17). The quarter the udder sampled had little effect upon the LPL determina- is in the mammary gland.

7. Relationship of Lipoprotein Lipase Activity to Lactation

A biopsy sample was obtained from a lactating Holstein producing 12 kg of 3.9% fat milk. One month after ation of lactation the animal was slaughtered and non- ating tissue was obtained. The two tissues (lactating

Table 17

ipoprotein Lipase Activity from Each of Four Quarters in One Mammary Gland[1]

uarter Sampled	LPL Activity[2]	Mean ± SE
Left front	142.6	
Right front	137.5	
Left rear	129.0	138.8 ± 3.7
Right rear	146.0	

All samples assayed simultaneously under identified incubation conditions. Conditions of assay were as shown in Table 3.

LPL activity = µeq. FFA released/hr./g. tissue

d non-lactating) were analyzed simultaneously for LPL tivity. Lipoprotein lipase activity was virtually absent the non-lactating tissue (20 as compared to 170 µeq. FFA leased/hr./g. tissue; non-lactating and lactating respectively). e low level of LPL activity from non-lactating mammary tissue rees with previous findings for non-lactating guinea pig mary tissue (McBride and Korn 1963, Robinson 1963a).

8. Summary of Characteristics of Bovine Mammary Lipoprotein Lipase

The activity of LPL in lactating bovine mammary gland ogenates was found to be dependent upon the concentration BSA, serum, and pH. Contrary to findings with other tissues rn 1959) the cations Ca++ and NH_4+ did not stimulate bovine mary tissue LPL activity and were found to be inhibitory.

eparin caused variable degrees of stimulation depending
pon its concentration and the tissue studied. The optimum
mount of serum for substrate activation was found to be
.125 ml/mg Ediol triglyceride. The greatest lipolytic
ctivity was achieved between pH 8.2-8.5. The majority
30%) of cellular lipolytic activity was associated with the
rticulate fraction. Lipoprotein lipase activity was
milar in all quarters of the udder, unaffected by freezing,
d greatly reduced in non-lactating tissue.

The following lines of evidence suggested that the
jority of the lipolytic activity determined on bovine
mmary homogenates in these studies was attributable to
oprotein lipase:

1) Eighty-three percent of the lipolytic activity was
pendent upon prior substrate activation by serum (Korn 1959).

2) Ninety percent of the lipolytic activity was inhibited
1.0 M NaCl, a specific known inhibitor of LPL (Korn 1959,
li and Shafrir 1969, Greten et al. 1969).

3) Eighty percent of the lipolytic activity was associated
h the particulate fraction of the cell [most of the mono-
ceride lipase activity is associated with the soluble
ion of the cell, Gorin and Shafrir (1964)].

4) A slight heparin stimulation (3-25%) was noted (Korn
', Robinson 1963b).

5) An alkaline pH optimum (8.2-8.5) was observed (Korn
).

6) Lipolytic activity was associated with lactation .e., little was found in non-lactating mammary tissue) cBride and Korn 1963, Robinson 1963a).

7) An apparent Km of 2.3 mM triglyceride was obtained, milar to that found for adipose tissue (Korn 1962b).

The following observations are suggested as possible nitations of the assay: 1) the previously mentioned lines evidence also indicate that 10-20% of the lipolytic activity bovine mammary tissue is due to a lipase other than oprotein lipase; 2) the high lipolytic activity in the ence of heparin and the variable response to exogenous arin suggests adequate but variable endogenous levels of s mucopolysaccharide in mammary tissue (if indeed a uirement exists); 3) demonstration of LPL activity in suggests milk contamination of tissue may cause variations he activity observed, but not more than 10%; 4) the lex nature of the substrate employed in *in-vitro* assays the necessity of activation of this substrate by a able biological fluid (serum) does not lend itself to fully controlled assays. Nevertheless, the assay was repeatable utilizing this substrate preparation.

The assay system developed does appear to reflect known protein lipase *in-vitro* and *in-vivo* responses and probably be considered as adequate as many of the assay systems for

reported in the literature. The final assay system
imized for the determination of bovine mammary lipoprotein
ase activity is shown in Table 3. The assay shown is for
ixed substrate, fixed enzyme, fixed time assay. In actual
lications to other systems, either the substrate or the
ogenate concentration should be varied to ensure enzyme
uration.

CHARACTERIZATION OF GLYCERIDE SYNTHESIS

1. Evaluation of Analytical Capabilities of the Assay System

McBride and Korn (1964b) stated that the extraction
tem used in this assay does not quantitatively extract
spholipids and monoglycerides. An estimate of the amount
monoglyceride and phospholipid extracted from a typical
eride synthetase incubation mixture by the heptane:
propanol:water:1.0 N NaOH (20:20:30:1) mixture, as compared
hloroform:methanol (2:1) was made. Heptane extractable
ds (mostly neutral lipids) and chloroform:methanol
actable lipids (all lipid classes) were separated by thin
r chromatography following incubation of mammary tissue
^{14}C-palmitate and appropriate cofactors. The spots
esponding to each lipid class were detected, scraped, and
ed. The distribution of label in the lipid classes is
1 in Table 18.

Table 18

Distribution of ^{14}C-Palmitate in Mammary Lipid Classes Following Extraction by Tow Methods[1]

Lipid Class	Heptane Extracted	Chloroform Extracted
	- Percent of Total Fatty Acid Esterified -	
Triglyceride	34	23
Diglyceride	51	36
Monoglyceride + Phospholipid	15	41

[1] Conditions of assay were those of Table 4. Data presented are from a total of three separate trials. Values for heptane extracted lipids are the averages from two tissues; 1120 and 445. Values for chloroform extracted lipids are from tissue A.

The comparison between heptane and chloroform (Table 18) as not conducted on the same animal and as such is only dicative of the general distribution of esterified fatty id in the two extraction procedures. In comparison to the loroform extraction, heptane contained only one-third as ch palmitate-1-^{14}C esterified as monoglyceride and spholipid. Although monoglycerides and phospholipids re not clearly separated on the chromatogram sheet proximately 60% of their combined activity was associated h the monoglyceride fraction (Chloroform extraction). se results indicated that less than 10% (.60 x .15) of palmitate-1-^{14}C esterified in heptane extractable lipids

in the routine assay could be attributable to palmitate esterified to phospholipids. These results also indicated that the relationship between the di- and triglycerides was constant regardless of the method of extraction.

The assay was very repeatable on the same homogenate, but when homogenates from the same tissue were prepared on different days more variability resulted. Table 19 show typical results from a single homogenate assay with five levels of substrate.

Table 19

Repeatability of Glyceride Synthetase Assay on a Single Homogenate[1]

	Concentration of Palmitate-1-^{14}C, mM				
Average Palmitate-1-^{14}C Esterification Rate (μmole/hr./g ± SE)	0.02	0.05	0.07	0.10	0.15
	1.05 ± 0.05	2.32 ± 0.10	2.81 ± 0.01	3.19 ± 0.06	3.12 ± 0.05

[1] Tissue 330. Conditions of assay were those shown in Table 4 except substrate was varied as indicated. Each substrate level was assayed in triplicate and esterification rate expressed as the average ± standard error of the mean.

The same tissue (330) was assayed a total of seven times on seven different days during a month's time period. A value of 2.56 ± 0.24 μmole palmitate esterified/hr./g tissue (range 1.7 to 3.3) was obtained by averaging these values.

All direct comparisons reported between tissues or several treatments on the same tissue were conducted during simultaneous incubations to reduce variability. Most of the values reported are average values of simultaneous duplicate incubations.

2. Cofactor Requirements

a) Incubation media components

Cofactor requirements recommended by McBride and Korn (1964b) for guinea pig mammary tissue were selected as a reference system for cofactor investigations. The µmoles of palmitate esterified per hour per gram of tissue by this system was designated 100 percent for comparative purposes. Palmitate esterification in response to varying the concentration of each cofactor while the other cofactors were held constant is given in Table 20.

The system was highly dependent upon an energy source (ATP), fatty acid activator (CoA), and fatty acid acceptor (α-GP). The system was also stimulated by $MgCl_2$ and to a lesser extent by NaF. Both $MgCl_2$ and NaF probably exerted their effect through ATP. Magnesium is a cofactor in the activation of fatty acid to its CoA derivative. Sodium fluoride possibly spared ATP by inhibiting an ATP'ase. In a separate study, α-monopalmitin was not an effective acyl acceptor and could not replace the requirement for α-glycerol

Table 20

Cofactor Requirements for Palmitate Esterification by Bovine Mammary Gland[1]

Component	Concentration	Relative Incorporation
	mM	%
ATP	0.00	10
	1.75	84
	[3.50]	100
	7.00	118
CoA	0.00	26
	0.10	98
	[0.20]	100
	0.40	114
α-GP	0.00	32
	10.00	109
	[20.00]	100
	40.00	112
$MgCl_2$	0.00	52
	0.10	76
	[0.20]	100
	0.40	110
NaF	0.00	80
	12.50	95
	[25.00]	100
	50.00	115

[1] Reference system values bracketed [ ]. Each value is the average of duplicate incubations with the same tissue source (1120). Reference system esterified 0.73 µmoles palmitate/hr./g. Two-tenths ml of a 1:8 homogenate (800 x g supernatant) was the enzyme source.

phosphate. All concentrations of α-monopalmitin (4-32 mM) were inhibitory to palmitate esterification.

Since each cofactor stimulated palmitate incorporation 10-18% at double its concentration in the reference system, all cofactors (except α-GP) were doubled. Increasing the concentrations of the cofactors two-fold double palmitate esterification. Each cofactor was subsequently investigated at higher concentrations without observing further increases in palmitate esterification compared to the revised system shown in Table 21. Magnesium chloride exhibited a broad optimum, eliciting no further stimulation or inhibition of palmitate esterification when tested at concentrations of 0.4-4.0 mM. Since 2.0 mM was similar to concentrations used by other investigators, the $MgCl_2$ concentration was arbitrarily raised from 0.2 to 2.0 mM. Further additions of energy to the system in the form of ATP inhibited palmitate esterification. The effects of ATP concentration on palmitate esterification are shown in Figure 11. This figure will also be referred to later during discussion of the effects of BSA and Dithiothreitol (DTT).

Various other cofactors were arbitrarily added to the revised system in a survey experiment to ascertain if further stimulation might be elicited. The rationale behind the addition of each compound is set forth in parenthesis following

Table 21

Palmitate Esterification in the Presence of Doubled Cofactor Concentrations[1]

Component	mM	μm Palmitate/hr./g	Relative Incorporation
ATP	3.5		
CoA	0.2		
α-GP	20.0	0.584	100%
$MgCl_2$	0.2		
NaF	25.0		
ATP	7.0		
CoA	0.4		
α-GP	20.0	1.212	208%
$MgCl_2$	0.4		
NaF	50.0		

[1] Each assay conducted in triplicate using the same homogenate (1120). Average values are reported. Each incubation was conducted for 60 minutes at 37°C pH 7.2. Two-tenths ml of a 1:8 homogenate (800 x g supernatant) was the enzyme source.

the name of each compound: NADH (source of reducing potential), glucose-6-phosphate (energy source, glycerol source), glutathione (sulfhydryl group protector), dithiothreitol (sulfhydryl group protector), bovine serum albumin (physiological presentation of FFA). The effects of these additions are shown in Table 22. Palmitate esterification in the presence of these additions is expressed as percent of palmitate esterified by the control system which is defined in the upper portion of the table.

Table 22

Palmitate Esterification in the Presence of Various Cofactors[1]

Addition	mM	% of Control
ATP, CoA, α-GP, $MgCl_2$, NaF } Control System	7.0, 0.4, 20.0, 2.0, 50.0	100
NADH	5.0	52
	10.0	29
G-6-P	1.5	93
	3.0	90
Glutathione	1.5	103
	3.0	103
Dithiothreitol	2.0	125
	4.0	136
	6.0	156
	8.0	155
BSA	2.0 mg	115
	10.0 mg	226
	20.0 mg	210
	30.0 mg	162
	40.0 mg	163

[1] Palmitate esterification is expressed as percent of that esterified by the control system. All values were determined on the same tissue homogenate (330). The value reported for the control system is the average of duplicate incubations. All other values are based upon one incubation. Cofactor concentrations were those of the control system plus the indicated additions. All incubations were conducted for 60 minutes at 37°C, pH 7.2. Two-tenths ml of a 1:8 tissue homogenate (800 x g supernatant) was the enzyme source.

The results of these trials demonstrated that both DTT and BSA stimulated palmitate esterification in this system. Similar results were obtained in three separate trials documenting the enhancement of palmitate esterification by BSA and Dithiothreitol. Although BSA and DTT were stimulatory separately and together, the stimulation was not additive (Table 23) and the probability existed that still another cofactor(s) was limiting. The cofactors most likely to be limiting were estimated to be ATP and/or CoA. The effect of increasing concentrations of CoA and ATP on palmitate esterification in the presence of DTT and BSA are shown in Table 23.

If BSA and DTT effects were strictly additive, an esterification of 228% (100 + 92 + 36) of the control value should have been observed in the stimulated system (Table 23). Instead an esterification of 191% of the control was observed upon addition of BSA and DTT together. This value was no higher than that observed when BSA was added alone. The addition of CoA to the BSA and DTT stimulated system did not alter palmitate esterification. However, the addition of 10.5 mM ATP caused the BSA and DTT stimulations to become completely additive, resulting in a 125% stimulation of palmitate esterification above the control values. The effects of BSA and DTT on CoA and ATP requirements can be

Table 23

Energy Dependent Stimulation of Palmitate Esterification by BSA and DTT[1]

System	Additions	Concentration	CPM Incorporation	Percent of Control
Control[2]	None	---	1223	100
Control	BSA	5.0 mg	2346	192
Control	DTT	4.0 mM	1655	136
Stimulated[3]	None	---	2341	191
Stimulated	CoA	0.6 mM	2399	196
Stimulated	CoA	0.8 mM	2361	193
Stimulated	CoA	1.0 mM	2388	195
Stimulated	ATP	10.5 mM	2753	225
Stimulated	ATP	14.0 mM	2518	206
Stimulated	ATP	17.5 mM	2475	202

[1] Each value reported is the average of two duplicate incubations. All values were determined on the same tissue homogenate (330). Each incubation contained the cofactors indicated. All incubations were conducted for 60 minutes at 37°C, pH 7.2. Two-tenths ml of a 1:8 homogenate (800 x g supernatant) was the enzyme source.

[2] Control system = ATP (7.0 mM), CoA (0.4 mM), α-GP (20.0 mM), $MgCl_2$ (2.0 mM), NaF (50.0 mM).

[3] Stimulated system = Control system + 5.0 mg BSA + 4.0 mM DTT.

noted in Figures 11 and 12. The cofactors and concentrations selected for routine assays of glyceride synthetase activity are shown in Table 4, Methods and Materials.

b) pH optimum

Conflicting data regarding pH optimum for palmitate esterification was obtained, depending upon the composition of the buffer used. A sharp 6.9-7.0 pH optimum was observed when 0.075 M Tris (hydroxy methyl aminoethane) buffer was used, while the pH optimum was 7.2-7.3 when 0.1 M sodium phosphate buffer was used (Figure 13). The pH optimum for the phosphate buffer was not as clearly indicated as the pH optimum for the Tris buffer. Nevertheless, when both buffers were tested simultaneously at pH 7.2 using the same tissue homogenate more palmitate was esterified by the incubation mixtures buffered by sodium phosphate (Figure 14). The pH of the incubation mixture for either buffer varied less than ± 0.1 unit during the course of a 60 minute incubation. The sodium phosphate buffer was selected for routine use since palmitate esterification was less variable at 0.1 pH unit from the optimum than when Tris buffered the incubation mixture.

The results of this pH study are similar to those conducted on goat mammary tissue (Pynadath and Kumar 1964) where potassium phosphate was found to provide a more favorable medium for glyceride synthesis than Tris. A pH optimum of 7.4 was observed for goat mammary tissue.

c) Other considerations

The effect of the composition of the buffer used to rinse, freeze, and homogenize the tissue is shown in Table 24.

Table 24

Comparison of Some Tissue Treatments Prior to Assay[1]

Prior to Freezing	Freezing Media	Homogenization Media	µmoles Palmitate/ hr./g
1) KCl[2]	None	KCl	1.87
2) KCl	None	Tris[3]	1.86
3) KCl	KCl	KCl	1.38
4) Sucrose-Tris[4]	None	KCl	1.45
5) Sucrose-Tris	None	Sucrose-Tris	1.85
6) Sucrose-Tris	Sucrose-Tris	Sucrose-Tris	0.95
7) KCl	None	KCl	2.79
8) KCl	None	KCl + 2.75 mM DTT	2.79

[1] Comparisons 1-6 conducted on different tissue source than comparisons 7 and 8.

[2] KCl = 0.15 M

[3] Tris = 0.05 M

[4] Sucrose-Tris = 0.25 M sucrose + .05 M Tris

The use of sucrose-Tris or DTT in tissue preparation had no beneficial effect upon the amount of palmitate esterified in the final assay. Potassium chloride (0.15 M) was selected to serve as both a rinse and homogenization medium. Since KCl was also used in preparation of mammary tissue for LPL assays this choice allowed one homogenate to serve as an enzyme source for both assays.

The effect of freezing on palmitate esterification is not clear. Several samples were analyzed prior to freezing and

contained more activity at a later date, but subsequent refinements of assay conditions weaken such comparisons. If any conclusions concerning the effect of freezing can be made, it would appear that frozen tissue does exhibit glyceride synthetase activity that is similar to or slightly more than that of fresh tissue.

3. Kinetics of Palmitate Esterification

The assay system (Table 4) was tested for its ability to esterify palmitate in response to increasing concentrations of homogenate and substrate and increasing length of incubation period. The esterification rate of palmitate followed a somewhat sigmoidal pattern between 0 to 12 mg tissue per 1.0 ml incubation mixture (Figure 15). The low esterification of palmitate at low homogenate concentrations is probably due to the micellar nature of the substrate (palmitate). Substrate inhibition caused by detergent properties of palmityl CoA depend upon the protein to detergent ratio in the incubation mixture (Abou-Issa and Cleland 1969). Esterification of palmitate increased in a linear manner between 3 to 9 mg tissue per ml of incubation mixture, presumably after substrate inhibition had been overcome. The amount of palmitate esterified was also linear from 0 to 60 minutes incubation time (Figure 16). Variable response was observed from 60 to 75 minutes depending upon the tissue source being studied. The substrate saturation curve for palmitate followed a hyperbolic form (Figures 14 and 17). A Lineweaver Burk

reciprocal plot of the data in Figure 17 is shown in Figure 18. The departure of reciprocal enzyme velocity from linearity was noted at high substrate concentrations, demonstrating non-correspondence (Christensen and Palmer 1967) due to substrate saturation. To avoid biasing the extrapolated data in the Lineweaver Burk plot, values obtained beyond the first level of substrate indicative of enzyme saturation were excluded from the calculation of the regression equation of the extended line. A Km of 0.13 mM and a maximum velocity (Vmax) of 7.89 μmoles palmitate esterified/hr./g tissue were obtained. Similar values were obtained when several different mammary tissue sources were assayed (Table 32) and will be discussed under the topic of substrate specificity. The Km determined for palmitate (0.13 mM) in these studies was similar to a 0.17 mM palmitate Km found for rat adipose tissue (Angel and Roncari 1967).

4. Subcellular Localization of Glyceride Synthetase Activity

A homogenate of bovine mammary tissue was separated into the fractions shown in Table 25. Each fraction was assayed for its ability to esterify palmitate into heptane extractable neutral lipids. The 80,000 x g supernatant and pellet correspond to the soluble and particulate fraction of the cell respectively. The 80,000 x g pellet was separated into "microsomal" (12,000 x g supernatant) and "mitochondrial"

Table 25

Subcellular Localization of Bovine Mammary Glyceride Synthetase Activity[1]

Fraction	Total Activity[2]	Protein[3]	Specific Activity[4]
800 x g Supernatant	238.0	14.2	16.8
80,000 x g Supernatant	2.0	9.8	0.2
80,000 x g Pellet	231.0	5.1	45.3
Resuspended 80,000 x g Pellet			
12,000 x g Supernatant	52.0	1.8	28.9
12,000 x g Pellet	198.5	2.7	73.5

[1] The values shown are averages of duplicate incubations from the same homogenate. Similar results were obtained in two further studies when the particulate fraction was sedimented at 100,000 x g (Table 26). Conditions of assay were those shown in Table 4, except enzyme source was varied as indicated.

[2] mμmoles palmitate esterified/hr./ml fraction assayed

[3] mg extractable protein/ml fraction assayed

[4] mμmoles palmitate esterified/hr./mg protein

(12,000 x g pellet) fractions. The activities in the latter two fractions should be considered tentative since the identity of the fractions was not rigorously established. The majority of the glyceride synthetase activity was associated with the particulate fraction of the cell, in agreement with previous findings for mammary tissue (McBride and Korn 1964b, Pynadath and Kumar 1964, Kuhn 1967a). The 12,000 x g pellet ("mitochondria") contained most of the particulate activity. This observation is in agreement with previous reports on

tissue from goat mammary gland (Pynadath and Kumar 1964), as well as rat adipose tissue (Roncari and Hollenberg 1967). Guinea pig glyceride synthetase activity was reported to be divided equally between mitochondria and microsomes (McBride and Korn 1964b), whereas GS activity in cat intestinal mucosa was predominantly microsomal in origin (Brindley and Hubscher 1965).

Glyceride synthesis in the particulate fraction of the cell has been shown to be stimulated by a supernatant factor(s) (Hubscher et al. 1967). The majority of this stimulation is believed to be due to the enzyme phosphatidate phosphohydrolase located in the soluble portion of the cell (Smith et al. 1967). A particle free supernatant fraction (100,000 x g for one hour) from mammary tissue was tested for its ability to stimulate glyceride synthesis in the particulate fraction (100,000 x g pellet) (Table 26). The ability of the particle free supernatant to stimulate glyceride synthesis is evident by comparing the sum of the total activity in the 100,000 x g supernatant and the 100,000 x g pellet (21.1 + 248.6 = 269.7) when assayed separately with their combined activity (367.4) when assayed together. Combining the two fractions resulted in a 36.2% stimulation in glyceride synthesis. Stimulation of particulate glyceride synthesis by the particle free supernatant can be interpreted as indirect evidence for the operation of the phosphatidic acid pathway in bovine mammary tissue.

Table 26

Palmitate Esterification in the Presence and Absence of the Particle Free Supernatant[1]

Fraction	Total Activity[2]	Protein[3]	Specific Activity[4]
800 x g supernatant	488.4	8.8	55.5
100,000 x g supernatant	21.1	4.6	4.6
100,000 x g pellet	248.6	2.2	113.0
Recombination of 100,000 x g supernatant and pellet	367.4	6.8	54.0

[1] The values shown are averages of duplicate incubations from the same homogenate. Similar results were obtained in an identical experiment (not shown). Conditions of assay were those shown in Table 4, except enzyme source was varied as indicated.

[2] Total activity = mμmoles palmitate esterified/hr./ml fraction assayed

[3] mg extractable protein/ml fraction assayed

[4] mμmoles palmitate esterified/hr./mg protein

Glyceride synthesis by the monoglyceride pathway in other tissues was not stimulated by the particle free supernatant (Hubscher et al. 1967). The true capacity of the 100,000 x g supernatant to stimulate glyceride synthesis cannot be estimated from this study since NaF, an inhibitor of phosphatidate phosphohydrolase in some studies (Hubscher et al. 1967) but not in others (Smith et al. 1967), was present in the incubation mixture. Hubscher et al. (1967) reported that glyceride synthesis by rat liver mitochondria was stimulated 300% by

the particle free supernatant in the absence of KF and only 60% in the presence of KF. Studies on palmitate esterification by the 800 x g supernatant of mammary tissue in the presence and absence of NaF (Table 20) have shown increased palmitate esterification when NaF was present in the incubation mixture. The maximum stimulation of palmitate esterification by recombination of the 100,000 x g supernatant and pellet would have been 96% (448.4 ÷ 248.6). This study (and that shown in Table 29) indicated that the phosphatidate phosphohydrolase of bovine mammary gland was not markedly inhibited by the presence of sodium fluoride in the incubation mixture. The absence of a large (300%, Hubscher et al. 1967) stimulation of palmitate esterification by the 100,000 x g supernatant may be due to the presence of a particle bound phosphatidate phosphohydrolase (Smith et al. 1967).

5. Characterization of Product

a) Exchange reaction

No palmitate-1-^{14}C was incorporated by boiled homogenates indicating that the radioactive label was not being incorporated into endogenous glycerides by simple non-enzymatic exchange. This homogenate was boiled for 60 seconds and then incubated with the usual cofactors plus palmitate-1-^{14}C.

b) Time course glyceride synthesis

Incorporation of 1-^{14}C palmitate into mono-, di-, and triglycerides in the heptane extractable lipids as a function

of time was investigated. The results are presented in two forms, one including the monoglycerides (Appendix Table 2) and one including just the di- and triglycerides (Table 27). Since monoglycerides are not quantitatively extracted by the heptane extraction procedure (McBride and Korn 1964b), their inclusion might obscure the relationship between the di- and triglycerides.

Table 27

Palmitate Esterification into Di- and Triglycerides as a Function of Time[1]

Glyceride Class		Minutes 15	30	45	60	120	150
Diglyceride	mμmoles[2]	0.87	1.63	2.68	3.96	8.56	11.47
	%	57	54	51	42	42	42
Triglyceride	mμmoles	0.66	1.39	2.55	5.41	11.81	15.99
	%	43	46	49	58	58	58
Total mμmoles esterified		1.53	3.02	5.23	9.37	20.37	27.46

[1] All values reported were obtained using the same tissue homogenate. Similar results with slightly different incubation conditions were obtained with a different tissue source (Table 28). Cofactors and concentrations were those shown for control system Table 23. Lipids were heptane-extracted as described in materials and methods.

The diglycerides contained the greatest amount of label during 0 to 45 minutes of incubation. After 45 minutes the triglycerides were found to contain 58% of the palmitate-1-^{14}C esterified. These incubations were continued for 120 and 150 minutes to ascertain if glyceride synthesis would proceed to

completion. Although the total incorporation of palmitate proceeded in a linear fashion to 150 minutes, the relationship of palmitate incorporation into di- and triglycerides remained constant from 60 to 150 minutes. In this situation excess acyl acceptor (α-GP) might mask the true extent of triglyceride formation by allowing a continual synthesis of new diglycerides, thus maintaining a constant relationship between the two classes. This possibility was tested by incubating for various time lengths in the presence of no acyl acceptor and a limited (5.0 mM) amount of acyl acceptor (Table 28). With no α-GP in the incubation mixture only endogenous acyl acceptors would be available for palmitate esterification. With a limited amount of α-GP present and palmitate in excess, glyceride synthesis should favor triglyceride formation. However, the relative percent palmitate esterified into triglycerides was not increased by decreasing the concentration of acyl acceptor in the assay system (Table 28). The main effect of limited acyl acceptor appeared to be that of decreasing total palmitate esterification, especially after 60 minutes of incubation time, as would be predicted from the results in Table 20. Increasing levels of α-GP augmented triglyceride formation but incubations up to 150 minutes did not enhance the percent of total palmitate esterified in triglycerides over that observed at 60 minutes. It was concluded that the concentration of acyl acceptor in the incubation mixture was not masking the true extent of triglyceride formation by this system.

Table 28

Time Course Glyceride Synthesis with Limited Concentrations of Acyl Acceptor[1]

Glyceride Class		No α-GP - - Minutes Incubation Time - - 30	60	90	120	5 mM α-GP - - Minutes Incubation Time - - 30	60	90	120
Diglyceride	mμmoles[2]	1.67	2.82	3.28	3.45	10.49	31.47	32.60	27.13
	%[3]	77	79	69	66	70	68	48	47
Triglyceride	mμmoles	0.49	0.75	1.49	1.53	4.51	14.67	34.90	30.25
	%	23	21	31	34	30	32	52	53
Total mμmoles esterified		2.16	3.57	4.77	4.98	15.00	46.41	67.50	57.38

[1] All values reported were obtained using the same tissue homogenate. Cofactors and concentrations (except α-GP) were those shown in Table 4. Lipids were heptane extracted as described in Materials and Methods.

[2] mμmoles = mμmoles palmitate esterified

[3] % = % of total mμmoles palmitate esterified

The third acylation may have been limiting the extent of triglyceride formation with this system. If the phosphatidic acid pathway is being utilized for glyceride synthesis in a tissue, the phosphate group on the 3rd position must be removed by the enzyme phosphatidate phosphohydrolase prior to the third acylation (Smith et al. 1967). Phosphatidate phosphohydrolase has been reported to be inhibited by the presence of fluoride ions (Coleman and Hubscher 1962). Since 50 mM NaF was used in this assay system, the effect of F^- on the incorporation of palmitate-1-^{14}C into glycerides was investigated. Duplicate

incubations of mammary homogenates were conducted under identical conditions except NaF was omitted from the incubation medium in one case. The reaction products were separated by thin layer chromatography, detected, scraped, and counted. The results are shown in Table 29.

Table 29

Glyceride Synthesis in the Presence and Absence of Sodium Fluoride[1]

	-NaF		+NaF	
Lipid Class	CPM[2]	%[3]	CPM	%
Monoglycerides and Phospholipids	193	3.3	271	3.8
Diglycerides	3784	65.0	4596	65.1
Triglycerides	1843	31.7	2192	31.0
TOTAL	5820	100.0	7059	100.0

[1] Values reported represent one determination on the same tissue homogenate. Conditions of assay were those shown in Table 4 except the -NaF incubation contained no NaF and the +NaF incubation contained 50.0 mM NaF. Lipids were heptane extracted as described in Materials and Methods.

[2] CPM = CPM palmitate-1-^{14}C esterified

[3] % = Percent of total palmitate-1-^{14}C esterified in each lipid class.

Two conclusions are evident from the data shown in Table 29. The presence or amount of sodium fluoride did not influence the relative extent of triglyceride formation by

this system, although NaF appeared to increase the extent of palmitate esterification. The 13% stimulation of palmitate esterification by 50 mM NaF agreed with previous findings (Table 20).

Some factor(s) appeared to be limiting the third acylation in this system. Excessive diglyceride formation and NaF inhibition of phosphatidate phosphohydrolase were ruled out as possible causes. A likely alternative would be obligatory requirement for a specific fatty acid to be esterified at the third position in the milk fat triglyceride molecule. Since the test system only employed one fatty acid (palmitic) this possibility seemed feasible. Further investigations concerning the requirement for a specific fatty acid will be discussed under the topic of substrate specificity.

c) Identity of mammary lipids

The relative distribution of palmitate-1-^{14}C among neutral and polar mammary lipids was investigated for two reasons: 1) to determine substrate distribution in the final product in this system and 2) to determine if labelling of various lipid classes might be indicative of possible intermediates in the pathway of glyceride synthesis.

Depicted in Figures 19 and 20 are separation and identification of chloroform:methanol (2:1) extracted neutral and polar lipids of mammary tissue from a lactating Angus cow. The predominant neutral lipids of this mammary tissue

based on size of the identified spot and intensity of color reactions were triglycerides, free fatty acids, and diglycerides. Cholesterol esters (near solvent front) and monoglycerides (near origin) were also detected in some instances but never in very high concentrations. With the solvent system shown in Figure 19 phospholipids remained at the origin. Four main classes of phospholipids were indicated by the colors that developed following spraying the chromatogram sheet with molybdate spray (Figure 20). These were tentatively identified as phosphatidyl ethanolamine, phosphatidyl choline, lyso-phosphatidyl ethanolamine or sphingomyelin, and phosphatidic acid. The spot indicated as phosphatidic acid was always very faint. The identity of this intermediate should be considered tentative due to lack of an authentic phosphatidic acid standard.

A series of incubations were conducted using appropriate cofactors and palmitate-1-^{14}C substrate after which the lipids were extracted with chloroform:methanol (2:1) separated and identified as previously described for the endogenous lipids. Following tentative identification of the lipid classes the corresponding lipid classes were counted to determine their content of palmitate-1-^{14}C.

Most of the label in neutral lipids was found in mono-, di-, and triglycerides (Table 30). The relatively high

Table 30
Distribution of Palmitate-1-^{14}C in Mammary Lipid Classes[1]

Lipid Class	CPM	Percent of Total Sheet Counts[2]	Percent of Esterified Fatty Acid Counts[3]
Phospholipids	152	2.0	14.0
Monoglycerides	230	3.0	21.0
Unidentified	158	2.0	14.5
1, 2-Diglycerides	230	3.0	21.0
1, 3-Diglycerides	106	1.4	9.7
FFA	6646	85.7	
Triglycerides	216	2.8	19.8
Cholesterol esters	0	0.0	
TOTAL	7739	99.9	

[1] Cofactors and concentrations were as follows: ATP (7.0 mM), CoA (0.4 mM), α-GP (20.0 mM), $MgCl_2$ (2.0 mM), NaF (50.0 mM). Enzyme source was 0.4 ml of a 1:8 mammary homogenate (800 x g supernatant). Incubations were conducted for 60 minutes at 37°C. Reaction was terminated by extracting the incubation mixture with chloroform:methanol (2:1). Similar results were obtained in three preliminary incubations with the same tissue.

[2] Total sheet counts includes all CPM found between origin and solvent front.

[3] Esterified fatty acid counts includes all lipid classes except FFA.

activity of the monoglycerides is difficult to explain based upon known mammary gland biosynthetic pathways. Kinsella (1968b) also noted that monoglycerides of bovine mammary cell cultures incubated with [$^{14}C_3$] glycerol had a high specific activity compared to other lipid classes. Although the 1, 3-diglyceride spot was visually larger and exhibited a more intense color reaction than the 1, 2-diglyceride spot the 1, 2-diglycerides contained twice as much label as the

1, 3-diglycerides. The 1-3 isomer may have resulted from the 1-2 isomer during lipid extraction procedures. Similar results were noted by Kinsella (1968b) for bovine mammary cell cultures.

Phospholipids accounted for 2.0% of the total label recovered from the chromatogram sheet. Neutral lipids (monoglycerides, diglycerides, triglycerides) accounted for 10.2% of the total label recovered from the chromatogram sheet. Fourteen percent of the esterified palmitate-1-^{14}C was found in phospholipids, leaving 86% in neutral lipids. During several early experiments in ninhydrin positive phospholipid was noted that was intensely labelled. However, FFA migrated in the polar lipid system with an R_f value of 0.65 compared to 0.53 for this particular ninhydrin reactive phospholipid. Labelling of this phospholipid may have been merely a reflection of FFA contamination since these two classes of lipid migrated to similar areas of the chromatogram sheet. A two dimensional thin layer chromatogram separated the FFA and phospholipids to areas of the plate remote from each other. The total number of counts corrected for quenching in phospholipids was 168 above background. This was about 2% of total recovered counts and 15% of total esterified fatty acid counts. The distribution of the palmitate-1-^{14}C is shown in Table 31. No one class of phospholipid was highly labelled. None of the phospholipids

Table 31

Distribution of Label in Polar Lipids Following Two Dimentional TLC[1]

R_f[2]	Molybdate[3] Reagent	Dragendorf[4] Reagent	Ninhydrin[5] Reagent	Tentative Identification	CPM Above[6] Background	% of Total Polar Lipid Counts
Origin						
.16	-	-	-	Phosphatidic Acid	10.2	6.1
.30	+	-	+	Sphingomyelin or Lyso-Phosphatidyl Ethanolamine	23.9	14.1
.38	+	+	-	Phosphatidyl Choline	27.0	16.1
.60	-	-	-	?	5.1	3.0
.79	+	-	+	Phosphatidyl Ethanolamine	65.6	39.0
.90	-	-	-	?	36.3	21.6
Solvent Front						

[1] First development was with Hexane:Ethyl Ether:Acetic Acid (80:20:1). Second development was 90° to the first development with chloroform:methanol:ammonium hydroxide (75:25:4).
[2] Relative migration of center of mass of each class in reference to the solvent front.
[3] Molybdate reagent - specific for all phosphate containing lipids.
[4] Dragendorf reagent - specific for all choline containing lipids.
[5] Ninhydrin reagent - specific for all amino-lipids.
[6] Corrected for quenching.

identified in this study were labelled with sufficient intensity to indicate that they were precursors of any major lipid class other than themselves.

d) Discussion of results

The percent of label incorporated into diglycerides decreased as the percent of label incorporated into triglycerides increased from 15 to 60 minutes of incubation time. This is consistent with a precursor-product relationship. However, the relationship between the two classes of lipids remained constant from 60 to 150 minutes. No more than 58% of the total di- and triglyceride label appeared in triglycerides regardless of length of incubation period. This value is greater than that found by Pynadath and Kumar (1964) for goat mammary tissue (24% triglyceride), about the same as McBride and Korn (1964b) found for guinea pig mammary tissue (57% triglyceride) and slightly less than Dils and Clark (1962) found for rat mammary tissue (63% triglyceride). Although this value (58%) compares favorably with those values previously reported for mammary tissue it is less than values reported for rat liver mitochondria (75% triglyceride) (Tzur et al. 1964) and rat adipose homogenates (84% triglyceride) (Roncari and Hollenberg 1967).

The extent of triglyceride formation by this system may be limited by a lack of specific fatty acids (Patton and McCarthy 1963b) or lack of a specific acyl acceptor

(Pynadath and Kumar 1964). Alternatively, lipolysis of newly formed triglycerides may prevent their accumulation (Vaughan and Steinberg 1965). This is unlikely because of the high concentration of F^- ions in the assay system. Fluoride ions are known to inhibit lipolysis in adipose tissue homogenates (Vaughan and Steinberg 1965). Finally a certain degree of cellular or membrane integrity destroyed by the homogenization procedure may be necessary for maximum or continued triglyceride synthesis. The role of cellular integrity in directing lipid synthesis is difficult to assess. Although this was a cell free system, the products formed resembled those found by Kinsella (1968a) using bovine cell cultures incubated with palmitate-1-^{14}C. Ten percent of the palmitate-1-^{14}C esterified by the cells in culture was found in phospholipid, 90% in neutral lipids. The major difference between palmitate-1-^{14}C esterification by bovine cell cultures and by this system was that 79% of the esterified palmitate was triglyceride with cell culture whereas only 20% was esterified as triglyceride with this homogenate.

Fourteen percent of the palmitate-1-^{14}C esterified was in phospholipid and 86% in neutral lipids. These values are similar to that of 30% phospholipid for guinea pig mammary tissue (Kuhn 1967a), 34% for rat liver mitochondria (Tzur et al. 1964), and 19% for rat adipose homogenates (Roncari and

Hollenberg 1967). The values of 86% for fatty acid incorporation into neutral lipids and 14% as phospholipid compares similarly to known compositional data of cow mammary tissue. Patton and McCarthy (1963b) listed the lipid composition of bovine mammary tissue bo be ~ 17% phospholipid, ~ 84% neutral lipid. The products synthesized by this system tended to resemble tissue lipid composition more than milk lipids.

The major classes of phospholipid that incorporated palmitate-1-^{14}C in this system were amino-phosphatides and a choline-phosphatide. Phosphatidyl ethanolamine and phosphatidyl choline are the two major phospholipid classes of milk and mammary tissue (Parsons and Patton 1967). The lipid identified as phosphatidyl choline (lecithin), while containing 16.1% of the palmitate-1-^{14}C incorporated into phospholipid, was never an especially active intermediate, as has been suggested by Patton et al. (1966b). The extent of palmitate incorporation into phosphatidyl choline is somewhat in agreement with Kinsella (1968b) who did not find this phospholipid to be highly labelled when bovine mammary cells were incubated with glycerol-^{14}C. However, phsophatidyl choline was the major phospholipid synthesized by these cells. Similar to the results of others (Patton et al. 1966a, Kinsella 1968b) phosphatidic acid was difficult to detect qualitatively and the area of the chromatogram sheet

where phosphatidic acid was expected to migrate never contained appreciable radioactivity. This observation should not necessarily be construed as evidence against the operation of the phosphatidic acid pathway. As suggested by Kinsella (1968b) the inability to detect phosphatidic acid may be due to its extremely rapid hydrolysis by the enzyme phosphatidate phosphohydrolase. The low specific activity of phosphatidic acid and the high specific activity of diglycerides synthesized by this system would be consistent with rapid hydrolysis of phosphatidic acid. The appreciable labelling of other classes of phospholipids known to be derived from phosphatidic acid (White et al. 1964) such as phsophatidyl ethanolamine and phosphatidyl choline at least imply the prior presence of phosphatidic acid if accepted pathways of phospholipid synthesis are functioning in this system.

The rather high activity of monoglycerides made in this system is difficult to explain. Monoglycerides and phospholipids were not well separated by the solvent system (hexane: ethyl ether:acetic acid, 80:20:1) used in these studies (Figure 19). Part of the monoglyceride activity could have been due to phospholipid contamination. Exogenous monoglyceride did not function as an acyl acceptor in this system, and a precursor-product relationship between monoglycerides and other lipid classes (Appendix Table 2) was not evident.

Monacyl glycerolphosphate may have been hydrolyzed by a phospholipase as suggested by Kinsella (1968b) producing monoglyceride.

In summary, the product produced by homogenates of bovine mammary tissue was similar in phospholipid and neutral lipid content to that of bovine tissue lipids. The product was different from tissue lipids with respect to the relative proportions of neutral lipids synthesized. Whereas tissue and milk glycerides are predominantly triglycerides only 20% of the total palmitate-1-^{14}C esterified or 58% of the palmitate-1-^{14}C esterified in di- and triglycerides was esterified into triglycerides. These values compare favorably with those reported in the literature for guinea pig, rat, and goat mammary homogenates but are lower than values reported for rat adipose and liver homogenates. No conclusive evidence was obtained for the operation of either the phosphatidic acid pathway or the monoglyceride pathway of glyceride synthesis. Although monoglyceride did not serve as an acyl acceptor in this system, monoglycerides were significantly labelled by palmitate-1-^{14}C. Although α-GP did serve as an acyl acceptor the phosphatidic acid intermediate was never highly labelled.

6. Substrate Specificity

The ability of the mammary tissue homogenates to esterify fatty acids of various chain lengths and degrees of unsaturation was investigated for the following reasons:

1) Shifts in the relative proportions of long chain fatty acids in the blood occur during milk fat depression. An alternation in the substrate presented to mammary gland enzymes could conceivably alter milk fat synthesis if fatty acid specificity does exist.

2) The results of the standard assay system utilizing 1-^{14}C-palmitate as the sole substrate could be altered if certain endogenous fatty acids present in the homogenate are stimulatory or inhibitory to glyceride synthesis.

3) Little information exists concerning the relative rates of esterification of various LCFA by bovine mammary tissue.

a) Individual fatty acids

Palmitic (C-16:0), stearic (C-18:0), oleic (C-18:1 *cis*), and linoleic (C-18:2 *cis-cis*) acids were tested for their ability to be esterified by the 800 x g supernatant of lactating bovine mammary tissue. Typical substrate saturation curves are shown in Figure 21. Oleic acid sometimes, but not always, exhibited substrate inhibition at high concentrations. Linoleic acid was not esterified at rates comparable to the other acids tested except for one instance. Linoleic acid

purchased from Hormel (The Hormel Institute, Austin, Minn.) was esterified at rates comparable to stearate at concentrations under 0.10 mM in one study (cow 330, 5/13/68) out of fifteen total trials. When this same linoleic acid (Hormel) was tested against another tissue (cow 642, 5/29/68) it proved to be inhibitory to its own esterification at concentrations above 0.05 mM. Linoleic acid purchased from Sigma (Sigma Chemical Co., St. Louis, Mo.) or Applied Sciences (The Anspec Co., Ann Arbor, Mich.) was never esterified as well as stearate and gave substrate saturation curves similar to that shown in Figure 21. Linolenic acid (C-18:3) tested at a later date than those shown in Figure 21 was incorporated by mammary homogenates at rates exceeding those of palmitate or oleate. For purposes of comparing enzyme affinities for the various fatty acids tested, Km values were derived by calculating Lineweaver-Burk regression equations for the data listed in Appendix Table 3. A total of four animals was used in these studies. Each value for Km and Vmax in Table 32 represents 2 to 4 determinations on different animals. The values listed in Table 32 are presented for comparative purposes within this study. The values were determined with only 3 to 5 concentrations of substrate and as such are strongly influenced by each observation that contributed to the calculated Lineweaver Burk regression equation. The Km values are different enough from each other, however, to

Table 32

Apparent Fatty Acid Affinities and Maximum Esterification Velocities for Bovine Mammary Tissue[1]

Fatty Acid	Km[2]	Vmax[3]
C-16:0 (4)[4]	0.13 ± 0.01	5.61 ± 1.65
C-18:0 (3)	0.32 ± 0.04	4.72 ± 1.33
C-18:1 (2)	0.24 ± 0.04	6.38 ± 1.22
C-18:2 (3)	0.50 ± 0.22	0.77 ± 0.34

[1] Conditions of assay were those shown in Table 4, except the concentration of each fatty acid was varied as shown in Appendix Table 3.

[2] Km = apparent concentration of fatty acid (mM) at one-half maximum velocity of esterification, average value ± SE.

[3] Calculated maximum velocity of esterification, μmoles/hr./g, average value ± SE.

[4] Values in parenthesis represent number of animals.

suggest that the enzyme(s) participating in mammary glyceride synthesis have different affinities for various fatty acids. The rather high Km for stearate is puzzeling since the mammary gland takes up large quantities of stearic acid from the blood (Barry et al. 1963). However, a large proportion of the stearic acid from blood is desaturated to oleic prior to esterification in milk fat (Lauryssens et al. 1961). If the Km of the glyceride synthetase complex for oleic acid is actually lower than that for stearic acid, the biological

desaturation of stearic acid to oleic acid may facilitate fatty acid esterification by the tissue. Alternatively, the high apparent Km for stearate may mean that part of stearate is being desaturated to oleic before esterification (as *in-vivo*) thus delaying the appearance of label in the product. The low apparent Km for palmitate may have biological significance also. Palmitate is the only acid of those tested that is known to be synthesized in the gland from acetate as well as removed from the blood (Jones 1969). The mammary gland glyceride synthetase complex may have a higher affinity (lower Km) for fatty acids synthesized *in-situ*. This would be consistent with the relatively high proportion of short chain fatty acids esterified in milk fat. Unfortunately, fatty acids of chain length shorter than C-16:0 were not tested in this system. Fatty acid esterification was tested in the presence and absence of the 100,000 x g supernatant fraction, to determine if endogenous acids present in the supernatant would influence fatty acid specificity of the particulate fraction. Esterification of C-16:0, C-18:0, C-18:1, C-18:2, and C-18:3 was measured at five substrate concentrations ranging from 0.0 to 0.3 mM fatty acid.[1] Similar substrate saturation curves to those of Figure 21 were obtained for all acids except linolenic acid (C-18:3). At low to intermediate substrate concentrations the esterification

[1] Part of this data has been presented previously (cow 3669, Appendix Table 3).

rate of linolenic acid was less than that of palmitic and oleic, but at high substrate concentrations the esterification rate of linolenic acid exceeded both palmitic and oleic esterification rates. Linolenic acid displayed a S-shaped substrate saturation curve in the presence of both the 800 x g supernatant and particulate fractions. The same relative order of fatty acid esterification was observed in the particulate fraction as in the 800 x g supernatant, although the differences were less pronounced (Figure 22). Apparent Km and Vmax values were calculated and listed in Table 33 for each acid, with the exception of C-18:3 which displayed unusual kinetics.

In all cases the enzymes of glyceride synthesis had a lower Km in the absence of the 100,000 x g supernatant (particulate fraction) than they did in the presence of the 100,000 x g supernatant (800 x g supernatant). Less substrate was required to saturate the particulate enzymes in the absence of the 100,000 x g supernatant. Soluble proteins present in the 800 x g supernatant may have bound free fatty acids added as substrate, thus decreasing their availability to the enzymes of glyceride synthesis, causing higher apparent Km values.

Table 33

Kinetic Parameters of Fatty Acid Esterification in the 800 x g Supernatant and Particulate Fractions of Mammary Homogenates[1]

Fatty Acid	800 x g Supernatant Km[3]	Vmax[4]	Particulate[2] Km[3]	Vmax[4]
C-16:0	0.14	6.94	0.09	3.79
C-18:0	0.50	5.68	0.17	1.79
C-18:1	0.21	7.63	0.18	4.02
C-18:2	0.86	1.43	0.35	0.13

[1] One homogenate of tissue 3669 was used for these studies. Conditions of assay were those shown in Table 4 except fatty acid and enzyme source were varied as described in text.

[2] Particulate = 100,000 x g pellet.

[3] Km = Apparent Km, mM

[4] Vmax = Calculated maximum velocity, μmoles fatty acid esterified/hr./g tissue

Observed *in-vitro* fatty acid esterification rates (C-16:0 ~ C-18:1 > C-18:0 > C-18:2) (Tables 32 and 33) were in general agreement with the fatty acid composition of mammary tissue and cream (C-16:0 ~ C-18:1 > C-18:0 > C-18:2) (Hilditch and Williams 1964). The esterification rate of C-18:3 (Figure 22) far exceeded its concentration in either tissue or milk. However ruminal hydrogenation of dietary linolenic acid may preclude significant quantities of this acid from ever reaching the mammary gland (Tove and Mochrie

1963, Davis and Sachan 1966, Kemp and Dawson 1968). The *in-vitro* esterification rates for linolenic acid observed in these studies indicated that mammary tissue possesses the capability to utilize linolenic acid for glyceride synthesis. Oil feeding and infusion experiments have also indicated that the degree of unsaturation of milk fat is increased when polyunsaturated fatty acids are provided to the animal in sufficiently large quantity to excape ruminal hydrogenation (Moore and Steele 1968).

A comparison can be made between *in-vitro* and *in-vivo* esterification rates for a representative fatty acid (palmitate) by a typical cow in a 24 hour day. If one assumes that (1) a cow possessed a 20 Kg udder, (2) this same cow produced 15.0 Kg of 3.0% fat milk per day (3) 100% of milk fat is triglyceride and (4) all the triglyceride was tripalmitin, certain calculations can be made which allow the comparison of *in-vitro* and *in-vivo* fatty acid esterification rates. Approximately 0.6 moles of tripalmitin would be synthesized per day, equal to 1.8 (3 x 0.6) moles of esterified palmitic acid. Dividing 1.8 moles of palmitate by 20 Kg of tissue produces an esterification rate of 0.09 moles of palmitate/Kg tissue/24 hours or 3.75 μmoles palmitate esterified/hr./g tissue. This value is similar to palmitate esterification rates (2.2 to 4.7) observed *in-vitro* (Appendix Table 3). However, the *in-vitro* assay system contained

cofactors in concentrations many times higher than those found in tissue. Baldwin et al. (1969) have found the concentration of α-GP in bovine mammary tissue to be 0.154 μmoles/g ~ 0.154 mM. The *in-vitro* assay system contained > 100 times (20 mM) as much α-GP as is present in tissue. When no α-GP was added to the *in-vitro* assay system, palmitate esterification was only 32% as great as when 20.0 mM α-GP was present (Table 20). Nevertheless, based on limited calculations, the *in-vitro* esterification rate for palmitate by this *in-vitro* system is similar to calculated *in-vivo* fatty acid esterification rates.

b) Fatty acid combinations

Brindley et al. (1967) found that unsaturated fatty acids in the particle free supernatant of cat intestinal mucosa and rat liver mitochondria were capable of stimulating glyceride synthesis. Since the standard assay developed for mammary tissue utilized palmitate as the sole substrate the concentration of FFA in the 800 x g supernatant and in the total assay media was investigated. Influences of endogenous FFA on the esterification of the exogenous substrate, palmitate were thus estimated.

Mammary tissue from three cows was extracted by the method of Dole and Meinertz (1960) and titrated for free fatty acids (Table 34). The FFA concentrations found agreed closely with those reported by Kuhn (1967b) for guinea pig mammary tissue.

Table 34

Concentration of Long Chain Fatty Acids in Mammary Tissue[1]

Cow	µmoles FFA/g tissue
329	4.7
330	3.0
642	3.6
Average	3.8

[1] 800 x g supernatant

The amount of homogenate routinely used in substrate specificity assays would contribute 0.08 µmoles FFA to the 2.0 ml assay volume, giving an endogenous FFA concentration of 0.04 mM. These endogenous FFA were also capable of being liberated from the homogenate (Appendix Table 4) presumably from the particulate fraction, (Appendix Table 5) during the course of an assay and esterified in the presence of cofactors (Appendix Table 6). Although endogenous FFA from the homogenate probably were released and esterified under routine assay conditions their total contribution to product would be small in a typical assay, assuming that the endogenous and exogenous fatty acid pools would equilibrate. For example, if 0.4 µmoles palmitate were added to the incubation mixture (typical amount added in a standard assay) then the 0.08 µmoles endogenous FFA present in 0.2 ml of a 1:8 homogenate

(Appendix Table 4) would be diluted by palmitate and would contribute 17% (.08 ÷ 48) of the total acids present. Endogenous acids would probably not constitute a significant portion of the fatty acids esterified at high substrate concentrations, but could be an important source of fatty acid at low substrate concentrations. If certain endogenous FFA are stimulatory to palmitate esterification as in the case of cat intestinal mucosa and rat liver mitochondria (Brindley et al. 1967) endogenous FFA could exert a further influence on palmitate esterification in the standard assay.

The possibility of stimulation of mammary gland palmitate esterification by various unlabelled FFA was investigated. In the first experiment, the esterification of 0.10 mM palmitate-1-^{14}C by mammary 800 x g supernatant was measured in the presence of 0 to 0.10 mM unlabelled fatty acids. No pronounced stimulation of palmitate-1-^{14}C esterification resulted from the addition of any fatty acid tested (Figure 23). However, linoleic acid markedly decreased palmitate esterification. Unlabelled palmitate and unlabelled oleate each decreased palmitate-1-^{14}C incorporation to the same extent. This agreed with previous results (Appendix Table 3) where palmitate and oleate were esterified at similar rates. The decrease in palmitate-1-^{14}C esterification in the presence of unlabelled oleate and palmitate was probably the result of dilution of specific activity of the palmitate-1-^{14}C substrate. Stearate and butyrate did not alter palmitate-1-^{14}C incorporation, indicating that these acids did not compete

with palmitate in the esterification process. The failure of stearate to compete with palmitate may be due to the higher apparent Km of stearate (Table 32) than palmitate in this system. The effect of linoleate in this study is difficult to explain. Since linoleate was not labelled the decreased palmitate-1-^{14}C esterification in the presence of linoleate may have been due to either preferential esterification of linoleate or to actual inhibition of palmitate esterification. In this experiment some of the fatty acids present individually at 0.05 mM were able to influence palmitate esterification. This concentration (0.05 mM) is similar to that calculated to be contributed by the endogenous FFA of the 800 x g supernatant in the standard assay. However, it is unlikely that any one endogenous acid would be present in these (0.05 mM) concentrations.

The effect of *cis-trans* isomerism on fatty acid esterification was tested with *cis*-9-octadecenoic and *trans* -11-octadecenoic acids (Appendix Table 7). Unlabelled *cis*-9-octadecenoic acid decreased palmitate-1-^{14}C esterification to a greater extent than did *trans*-11-octadecenoic acid, indicating that the *cis* isomer (oleic) of C-18:1 was esterified more readily than the *trans* isomer (vaccenic) of C-18:1. Although the value for palmitate esterification in the presence of 0.02 mM vaccenic acid was greater than that

obtained when palmitate-1-^{14}C was incubated alone (2.26 vs 2.10), the results of another trial (Table 37) did not show an increased palmitate esterification at low concentrations of vaccenic acid.

To test the possibility that a combination of several fatty acids present in the incubation mixture simultaneously might be stimulatory to palmitate esterification, 0.10 mM palmitate-1-^{14}C was incubated with various combinations of unlabelled stearic, oleic, linoleic, and butyric acids. The results observed with combinations of fatty acids were similar to those of the previous experiment (Appendix Table 8). No combination of unlabelled acid caused greater esterification of palmitate-1-^{14}C than did palmitate-1-^{14}C alone.

Further studies were conducted alternating the fatty acid that contained the ^{14}C-label. Palmitate-1-^{14}C, stearate-1-^{14}C, oleate-1-^{14}C, and linoleate-1-^{14}C were all incubated individually with each of the unlabelled analog fatty acids. In this manner the alteration of esterification of one of a pair of acids when incubated together could be more accurately assessed. For example, if the esterification of fatty acid A-^{14}C was decreased by the presence of fatty acid B, and the esterification of fatty acid B-^{14}C was increased by presence of fatty acid A, one could conclude

that the decreased esterification of A-^{14}C was due to increased esterification of B. Alternatively, if A-^{14}C esterification was decreased by B, but B-^{14}C was not increased in the presence of A, one could conclude that fatty acid B was inhibitory to the esterification of fatty acid A. If each unlabelled acid decreased the other labelled acid's incorporation to the same extent, this would mean the two acids competed with each other (for enzyme binding sites) to the same extent. By the same logic, if the esterification of fatty acid A-^{14}C was decreased by acid B but B-^{14}C esterification was not decreased by A one could conclude that the enzymes involved have a greater affinity for fatty acid B. These experiments would have been easier to interpret if ^{3}H and ^{14}C fatty acids had been available. The results of this "label switch" experiment are shown in Table 35.

Each labelled acid (except linoleic acid) when incubated with its unlabelled analog caused approximately a 50% decrease in incorporation of label. Linoleic acid inhibited its own incorporation by 79%. No combination of acids resulted in an increased esterification of any acid above that of the sum of the acids incubated alone. Stearate did not decrease palmitate-1-^{14}C incorporation appreciably, but palmitate markedly decreased stearate-1-^{14}C incorporation. This implied that the enzymes of glyceride synthesis had greater affinity for palmitate than stearate. Oleate and

Table 35

Competition Between Fatty Acids During Glyceride Synthesis[1]

Labelled Acid	mM	Unlabelled Acid	mM	μmoles FA/ hr./g[2]	% of Control[3]
Palmitate-1-^{14}C	.10	None		2.33	100
Stearate-1-^{14}C	.10	None		0.66	100
Oleate-1-^{14}C	.10	None		2.60	100
Linoleate-1-^{14}C	.10	None		1.23	100
Palmitate-1-^{14}C	.10	Palmitate	.10	1.48	64
Palmitate-1-^{14}C	.10	Stearate	.10	2.19	94
Palmitate-1-^{14}C	.10	Oleate	.10	1.40	54
Palmitate-1-^{14}C	.10	Linoleate	.10	0.81	35
Stearate-1-^{14}C	.10	Stearate	.10	0.32	49
Stearate-1-^{14}C	.10	Palmitate	.10	0.21	32
Stearate-1-^{14}C	.10	Oleate	.10	0.30	46
Stearate-1-^{14}C	.10	Linoleate	.10	0.18	27
Oleate-1-^{14}C	.10	Oleate	.10	1.40	54
Oleate-1-^{14}C	.10	Palmitate	.10	1.48	57
Oleate-1-^{14}C	.10	Stearate	.10	2.36	91
Oleate-1-^{14}C	.10	Linoleate	.10	0.38	15
Linoleate-1-^{14}C	.10	Linoleate	.10	0.26	21
Linoleate-1-^{14}C	.10	Palmitate	.10	0.68	55
Linoleate-1-^{14}C	.10	Oleate	.10	0.35	26
Linoleate-1-^{14}C	.10	Stearate	.10	1.12	91

[1] The values reported are from one trial. These data are supported by several other trials conducted under slightly different experimental conditions (Table 39, Appendix Table 8). Conditions of assay were those shown in Table 4, except fatty acid was varied as indicated.

[2] This rate refers to the esterification of the fatty acid-1-^{14}C, not total fatty acid.

[3] The esterification rate of each fatty acid-1-^{14}C at 0.10 mM (incubation alone) is referred to as 100%.

palmitate each depressed the other acid's incorporation to a similar extent (54 to 57%), indicating that the enzymes involved have similar affinities for these two acids. Although no stimulation of fatty acid-1-^{14}C esterification was observed in this experiment, linoleate appeared to inhibit the esterification of all the other fatty acids. In every case linoleate exerted an effect far greater than would have been predicted based upon the relative rate of esterification of linoleate when tested as the sole substrate. The effect of linoleate did not appear to be stimulatory, since in no case was increased incorporation of labelled linoleate observed.

Although combinations of fatty acids did not stimulate each others esterification the possibility existed that certain acids might be somewhat additive in their combined esterifications. In order to assess the degree to which combinations of fatty acids were additive in fatty acid esterification, equal specific activity substrates were prepared. By employing equal specific activity fatty acid-1-^{14}C substrates quantitative interpretation of total fatty acid esterification was facilitated. Each acid was incubated by itself at 0.10 and 0.20 mM concentrations and then in various 0.10 mM combinations with other fatty acids. Figure 24 illustrates esterification of some selected combinations of fatty acids from Table 36. Since all acids were ^{14}C-labelled, only absolute amounts of fatty acids

Table 36

Total Fatty Acid Esterification Employing Equal Specific Activity Fatty Acids[1]

Fatty Acid-1-^{14}C	mM	μmoles FA/hr./g[2]
Palmitate	.10	1.55
Palmitate	.20	1.74
Stearate	.10	0.54
Stearate	.20	1.24
Oleate	.10	2.33
Oleate	.20	2.23
Linoleate	.10	0.64
Linoleate	.20	0.29
Palmitate +	.10	
Stearate	.10	1.90
Palmitate +	.10	
Oleate	.10	2.00
Palmitate +	.10	
Linoleate	.10	0.81
Stearate +	.10	
Oleate	.10	2.67
Stearate +	.10	
Linoleate	.10	0.76
Oleate +	.10	
Linoleate	.10	0.56
Palmitate +	.10	
Stearate +	.10	
Oleate	.10	2.24
Stearate +	.10	
Oleate +	.10	
Linoleate	.10	0.69
Palmitate +	.10	
Oleate +	.10	
Linoleate	.10	0.57
Palmitate +	.10	
Stearate +	.10	
Oleate +	.10	
Linoleate	.10	0.57

[1] The values reported are from one trial. These data are supported by several other trials conducted under slightly different experimental conditions (Table 39, Appendix Table 8). Conditions of assay were those shown in Table 4, except fatty acid was varied as indicated.

[2] Refers to total μmoles of fatty acid-1-^{14}C esterified for the acid(s) shown.

esterified could be calculated. The contribution of each acid to this total could not be calculated. For comparative purposes, in Figure 24 the esterification of palmitate-1-^{14}C at 0.20 mM (1.74 µmoles palmitate esterified/hr./g tissue) was designated 100%. The esterification rates of all other combinations were expressed as a percent of this value. Combinations of palmitate, stearate, and oleate all resulted in greater total esterification of fatty acid than palmitate alone. The greatest esterification of fatty acids was observed when stearate plus oleate were incubated together. In this experiment, and other similar ones, the esterification of oleate decreased at higher concentrations of acid (i.e., oleate at 0.10 mM = 2.33, oleate at 0.20 mM = 2.23). Stearate did not exhibit substrate inhibition. If the mammary gland stearate desaturase system (Lauryssens et al. 1961) was operating in this assay system, the facilitation of fatty acid esterification by the stearate-oleate couple might be explained by oleate generation from stearate. The inhibitory nature of oleate at 0.20 mM would be avoided when oleate concentration was reduced to 0.10 mM and supplied gradually by desaturation of stearate. Generation of oleate from stearate is not a satisfactory explanation for the beneficial effect of stearate in the palmitate-stearate couple. The combination of stearate and palmitate resulted in a combined esterification that was greater than either of the acids alone.

Although the combination of palmitate and oleate resulted in a greater esterification than palmitate alone, the combined esterification was less than that of oleate alone. Similar results for palmitate-oleate combinations have been reported for guinea pig mammary tissue (Korn 1967a). The additive nature of stearate and the competetive nature of palmitate and oleate suggested that stearate is incorporated by a different set of enzymes (i.e., acyltransferase) than are palmitate and oleate which appear to compete at some step for a common enzyme associated with fatty acid esterification. The higher Km observed for stearate (Table 32) than for palmitate or oleate agreed with these observations.

Two possible explanations for the failure to observe true stimulation[1] of fatty acid-1-^{14}C esterification by combinations of fatty acids were investigated. The studies conducted previously were assayed at near saturating concentrations of palmitate. Conceivably, stimulation might have been masked by saturating concentrations of fatty acid in the assay system. Table 37 presents the results from a series of assays conducted at less than saturating concentrations of palmitate-1-^{14}C (0.05 mM). No stimulation was observed under these conditions.

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[1] True stimulation is used in the sense that the resulting esterification of a combination of fatty acids would be greater than the sum of the rates of both acids alone.

Table 37

Unlabelled Fatty Acid Effect on Palmitate-1-^{14}C Incorporation When Both Acids Are Present at Low Concentrations[1]

Labelled Acid	mM	Unlabelled Acid Addition	mM	µmoles Palmitate/ hr./g
Palmitate-1-^{14}C	.05	None	---	1.81
Palmitate-1-^{14}C	.05	Stearate	.02	1.76
Palmitate-1-^{14}C	.05	Oleate	.02	1.79
Palmitate-1-^{14}C	.05	Linoleate	.02	1.44
Palmitate-1-^{14}C	.05	Trans-Vaccenic	.02	1.79
Palmitate-1-^{14}C	.05	Butyrate	.02	1.73

[1] The values reported are the results of one trial. Conditions of assay were those shown in Table 4, except fatty acid was varied as indicated.

All previous investigations were conducted using the 800 x g supernatant as the enzyme source. Brindley et al. (1967) observed that palmitate esterification by cat intestinal mucosa and rat liver mitochondria was not stimulated as much or as consistently by unsaturated fatty acids in the presence of the 100,000 x g supernatant as in its absence. This suggested that unsaturated fatty acids present in the 100,000 x g supernatant obscurred the stimulatory effect of exogenous unsaturated fatty acids. The particulate fraction of mammary tissue was separated from the 100,000 x g supernatant and

tested for fatty acid stimulation of palmitate esterification. The results are shown in Table 38. No stimulation of palmitate esterification was observed in the absence of the particle free supernatant, agreeing with studies just presented that used the 800 x g supernatant.

Table 38

Palmitate Esterification in the Absence of the Particle Free Supernatant[1]

Fatty Acid-1-^{14}C	mM	Predicted[2] Incorporation	Observed Incorporation	Percent[3] of Predicted
			- - CPM - -	
C-16:0	0.30		1890	
C-18:0	0.02		110	
C-18:1	0.02		250	
C-18:2	0.02		30	
C-18:3	0.02		220	
C-16:0 + C-18:0	0.30 + 0.02	2000	1820	91
C-16:0 + C-18:0	0.30 + 0.02	2140	2040	95
C-16:0 + C-18:2	0.30 + 0.02	1920	1760	92
C-16:0 + C-18:3	0.30 + 0.02	2110	2300	92
C-16:0 at 0.30 mM plus all other acids at 0.02 mM		2500	1900	75

[1] Values reported are the results of one trial. Conditions of assay were those shown in Table 4 except the enzyme source was the 100,000 x g pellet and fatty acids were varied as indicated.

[2] Predicted incorporation = Appropriate sum of observed individual incorporations.

[3] Percent of predicted = observed ÷ predicted.

In summary, the cooperative effects of various fatty acids on glyceride synthesis were observed to be partially additive but never stimulatory. One fatty acid, linoleate, behaved in a manner different from the other acids tested. The inhibitory nature of linoleic acid was investigated further.

c) Linoleate inhibition

Linoleic acid has been demonstrated to increase in serum (Davis and Sachan 1966) and milk fat (Beitz and Davis 1964) of cows exhibiting nutritionally elicited milk fat depression. In light of these observations *in-vitro* inhibition of fatty acid esterification by linoleic acid was investigated (Figure 24).

Linoleate inhibition was investigated by two approaches using equal specific activity palmitate-1-^{14}C and linoleate-1-^{14}C acids: (1) Esterification of total fatty acid was measured at constant palmitate-1-^{14}C concentrations and increasing linoleate-1-^{14}C concentrations; (2) Esterification of total fatty acid was measured at constant linoleate-1-^{14}C concentrations and increasing palmitate-1-^{14}C concentrations. Approach number one was employed to determine if a critical concentration of linoleate existed, past which inhibition would result. Approach number two was conducted to allow appraisal of the type of inhibition (i.e., competetive-noncompetetive) caused by linoleic acid.

Figure 25 shows inhibition of palmitate esterification as a function of linoleate concentration, using four cows and three sources of linoleic acid. Three of the four mammary tissue sources tested behaved similarly,[1] exhibiting slight inhibition of palmitate esterification from 0 to 0.10 mM linoleate. Past 0.10 mM linoleate, inhibition became severe. Transformation of the data used to plot the upper three curves shown in Figure 25 into 1/V vs [linoleate] plots is shown in Figure 27. In all three cases the plots of 1/V vs [i] were linear from 0 to 0.10 mM linoleate. In two out of three cases slight departure from linearity was observed between 0.10 mM to 0.20 mM linoleate, with marked non-linearity evident past 0.20 mM. With one tissue (32169) the inhibition curve was linear to 0.20 mM linoleate. Straight line 1/V vs [i] plots are consistent with normal competetive or non-competetive inhibition (Dixon and Webb 1964). Zahler and Cleland (1969) state that detergent effects of fatty-acyl-CoA micelles is consistent with nonlinear plots of 1/V vs [i] and by a marked departure from linearity occurring between inhibitor concentrations where inhibition does and does not occur. The results of the inhibition of fatty acid esterification by linoleate suggested that the inhibition observed (Figure 25) between 0.0 and 0.10 mM linoleate was not due to

[1] The fourth tissue (642) was tested against linoleic acid from Hormel. This linoleic acid was always more inhibitory than linoleic acid from other sources.

detergent action. The slight departure from linearity of 1/V vs [linoleate] plots between 0.10 and 0.20 mM may be due to slight detergent inhibition. The marked departure from linearity observed past 0.2 mM linoleate may be largely due to detergent action of linoleate micelles. It appears unlikely from the above considerations that inhibition of fatty acid esterification by linoleic acid can be attributed entirely to enzyme-fatty acid detergent effects.

Since a saturating concentration of palmitate was employed in these studies (0.20 mM) it could be argued that a major portion of linoleate inhibition might merely be due to a total fatty acid substrate inhibition. To test this possibility, fatty acid esterification was measured using palmitate-1-^{14}C, 0.20 mM, as a control while adding increasing quantities of oleate-1-^{14}C, palmitate-1-^{14}C and linoleate-1-^{14}C to identical control flasks. In this manner total fatty acid esterification was measured at 0.20, 0.25, 0.30, 0.35, and 0.40 mM total fatty acid in the incubation mixture. The results of this experiment are shown in Figure 26. Linoleic acid exhibited an entirely different behavior than either oleate or palmitate. Palmitate esterification was constant to 0.35 mM palmitate concentrations. Oleate plus palmitate showed increasing esterification of fatty acid to 0.35 mM total fatty acid. Oleate plus palmitate were partially additive with respect to total fatty acid esterification, confirming previous

results (Table 36). As noted previously, linoleate had little effect on palmitate esterification until its concentration exceeded 0.10 mM when marked inhibition occurred. All acids began to inhibit their incorporations past 0.35 mM total acid. From the results just discussed, only a small portion of linoleate inhibition can be attributed to "total acid" substrate inhibition.

The type of inhibition of glyceride synthesis resulting from the presence of linoleic acid in the incubation medium is not clear. Appendix Table 9 lists data from inhibition studies with three cows and three sources of linoleic acid. In one instance[1] (cow 642) inhibition by 0.10 mM linoleate was not relieved by increasing palmitate concentrations. Variable response to increasing palmitate concentrations was observed with the other two cows (333, 3669). Comparison of 1/S vs 1/V plots of the data shown in Appendix Table 9 did not clearly indicate the type of inhibition exerted by linoleate.

Abou-Issa and Cleland (1969) have reported that substrate inhibition caused by the detergent properties of palmityl-CoA micelles is dependent on the protein (enzyme) detergent (Acyl-CoA) ratio in the assay. Investigation of the effect of concentration of homogenate in the assay mixture on the inhibition caused by linoleate did not reveal a protein-detergent interaction (Table 39).

[1] Linoleic acid used in studies on 642 was from Hormel.

Table 39

Relationship Between Concentration of Homogenate and Linoleate Inhibition[1]

ml Homogenate[2]	Palmitate-1-^{14}C	Linoleate-1-^{14}C	CPM[3]
.2	0.20	0	928
.2	0.20	0.10	866
.5	0.20	0	1894
.5	0.20	0.10	1454
1.0	0.20	0	2372
1.0	0.20	0.10	2150

[1] Assay conditions similar to those described in Table 4 except the concentration of homogenate was varied and linoleate-1-^{14}C was included as indicated.

[2] 1:8 mammary homogenate, cow 445, 5/28/69.

[3] Counts per minute fatty acid esterified.

Thin layer chromatography of the reaction products of a linoleate inhibition study was conducted to ascertain if inhibition was the result of decreased esterification in a specific lipid class (Table 40). Linoleic acid caused a greater percentage of fatty acids to esterify into the phospholipid and monoglyceride fraction than palmitate alone. This is consistent with the greater content of linoleic acid in mammary tissue phospholipids than in neutral lipids (Kinsella and McCarthy 1968b). When linoleate-1-^{14}C was included with palmitate-1-^{14}C in the reaction mixture, less fatty acid-1-^{14}C was esterified into di- and triglycerides, and more fatty acids were esterified into monoglycerides and

Table 40

Thin Layer Chromatography of Reaction Products of Glyceride Synthesis Employing Palmitate-1-^{14}C, Linoleate-1-^{14}C, and Palmitate-1-^{14}C + Linoleate-1-^{14}C as Substrates[1]

Lipid Class	Palmitate-1-^{14}C CPM[2]	Palmitate-1-^{14}C %[3]	Palmitate-1-^{14}C + Linoleate-1-^{14}C CPM	Palmitate-1-^{14}C + Linoleate-1-^{14}C %	Linoleate-1-^{14}C CPM	Linoleate-1-^{14}C %
Phospholipid + Monoglyceride	162	13.5	268	23.7	65	30.8
Diglyceride	817	68.0	686	60.7	98	46.4
Triglyceride	216	18.0	158	14.0	43	20.4
Cholesterol esters	6	0.5	18	1.6	5	2.4
Total	1201	100.0	1130	100.0	211	100.0

[1] Tissue from cow 445, 5/28/69 was used for this study, each acid present at 0.20 mM. Lipids were extracted by heptane: isopropanol:water:1.0 N NaOH (40:40:30:1). Fatty acids employed were of equal specific activity. Conditions of assay were as shown in Table 4, except linoleate-1-^{14}C was added as indicated.

[2] CPM = Counts per minute from esterified fatty acid.

[3] % = CPM esterified in each lipid class ÷ total CPM in esterified lipids.

phospholipids compared to the incubation conducted with palmitate-1-^{14}C alone. The total CPM listed for each chromatogram sheet is not a quantitative estimation of fatty acid esterification since the reaction products of each flask were not quantitatively transferred to the chromatogram sheet. Identical incubations extracted and assayed by the standard

method (described in materials and methods) exhibited the following activities: palmitate 2840 CPM, linoleate plus palmitate 1644, and linoleate 384 CPM. From the results of this study linoleate appeared to inhibit palmitate esterification of both di- and triglycerides.

In summary linoleate was poorly esterified by mammary tissue homogenates, and inhibited the esterification of palmitate, stearate, and oleate by these same homogenates. In three out of four cows tested linoleate exhibited a similar type of inhibition. Both oleic (C-18:1) and linolenic (C-18:3) were esterified by this system (Figure 22) and were not inhibitory to the esterification of other acids. The effects attributable to linoleate (C-18:2) cannot be explained by the fact that linoleate is an unsaturated fatty acid. Examination of linoleate inhibition of fatty acid esterification by mammary tissue homogenates for characteristic acyl CoA detergent effects indicated that the inhibitory nature of linoleic acid cannot be explained entirely on the basis of non-specific detergent inhibition of enzyme action. Inhibition of fatty acid esterification by linoleate was consistently observed with all animals tested and all sources of linoleic acid employed. A summary of linoleic acid inhibition observed using mammary tissue from nine cows is listed in Appendix Table 10. The possible physiological significance of linoleate inhibition will be discussed further under the topic of "Milk Fat Depression."

d) Relationship of butyrate esterification to milk fat synthesis

Ruminant milk fat is unique in its relatively high content of short chain fatty acids. Butyrate comprises approximately 10 mole percent of the fatty acids esterified in milk fat triglycerides (Hilditch and Williams 1964). Patton and McCarthy (1963b) have proposed that the esterification of butyrate may be a completing step in the synthesis of a portion of milk fat triglycerides. Butyrate-1-^{14}C was tested for its ability to be esterified by this *in-vitro* system. Butyrate was not esterified significantly (0.04 µmoles/hr./g compared to 3.0 µmoles/hr./g for palmitate) when the standard assay conditions (as described in Materials and Methods) were used. The same system that esterified palmitate, stearate, oleate, and linolenate esterified butyrate at only 1 to 2% of the rates observed for the long chain fatty acids. Numerous attempts to arrive at *in-vitro* conditions conducive to butyrate esterification were unsuccessful. A summary of the experimental approaches used in trying to solve this problem are listed in Appendix Table 11.

Pynadath and Kumar (1964) have reported similar negative results for studies on butyryl-CoA esterification by goat mammary tissue. The failure of butyrate to be esterified by mammary homogenates in these studies (Appendix Table 11) along with the observation that triglyceride formation is not complete when palmitate is the sole substrate (Table 28),

agrees with the proposal (Patton and McCarthy 1963b) that butyrate may be necessary for a third acylation in milk fat triglyceride synthesis. The observation that mammary tissue 1, 2-diglycerides contain only small quantities of butyric acid compared to triglycerides (Patton and McCarthy 1963b) also suggests that the build up of diglycerides by this system may be due to the lack of a specific fatty acid (i.e., butyrate) necessary for a third acylation. The maximum extent of palmitate esterification into triglyceride by this system was 58%. This leaves 42% of palmitate-1-^{14}C in diglycerides which may require a short chain fatty acid such as butyrate for a third acylation to triglyceride. This is in fair accord with analytical data indicating that 50% of milk fat triglycerides contain a mole of short chain fatty acid per triglyceride molecule esterified predominantly to the 3 position (Kuksis and Breckenridge 1968, Breckenridge and Kuksis 1968).

7. Summary of Characteristics of Bovine Mammary Glyceride Synthesis

The esterification of palmitic acid by homogenates of bovine mammary tissue exhibited characteristics similar to fatty acid esterification previously described for rat mammary tissue (Dils and Clark 1962), goat (Pyndath and Kumar 1964), and guinea pig (McBride and Korn 1964b, Kuhn 1967a). The cofactor requirements, with the exception of ATP and DTT, were

similar to those found for mammary tissue of other species. The ATP requirement for bovine mammary tissue fatty acid esterification was approximately twice as high as that found for rat, guinea pig, or goat mammary tissue. However, previous investigatiors of mammary gland fatty acid esterification have not included DTT in the incubation media. The ATP requirement was increased 20% (Figure 11) in the presence of DTT, presumably because of increased ATP requirements due to accelerated fatty acid esterification. Dithiothreitol probably provided a more favorable environment for fatty acid esterification due to its sulfhydryl group protecting capabilities. Dithiothreitol may have exerted its protective effect directly on enzyme sulfhydryl groups (rather than CoA) since the CoA requirements were not altered by the presence or absence of DTT in the incubation mixture (Figure 12).

Palmitate esterification exhibited a pH optimum (7.2) near neutrality (Figure 13) and the activity was localized to the extent of 90% in the particulate fraction of the cell (Table 26). An apparent Km for palmitate of 0.13 mM was observed. This value was similar to that for palmitate esterification in rat adipose tissue (Angel and Roncari 1967). Mammary homogenates exhibited different affinities and esterification velocities toward fatty acids of different chain lengths and degrees of unsaturation (Table 32).

Combinations of certain fatty acids resulted in modest increases in fatty acid esterification compared to rates observed when each of the acids was incubated alone. However, no combination of fatty acids resulted in an esterification rate greater than the sum of the rates observed when each acid was incubated alone (Table 36). Linoleate was inhibitory to fatty acid esterification in this system. Although butyrate is found esterified in ruminant milk fat triglycerides, all attempts to demonstrate butyrate esterification by this system failed (Appendix Table 11). This observation along with that of the inability of this system to form greater than 58% triglyceride (Table 27) suggested that short chain fatty acid esterification may be necessary for a third acylation in milk fat synthesis.

An analysis of mammary tissue for FFA revealed a concentration of ~ 3.8 mM (Table 34). This value was several times greater than the apparent Km values (0.13 to 0.50) observed for fatty acid esterification (Table 32), indicating that fatty acid may not be limiting to glyceride synthesis *in-vivo*. There are, however, indications that α-GP may limit fatty acid esterification *in-vivo*. Kuhn (1967b) found that the concentration of α-GP in guinea pig mammary tissue was considerably below its Km for glyceride synthesis. Baldwin et al. (1969) reported the α-GP concentration in bovine mammary tissue to be ~ 0.154 mM, much less than the concentration

of α-GP found to be optimum for palmitate esterification (10 to 20 mM) in these studies. These results indicate that the amount of acyl acceptor may limit the extent of glyceride synthesis *in-vivo* while the amount of fatty acid probably does not.

C. MAMMARY ENZYME AND LONG CHAIN FATTY ACID MEASUREMENTS OF COWS FED RESTRICTED ROUGHAGE - HIGH GRAIN RATIONS

1. Experiment One - Nine Cow Study

Nine lactating Holstein cows were assigned to three 3 x 3 latin squares (as described in Methods and Materials) to study the effect of sequential ration changes upon certain mammary, liver and adipose enzyme activities. The rations fed were: normal ration (N), restricted roughage - high grain (RR), a typical ration that is likely to cause decreased milk fat yields, and restricted roughage - high grain plus MgO (RR + MgO). This additive has been shown to be effective in preventing depressed milk fat yields when cows are fed a restricted roughage - high grain ration.

The results reported will be concerned with measurement of the enzymes lipoprotein lipase (LPL) and glyceride synthetase (GS) in mammary tissue. Lipolytic activity towards "activated" Ediol is termed LPL activity and the esterification of palmitate into heptane extractable neutral lipids is termed GS activity. Serum, cream and mammary tissue fatty acid compositions will also be presented. A discussion of serum lipoprotein composition and enzyme response to ration in liver and adipose tissue from these same cows is published

elsewhere (Benson 1969). Each of the nine animals were fed the three previously described rations in the sequence shown in Appendix Table 12. Data on milk production and composition is shown in Appendix Table 13 and that on feed consumption Appendix Table 14.

Individual values for enzyme activities, tissue protein, tissue hydroxyproline, and milk fat tests of each cow are reported in Appendix Table 12.

No significant differences in enzyme response to ration were observed (Table 41).

Table 41

Mammary Gland Enzyme Activities in Cows Fed Three Rations, Experiment I

Parameter measured	Ration[1] N	RR	RR + MgO
Glyceride Synthetase[2]			
µmoles/hr./g tissue	1.9 ± 0.2	1.9 ± 0.2	2.1 ± 0.1
moles/hr./µg protein	24.7 ± 2.7	24.1 ± 3.3	27.5 ± 1.4
Lipoprotein Lipase[3]			
µeq. FFA/hr./g tissue	425.0 ± 45.0	378.0 ± 83.0	432.0 ± 54.0
µeq. FFA/hr./mg protein	5.6 ± 0.5	5.1 ± 1.2	5.8 ± 0.7
Fat Test (%)[4]	3.0 ± 0.1	2.5 ± 0.2	3.0 ± 0.1

[1] Rations: N = normal, RR = restricted roughage-high grain, RR + MgO = restricted roughage-high grain + MgO.

[2] Conditions of assay were those shown in Table 4 except palmitate was present at 0.30 mM.

[3] Conditions of assay were those shown in Table 3.

[4] Statistically significant ($P < 0.04$) by least squares method of analysis.

Two cows, 642 and 341, exhibited markedly decreased enzyme activity when receiving the RR ration. These assays were subsequently repeated, using a different homogenate from the same tissue. Similar low values for enzyme activity were again observed. Three possible explanations for these observations were feasible: 1) The decreased activity was a genuine response to ration; 2) The biopsy sample contained a disproportionate amount of inert connective tissue, or 3) The tissue somehow lost activity. These responses did not seem to be related to an especially severe milk fat depression. Biopsy specimens from cow 341 did contain less extractable protein per gram of tissue when receiving the RR ration than when receiving the other rations. However, those from cow 642 did not. Hydroxyproline values were not significantly different between treatment groups (Appendix Table 12) indicating similar amounts of connective tissue in all biopsy samples. Cow 341 exhibited an extremely high value (100 times normal) for adipose tissue fatty acid esterification coincident with depressed mammary tissue fatty acid esterification but tissue from cow 642 did not (Benson 1969). Consideration of all lines of evidence suggested that the decreased activity of mammary LPL and GS enzyme in cow 642 was probably not related to response to ration, but that of cow 341 might have been. The lack of a similar response by the other seven cows suggested that the response by 341 was atypical.

However, some basis for such a response may be attributable to the failure of cow 341 to consume the small allotment of hay (Appendix Table 13) when fed the RR-HG ration.

In this study the activities of LPL and GS (irrespective of ration) were positively correlated with each other, as well as with milk fat production and extractable tissue protein. These same enzyme activities were negatively correlated with hydroxyproline content of the tissue (Table 42). Although the correlations were not high, they do support the contention that the *in-vitro* assay systems were at least somewhat representative of *in-vivo* occurrences.

Table 42

Correlation of Some Mammary Gland Parameters with *In-Vitro* Enzyme Activities[1]

Enzyme Activity per gram of tissue	Parameter Correlated With	Correlation Coefficient
GS	LPL	0.62
GS	lbs milk fat/day	0.39
GS	mg protein/g tissue	0.42
GS	mg hydroxyproline/g tissue	-0.33
LPL	lbs milk fat/day	0.28
LPL	mg protein/g tissue	0.06
LPL	mg hydroxyproline/g tissue	-0.23

[1] N = 26; r values > 0.37 significant at $P < 0.05$.

Correlation of mammary gland GS and LPL activities with several serum parameters from the study of Benson (1969) did not reveal any significant correlations between triglyceride uptake by the mammary gland and mammary LPL or GS activities (Table 43). The lack of significant correlation between

Table 43

Correlation Between Mammary Gland Enzyme Activities and Serum Triglyceride Measurements[1]

Parameter[2]	Mammary Gland Arteriovenous Differences[3]	Correlation Coefficient
Mammary LPL	Serum TG-AV	-0.07
Mammary LPL	DSPLP TG-AV	0.04
Mammary GS	Serum TG-AV	0.17
Mammary GS	DSPLP TG-AV	0.15
DSPLP-TG	DSPLP TG-AV	0.60
Milk fat test (%)	DSPLP TG-AV	-0.13

[1] Serum parameters are from the study of Benson (1969); N =26; r values > 0.37 significant at $P < 0.05$.

[2] Enzyme activities used to calculate these correlations were expressed on a per gram of tissue basis. DSPLP-TG = dextran precipitable lipoprotein triglyceride arterial concentration.

[3] Arteriovenous differences used to calculate these correlation coefficients were expressed as mg triglyceride/100 ml serum. DSPLP TG-AV = dextran precipitable lipoprotein triglyceride arterial concentration minus venous concentration. Serum TG-AV = serum triglycerides, arterial concentration minus venus concentration.

mammary LPL and triglyceride uptake by the mammary gland suggested that this enzyme may be present in excess and that triglyceride fatty acid uptake by the mammary gland is more responsive to arterial lipoprotein triglyceride concentrations (Huber et al. 1969, Benson 1969) than to the amount of LPL activity in mammary tissue homogenates. The lack of a strong positive correlation between triglyceride fatty acid uptake and mammary GS activity agreed with previous estimations

(Summary, Characterization of Glyceride Synthesis) that fatty acid concentration may not limit glyceride synthesis in the mammary gland. The low correlation (-0.13) between dextran sulfate precipitable lipoprotein (DSPLP) triglyceride uptake and milk fat test also agreed with this concept.

Although mammary enzymes did not appear to respond to ration, increased GS and LPL activities were noted in the adipose tissue from these same cows (Benson 1969). Liver GS responded similarly to GS of mammary tissue. A comparison of enzyme response to ration in the three tissues studied is presented in Figures 28 and 29. The results of those studies supported the concept (Opstvedt et al. 1967, Baldwin et al. 1969) that the feeding of restricted roughage-high grain diets caused increased activity of adipose enzymes associated with lipid metabolism, while at the same time causing little or no effect upon the same enzymes in the mammary gland. Baldwin et al. (1969) have suggested that milk fat depression may be partially attributable to a decreased availability of long-chain fatty acids for milk fat synthesis due to increased uptake and deposition of these LCFA by adipose tissue. However, no conclusive evidence exists suggesting that there is a decreased uptake of triglyceride fatty acid by the mammary gland under the conditions of milk fat depression. To the contrary, Huber et al. (1969) observed no decrease in heparin precipitable lipoprotein triglyceride arteriovenous differences

by the mammary gland under the conditions of milk fat depression. Similarly, Benson (1969) found no significant decrease in either dextran precipitable lipoprotein triglyceride or serum triglyceride mammary gland arteriovenous differences in this study (Table 44).

Table 44

Serum and Lipoprotein Triglyceride Concentrations and Mammary Gland Uptake[1]

Component	Ration N mean ± SE	Ration RR mean ± SE
	- - mg/100 ml - -	
Serum triglycerides		
Arterial concentration	14.3 ± 1.3	13.0 ± 1.3
A-V difference	5.3 ± 0.9	4.2 ± 1.3
DSPLP triglycerides		
Arterial concentration	6.1 ± 0.8	7.5 ± 0.5
A-V difference	3.4 ± 0.5	4.0 ± 1.1

[1] Data from Benson (1969)

A consideration of the results just presented suggested that although LCFA acid uptake and esterification appeared to be increased in adipose tissue, a LCFA acid deficiency did not exist at the mammary gland. Failure to observe decreased mammary LPL or GS activity in this study under *in-vitro* assay conditions does not necessarily mean that the activity of these enzymes were not affected *in-vivo*. As stated by Baldwin et al. (1969) shifts in tissue levels of

enzyme substrates can occur which may produce metabolic changes in the absence of enzymatic adaptations.

To assess whether an alteration in LCFA substrate presented to the mammary gland had occurred, serum (precursor), mammary tissue (intermediate product) and cream (final product) were analyzed for long chain fatty acids (Table 45). Total serum LCFA showed little change in response to ration although

Table 45

Weight % LCFA in Serum, Cream and Mammary Tissue, Experiment I

	Serum[1]			Cream[2]			Mammary[3]		
Fatty Acid	N	RR	RR + MgO	N	RR	RR + MgO	N	RR	RR + MgO
C-14:0	8 ± 1	8 ± 1	9 ± 1	17 ± 1	19 ± 1	18 ± 1	5	7	6
C-16:0	19 ± 1	19 ± 2	18 ± 1	38 ± 2	32 ± 2	33 ± 2	45	35	32
C-18:0	29 ± 1	28 ± 1	28 ± 1	12 ± 1	13 ± 1	13 ± 1	13	13	15
C-18:1	13 ± 1	12 ± 1	12 ± 1	29 ± 2	30 ± 2	31 ± 2	29	35	37
C-18:2	31 ± 2	33 ± 2	33 ± 2	4 ± 1	6 ± 1	5 ± 1	8	11	10

[1] N = 9, tail serum
[2] N = 7, cream samples for 333 and 340 were lost
[3] N = 9, 800 x g supernatant. Samples were pooled by treatment prior to analyses.

a slight increase in linoleic acid (C-18:2) was noted in the serum of cows receiving RR or RR + MgO rations. Cream LCFA from cows fed RR or RR + MgO rations showed a decrease in palmitic acid and an increase in linoleic acid. Although the increase in cream linoleic acid was only 2% in the case of the

case of the RR group, this amounted to a 50% increase above normal concentrations of cream linoleic acid. Mammary tissue LCFA changes were similar to those of cream. Palmitic acid decreased and oleic and linoleic acids increased in mammary homogenates of cows fed restricted roughage rations. Mammary tissue lipids were further separated into phospholipids, triglycerides and free fatty acids to allow LCFA determinations of each lipid class (Table 46). Each lipid class reflected

Table 46

Weight % LCFA in Mammary Lipids, Experiment I[1]

Fatty Acid	Phospholipids			Triglycerides			Free Fatty Acids		
	N	RR	RR + MgO	N	RR	RR + MgO	N	RR	RR + MgO
C-14:0	5	3	5	7	6	8	8	4	6
C-16:0	29	22	24	34	31	34	25	20	22
C-18:0	16	21	18	25	25	25	9	11	11
C-18:1	38	38	39	32	33	29	48	50	50
C-18:2	12	16	14	2	5	4	10	15	11

[1] N = 9, mammary tissue 800 x g supernatants pooled by treatment, prior to analyses.

the same general pattern observed in cream and mammary tissue (Table 45), i.e., tissue from RR rations manifested decreased palmitic and increased linoleic acid weight prevent. Of particular significance was the change in LCFA composition of mammary tissue free fatty acids. Both myristic (C-14:0) and palmitic (C-16:0) acids were decreased 4 to 5% in tissue from the RR group while linoleic acid increased 5%. Both myristic and palmitic acids are synthesized within the mammary gland

from acetate and ß-hydroxybutyrate. A decreased weight percent of these acids could be a result of either: 1) no change in their concentrations but increased amounts of longer chain fatty acids or 2) actual decreased concentrations of myristic and palmitic and no change in the concentrations of the other acids. The lack of an increased uptake of triglyceride LCFA by the mammary gland of cows fed RR rations (Table 44) favored the latter viewpoint. These results are consistent with, and perhaps indicative of, decreased mammary synthesis of fatty acids when cows were fed RR rations.

The increased weight percent C-18:2 of mammary tissue FFA, presumably the substrate pool for mammary glyceride synthetase, was of interest in view of the *in-vitro*-inhibitory nature of linoleic acid (Figure 24). Although linoleic acid did not increase extensively in the total serum of cows fed restricted roughage rations (Table 45) shifts in the relative proportions of LCFA in a specific fraction of serum lipids might have been obscurred by the total LCFA composition of serum lipids. Bovine plasma low-density lipoproteins contained only 10% of the total esterified fatty acids in tail plasma (Emery et al. 1965) but contributed the majority of the fatty acids transferred from blood to milk fat (Table 44, Benson 1969). In this study (Benson 1969), cows receiving normal rations contained greater weight percent C-18:2 in mammary venous blood than in tail blood for DSPLP triglycerides

and cholesterol esters, indicating that C-18:2 was not removed from DSPLP triglycerides to the degree that the other fatty acids were. However, in the same cows fed RR rations, linoleic acid increased markedly in arterial DSPLP cholesterol esters and decreased in mammary venous blood indicative of possible increased transfer of linoleic acid to mammary tissue of cows fed restricted roughage rations. Free fatty acid concentrations on mammary homogenates from individual cows were not measured in the nine cow experiment just described. A follow up experiment was conducted to allow measurement of FFA concentrations in mammary tissue of cows fed restricted roughage and normal rations. This would permit a quantitative assessment of mammary tissue linoleic acid changes as a function of ration.

2. Experiment Two - Two Cow Study

Two lactating Holstein cows, were assigned tratements according to a 2 x 2 latin square design, as described in Methods and Materials. Feed consumption and milk production values are shown in Appendix Table 15. Confirming the results of the previous experiment, only slight differences in mammary LPL or GS activities were noted when cows received either RR or N rations (Table 47). Tissue samples from experiment II contained less extractable protein than those from experiment I, giving rise to higher enzyme activities than those of experiment I when expressed on a protein basis. Although tissue from cow 445 N exhibited less activity on a per gram of tissue

basis than 445 RR, the values were almost identical when expressed on a protein basis.

Table 47

Enzyme Activities of Cows Fed Restricted Roughage-High Grain or Normal Rations, Experiment II[1]

Parameter	Cow 445 N	Cow 445 RR	Cow 444 N	Cow 444 RR
Glyceride synthetase[2]				
μmoles/hr./g tissue	1.5	2.3	2.0	2.0
μmoles palmitate/hr./μg protein	51.4	48.0	39.0	36.4
Lipoprotein lipase[3]				
μeq. FFA/hr./g tissue	393.0	526.0	541.0	519.0
μeq. FFA/hr./mg protein	13.6	10.8	10.3	9.6
Fat test (%)	3.9	3.4	3.4	1.2

[1] N = normal ration, RR = restricted roughage - high grain

[2] Conditions of assay were as described in Table 4

[3] Conditions of assay were as described in Table 3.

In this experiment cow 445 did not manifest a decreased milk fat test (Table 47) even though she consumed almost identical quantities of ration as cow 444 (Appendix Table 15). A comparison of fatty acid composition of cream, mammary tissue, serum, and rumen fluid might be useful in explaining the difference in response observed. Both cows exhibited a similar LCFA cream composition when fed normal rations (Table 48).

Table 48

Fatty Acid Composition of Cream, Experiment II

Fatty Acid	445 N	445 RR	444 N	444 RR
	- - wt % - -			
C-16:0	33.5	29.1	35.3	26.9
C-18:0	18.3	24.7	18.0	6.1
C-18:1	46.1	42.7	44.5	59.4
C-18:2	2.1	3.5	2.2	7.7

However, differences were observed in cream LCFA composition when both animals were fed RR rations. Cream samples from both animals decreased in weight percent C-16:0 and increased in weight percent C-18:2. A greater response was noted for cow 444 in each instance. Different responses were observed with respect to stearic and oleic acids. Whereas stearate increased in the cream of cow 445, it decreased drastically in cow 444. Oleate decreased in cow 445 and increased in cow 444.

Similar changes in LCFA composition of mammary lipid classes were also observed (Table 49). A decrease in palmitate and an increase in linoleate was found for both cows fed RR rations when compared to the normal ration. In every instance the degree of such changes was greater in the cow that showed milk fat depression (444) than in the one that did not (445).

Table 49

Fatty Acid Composition of Triglycerides, Phospholipids and FFA of Mammary Homogenates, Experiment II

Lipid Class	Fatty Acid	445 N	445 RR	444 N	444 RR
			- - wt % - -		
Triglycerides	C-16:0	31.9	27.3	39.4	31.0
	C-16:1	1.0	1.4	0.8	3.7
	C-18:0	21.6	22.3	18.1	10.3
	C-18:1	41.8	43.5	37.3	47.3
	C-18:2	3.7	5.9	4.1	7.7
Phospholipids	C-16:0	23.6	20.3	23.3	21.8
	C-16:1	0.7	0.5	0.7	1.3
	C-18:0	16.1	16.5	15.3	12.3
	C-18:1	48.3	49.5	45.4	44.1
	C-18:2	11.3	13.1	15.3	20.5
FFA	C-14:0	9.1	7.0	12.9	7.6
	C-16:0	25.4	19.5	34.7	22.9
	C-16:1	1.9	2.6	1.3	5.4
	C-18:0	10.1	8.8	10.0	5.7
	C-18:1	51.8	60.0	39.7	55.4
	C-18:2	1.6	2.1	1.5	3.0

When comparing RR rations to normal rations, all classes of mammary lipids from cow 444 decreased in stearic acid. Triglycerides and FFA increased in oleic acid. Mammary triglycerides and phospholipids of cow 445 did not show the same stearic-oleic shift, but did exhibit increased oleic acid in the FFA fraction.

Rumen fluid samples were analyzed for LCFA in an attempt to detect the origin of the unsaturated fatty acids appearing in milk and mammary lipids of cows fed RR rations (Table 50).

Table 50

Fatty Acid Composition of Strained Rumen Fluid of Cows Fed Normal and Restricted Roughage-High Grain Rations

Fatty Acid	445		444	
	N	RR	N	RR
	- - wt % - -			
C-16:0	23.2	16.0	23.1	21.8
C-18:0	60.1	24.8	52.4	42.9
C-18:1	12.7	25.6	14.8	27.4
C-18:2	3.8	33.6	9.7	7.9

Lesser concentrations of unsaturated fatty acids were found in cream and mammary tissue of cow 445 when fed the RR ration than for cow 444, however, a dramatic increase in oleic and linoleic acids occurred in the rumen fluid of cow 445 when fed the RR ration. Cow 444, on the other hand, when fed the RR ration had a greater proportion of unsaturated fatty acids in cream and mammary tissue than cow 445 but had less unsaturated fatty acids in rumen fluid than did cow 445. Both animals showed a similar increase in oleic acid in their rumen fluid when fed the RR ration, but rumen fluid from cow 444 contained less linoleic acid than did rumen fluid from cow 445. Similar changes in LCFA of rumen fluid were observed in the LCFA of DSPLP triglycerides and cholesterol esters of the serum (i.e., C-18:2 increased in triglycerides and cholesterol esters of

cow 445 when fed RR ration, but did not increase in cow 444 on RR ration) (Benson 1969).

Although linoleic acid did not increase in the DSPLP fatty acids when cow 444 was fed a RR ration, the weight percent of linoleic acid increased in the mammary tissue of this cow (Table 49). In view of the *in-vitro* concentration dependant inhibition of fatty acid esterification by linoleic acid (Figure 25) FFA concentrations in mammary homogenates were measured. By changing weight percent linoleic acid to mole percent and applying appropriate correction factors for short chain fatty acid extraction by the Dole procedure, an estimate of linoleic acid concentrations in mammary tissues can be made (Table 51).

Although linoleic acid concentrations in mammary tissue of both cows increased ca 60% when fed RR rations, the absolute concentration of C-18:2 was 75% greater in the tissue of cow 444 than cow 445. The *in-vivo* linoleate concentrations for cow 445 (0.05 or 0.08 mM), regardless of ration fed, were not in the range of severe *in-vitro* linoleate inhibition (Figure 25). This same animal (445) did not exhibit milk fat depression when fed the RR ration. However, cow 444 did exhibit milk fat depression. The mammary tissue concentration of linoleate for cow 444 when fed a normal ration was 0.09 mM, not in the inhibitory range (Figure 25). However, when 444

Table 51

Linoleic Acid Concentrations in Mammary Tissue of Cows Fed Restricted Roughage-High Grain and Normal Rations[1]

Cow	Ration	Concentration of FFA[2]	Mole % Linoleic Acid	Concentration of Linoleic Acid
		(μeq./g)		(μmoles/g)
445	N	3.1	1.6	.05
	RR	4.0	2.0	.08
444	N	6.4	1.4	.09
	RR	5.1	2.8	.14

[1] Each sample was analyzed in triplicate. Values reported are average values.

[2] According to Hilditch and Williams (1964) mammary tissue lipid contains 12.8 mole % fatty acids of carbon chain length < 14. Applying appropriate distribution coefficients (f) for each fatty acid in heptane-isopropanol (Dole and Meinertz 1960) suggests that ~ 6.0% of the FFA detected in mammary tissue by the Dole procedure could be attributable to fatty acids of carbon chain length < 14. Values for FFA determined by Dole procedure were reduced by 6.0% before calculating concentrations of linoleic acid in mammary tissue.

was fed a RR ration tissue linoleate concentration was 0.14 mM, close to the range of severe linoleate inhibition of palmitate esterification. These results are not intended to imply that the *in-vitro* concentrations of linoleate shown to be inhibitory to palmitate esterification necessarily exist *in-vivo*. Rather, they are intended to show that the magnitude of change of linoleic acid concentrations in mammary tissue are sufficient, compared to an *in-vitro* system, to cause inhibition of fatty acid esterification.

The results presented for cows 445 and 444 do not have sufficient degrees of freedom to allow a meaningful estimate of statistical significance. These results (decreased palmitic and increased oleic and linoleic acids in cream and mammary tissue, no change in *in-vitro* LPL or GS activity in response to ration) did, however, agree with the results of the previous nine cow study (Experiment I).

A one cow pilot study was conducted to investigate the effect of intra-ruminally administered linoleic acid on milk fat percent. The administration of 500 to 700 ml (one dose per day for 11 days) of 75% linoleic acid (General Biochemicals, Chagrin Falls, Ohio) caused a 30% decrease in milk fat test without decreasing milk yield. Similar results were obtained when the experiment was repeated with safflower oil (ca 75% linoleic acid). Analysis of a mammary biopsy sample obtained when milk fat test had decreased from 3.2 to 0.6% following daily administration of 700 to 1000 ml safflower oil for 15 days indicated 0.5 µmoles of linoleate/g tissue present. Although a linoleate concentration value for this tissue when the animal was fed a normal ration was not available, such a concentration of linoleate would be consistent with severe *in-vitro* linoleate inhibition of palmitate esterification. A third oil, coconut oil (0.8% linoleic acid), was administered under the same conditions as was safflower oil as a non-linoleic acid control in these experiments. This oil also

depressed milk fat test ca 20% indicating that at least a portion if not all of the decreased milk fat percent observed in response to linoleic acid administration might have been due to a non-specific oil effect on ruminal fermentation. However, Steele and Moore (1968e) demonstrated that supplemental myristic acid in sheep rations decreased crude fiber digestibility. Five percent lauric acid added to the ration was extremely effective in decreasing milk fat yield in dairy cows (Steele and Moore 1968d). Since lauric and myristic acids are the major fatty acids of coconut oil, the selection of coconut oil as a control treatment in this experiment may have been a poor non-linoleic acid control. Arguing against a non-specific oil effect on rumen fermentation are the data of Shaw and Ensor (1959) who found that 300 g of linoleic acid fed in the ration decreased milk fat yield within 63 hours after feeding.

3. Discussion of Feeding Experiments

Lipoprotein lipase and glyceride synthetase assays of mammary tissues from cows fed normal, restricted roughage-high grain, and restricted roughage-high grain plus MgO rations revealed no significant treatment effect on enzyme activity. These same treatments did appear to exert an effect upon LPL and GS activities in adipose tissue (Benson 1969). Increased LPL and GS activity in adipose tissue of

cows receiving RR rations with little or no change in these same enzymes of the mammary gland agreed with similar studies (different enzymes) by Opstvedt et al. (1967) and Baldwin et al. (1969). These results are also consistent with the glucogenic theory of milk fat depression (i.e., RR rations favor a fattening type of metabolism to the exclusion of milk fat synthesis). However, the central point of the glucogenic theory assumes that stimulation of a fattening type of metabolism would of necessity cause a shortage of fatty acids to the mammary gland. This major point was not substantiated by the results of this study (Benson 1969) or previous studies (Huber et al. 1969). Baldwin et al. (1969) found increased concentrations of α-GP in mammary tissue of cows fed all concentrate diets, indicating that fatty acid acceptor was not responsible for decreased fat synthesis. Such data does not necessarily mean that a decreased mammary gland utilization of blood LCFA does not occur. Opstvedt and Ronning (1967) found that the reduction in milk fat yield observed in cows fed all concentrate diets was due to a reduction in the amounts of all major milk fatty acids, particularly long chain saturated fatty acids derived from blood. McCarthy et al. (1966) have proposed that when exposed to a supply of altered lipids (as in milk fat depression) the mammary gland will efficiently utilize only those lipids which fit the normal pattern of milk fat triglyceride composition.

These studies revealed that the fatty acid pool of mammary tissue was altered in such a manner as to reflect the changes in fatty acid composition of blood lipids when cows were fed RR-HG rations. A possible deficiency of stearic and palmitic acids or an oversupply of oleic and linoleic acids might have resulted in a decreased efficiency of milk fat synthesis. Several *in-vitro* observations on fatty acid esterification by mammary homogenates supported this possibility.

Although stearic acid alone was not esterified as rapidly as palmitic or oleic acids, combinations of oleic and stearic or palmitic and stearic were more additive in their esterification rates than other fatty acid combinations (Table 36). A decrease in stearate in mammary tissue FFA was observed in cow 444 coincident with decreased milk fat secretion. Only a slight decrease in stearate was observed in mammary tissue of cow 445 under the same conditions, but not exhibiting decreased milk fat secretion. No decrease in stearic acid in mammary tissue FFA of cows fed RR rations was observed in experiment I (Table 46). However, the degree of milk fat depression in experiment I was not as severe (2.5% fat) as that observed in cow 444 (1.6% fat).

Oleic acid increased in mammary tissue FFA of cows fed RR rations in both experiment I and II. In view of the rapid *in-vitro* esterification rates of C-18:1 (*cis*) it is difficult to comprehend how increased concentrations of oleic acid could

decrease fatty acid esterification. However the gas chromatographic procedure used in this study would not detect the difference between C-18:1 (*cis*) or C-18:1 (*trans*) acids. Part of the increased C-18:1 in serum DSPLP's (Benson 1969) cream, and mammary tissue may have been due to an increased production of the *trans* isomer of C-18:1 (*trans*-9-octadecenoic or *trans*-11-octadecenoic) Storry and Rook (1965) observed a dramatic increase in the proportion of *trans*-octadecenoic acid in milk fat of cows fed RR-HG diets. Normal ruminal hydrogenation of linoleic acid may be less complete when cows are fed RR-HG rations resulting in an increasing production of the *trans*-C-18:1 intermediate (Storry and Rook 1965a, Katz and Keeney 1966). *Trans* isomers of C-18:1 may not be utilized as well for milk fat synthesis as *cis* isomers. *In-vitro* competition studies using unlabelled *trans*-vaccenic acid (*trans*-11-octadecenoic acid) or oleic acid (*cis*-9-octadecenoic acid) with labelled palmitate supported this concept (Appendix Table 7). If a decreased stearic acid supply to the udder does exist under the conditions of milk fat depression (Benson 1969), the intramammary formation of oleic acid (*cis*-9-octadecenoic) by desaturation of stearate (Lauryssens et al. 1961) may be impaired. Decreased *cis* 18:1 coupled with increased *trans* 18:1 could be less favorable to milk fat synthesis. Acyl transferases in rat liver (Lands 1965b) and erythrocyte membranes (Waku and Lands 1968) discriminate sharply between *cis-trans* isomers of C-18 fatty acids.

Linoleic acid increased in Mammary tissue FFA and cream lipids in both experiments I and II. The magnitude of the increase in mammary tissue FFA in a limited study (2 animals) was consistent with both the failure of one animal (cow 445) to exhibit milk fat depression and the depression in milk fat percent observed in the other animal (cow 444) when fed RR-HG rations. Linoleic acid was also effective in depressing milk fat percent when administered to a lactating cow. However, so was coconut oil, an oil low in linoleic acid. The results from feeding experiments were consistent with the *in-vitro* inhibitory nature of linoleic acid. In two out of the three linoleic acid sources tested only limited esterification of linoleic acid into glycerides was observed. However, linoleic acid has been demonstrated to be rapidly incorporated into milk fat when oils rich in linoleic acid were infused directly into the blood of lactating cows (Storry and Rook 1965, Tove and Mochrie 1963). Linoleic acid may exert inhibitory effects on lipid metabolism in the mammary gland other than on fatty acid esterification. Decreased proportions of palmitate and myristate were observed in mammary tissue FFA when cows were fed RR-HG rations in both experiment I and II. Both of these fatty acids can be synthesized from acetate and ß-hydroxybutyrate by the mammary gland (Jones 1969). Palmquist et al. (1969) reported a decreased specific activity of milk fat synthesized from intra-mammary infused acetate-^{14}C by cows receiving RR

rations. This implied that perhaps acetate utilization by the mammary gland was imparied during milk fat depression. Although linoleate was not tested for its effect on fatty acid synthesis by mammary homogenates, studies with mice have indicated that livers from animals fed a high linoleic acid diet possessed decreased capability to synthesize fatty acids, while those from animals fed linoleic acid deficient diets possessed increased capabilities for fatty acid synthesis (Allman and Gibson 1965, Sabrni et al. 1969). Dual inhibition of mammary gland fatty acid synthesis and esterification by fatty acid(s) produced in excess under the conditions of milk fat depression would constitute an extremely effective mechanism of decreasing milk fat synthesis.

The mechanism whereby MgO prevents milk fat depression was not apparent from these studies. There was no significant treatment differences with respect to LPL and GS activities between the three rations fed. In general, fatty acid compositional shifts in mammary lipid classes (Table 46) were similar to those of the RR group, but were less pronounced. Benson (1969) observed an increased mammary gland arterial venous difference for DSPLP triglyceride when cows received MgO, agreeing with previous studies (Huber et al. 1969) indicating that MgO may increase transfer of blood fat to milk fat.

CHAPTER V

SUMMARY

In-vitro assay systems were devised to allow the measurement of lipoprotein lipase and glyceride synthetase activity in bovine mammary tissue. Certain characteristics of fatty acid uptake and esterification were studied prior to investigating the involvement of these enzymes in a metabolic aberration of bovine lipid metabolism, milk fat depression.

Lipoprotein lipase activity was present in lactating tissue, but absent in non-lactating tissue. The majority of the subcellular lipolytic activity was associated with the particulate fraction of the cell and was strongly dependent upon prior activation of the coconut oil substrate with serum. A lipase with properties similar to tissue lipoprotein lipase composed the majority of milk lipase activity toward serum-activated coconut oil. Mammary tissue lipoprotein lipase activity was not correlated with lipoprotein triglyceride uptake by the mammary gland, but was positively correlated with milk fat production.

Similarly, the majority of the subcellular fatty acid esterifying activity was associated with the particulate fraction of the cell. Fatty acid esterification was strongly dependent upon ATP, CoA, α-GP, and Mg++. The system was also stimulated by NaF, DTT and bovine serum albumin.

Although palmitate, stearate, oleate, and linoleate were all esterified at rates consistent with their content in milk fat, butyrate was poorly esterified by this system. This observation plus the fact that only 58% of the total palmitate-1-^{14}C esterified in di- and triglycerides was esterified as triglyceride agreed with the suggestion (Patton and McCarthy 1963b) that bovine mammary tissue may require a short chain fatty acid for a third acylation in milk fat synthesis. Certain combinations of fatty acids were partially additive in their combined esterifications. No combination of fatty acids yielded an esterification rate greater than the sum of that observed when each fatty acid was incubated alone.

Stearic acid was particularly complimentary to the esterification of oleic and palmitic acids. Unlabelled *trans* vaccenic acid did not compete with labelled palmitate as efficiently as unlabelled oleic acid, indicating that mammary gland enzymes may prefer the *cis* isomer of C-18:1. Linoleic acid behaved differently than the other acids tested. Although poorly esterified itself, linoleate also inhibited

the esterification of other fatty acids. Investigation of the inhibitory nature of linoleic acid suggested that not all of the *in-vitro* inhibition could be attributed to simple non-specific detergent effects.

Mammary gland lipoprotein lipase and glyceride synthetase activities did not change when cows were fed normal, restricted roughage-high grain or restricted roughage-high grain plus MgO rations. However, these enzymes showed an increased activity in adipose tissue of the same cows fed restricted roughage rations (Benson 1969). Fatty acid compositional studies of mammary lipids and cream suggested that a much different array of long chain fatty acids were being presented to mammary enzymes involved in fatty acid esterification. Extention of *in-vitro* studies to *in-vivo* fatty acid compositional changes suggested three possible mechanisms whereby fatty acid esterification might be decreased under the conditions of milk fat depression. A stearic acid deficiency may exist resulting in reduced esterification of other acids and/or reduced formation of oleic acid from stearic. If a portion of the large increase in C-18:1 fatty acids in mammary lipids is a *trans* isomer such as *trans*-vaccenic, the *trans* isomers may not be as well utilized for milk fat synthesis as the *cis* isomers. The increased concentrations of linoleic acid found in mammary FFA of cows receiving restricted roughage-high grain rations may also have physiologic significance if linoleate is as inhibitory *in-vivo* as it was *in-vitro*.

The highly ordered structure of milk fat triglycerides and the marked shift in composition of the long chain fatty acids presented to the mammary gland under the conditions of milk fat depression together with the *in-vitro* fatty acid specificities observed suggested that restricted roughage-high grain rations may impair fatty acid utilization by the mammary gland at a time when adipose tissue is incapable of releasing fatty acids that might allow a compensatory uptake of preferred fatty acids by the mammary gland. The net result may be a reduced utilization of a non-ideal array of long chain fatty acids by the mammary gland for milk fat synthesis.

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APPENDIX A

FIGURES

Figure 1. Release of FFA by lipoprotein lipase as a function of concentration BSA in incubation mixture. Conditions of assay were as described in Table 3 except BSA was varied. Similar results were obtained in another study at a lower pH (7.9).

Figure 2. Decrease in FFA released by lipoprotein lipase with increasing concentration of $CaCl_2$ or NH_4SO_4. Assay conditions were as described in Table 3 except for additions indicated. Similar results were obtained in another experiment conducted at lower (40.0 mg) BSA concentrations.

Figure 3. Release of FFA by lipoprotein lipase as a function of the percent serum used to activate Ediol. All assays were conducted at equal Ediol concentrations in the incubation mixture. Conditions of assay were those shown in Table 3 except the percent serum in the substrate was varied as indicated.

Figure 4. Release of FFA by lipoprotein lipase as a function of pH of the incubation mixture. Assay conditions were those of Table 3 except the pH of the incubation mixture was varied as indicated. Similar results were obtained in another experiment conducted over a narrower pH range (pH 7.3 to 8.3).

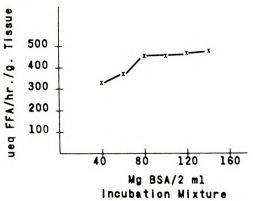


Figure 1

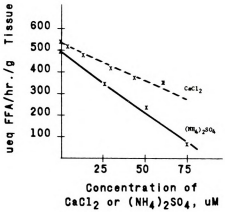


Figure 2

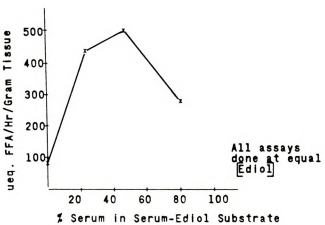


Figure 3

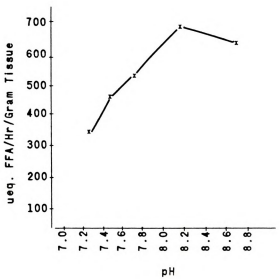
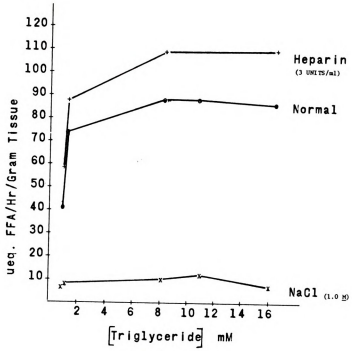


Figure 4

Figure 5. Release of FFA in the presence of Heparin and Sodium Chloride. Normal values represent simultaneous incubations in the absence of Heparin and NaCl. Heparin was present at all concentrations of substrate at 3 units/ml incubation mixture. NaCl was present in 1.0 M concentrations at all concentrations of substrate. Conditions of assay were those shown in Table 3 except substrate, Heparin, and NaCl were varied as indicated. Similar results were obtained in another trial using 0.3 units Heparin/ml incubation mixture and 0.25 M NaCl.

Figure 6. Release of FFA in response to increasing homogenate concentration. Concentration of homogenate is expressed as mg of tissue the homogenate was prepared from per ml incubation mixture. Conditions of assay were as described in Table 3 except amount of homogenate was varied and time of incubation was 60 minutes. Similar results were obtained in another trial with slightly different cofactor levels (40.0 mg BSA).

Figure 7. Free Fatty Acid Release as a Function of Incubation Time. Conditions of assay were as described in Table 3 except incubation time was varied as incubated. Similar results were obtained under slightly different cofactor concentrations (40.0 mg BSA).



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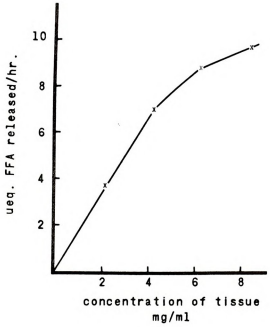


Figure 6

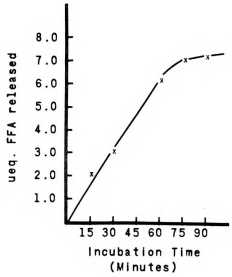


Figure 7

Figure 8. Release of FFA in response to increasing substrate concentration. Substrate is "activated" Ediol and is expressed on the basis of the triglyceride content of the Ediol portion of the substrate. Conditions of assay were those shown in Table 3 except substrate was varied as indicated.

Figure 9. Lineweaver Burk plot of data shown in Figure 8. Values of 2.3 mM triglyceride and 532 µeq. FFA released/hr./g tissue (corresponding to the apparent Km and maximum velocity, respectively) were obtained from this figure.

Figure 10. Lipolytic activity (µeq. FFA released/hr./ml) of skim milk in the presence of three substrate preparations. Serum-Ediol represents Ediol that has been "activated" by serum prior to assay. Ediol represents "non-activated" Ediol. Serum-Ediol + 1.3 M NaCl represents 1.3 M NaCl present at all concentrations of triglyceride from "activated" Ediol. The incubation mixture contained 100 mg BSA, 0.5 ml skim milk, and Ediol triglyceride as indicated. The incubation volume was 3.0 ml. Flasks were incubated at 37°C for one half hour.

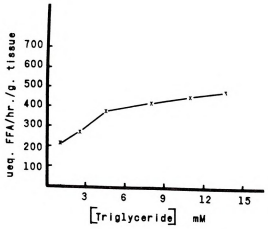
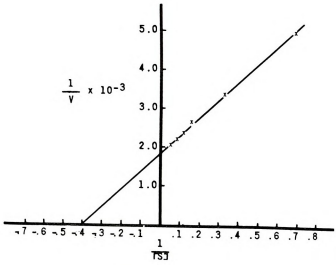


Figure 8



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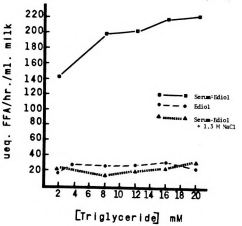

Figure 10

Figure 11. Relative esterification of palmitate at five concentrations of ATP in the incubation mixture. Palmitate esterification at 7.0 mM ATP is represented at 100% to allow comparison of esterification rates in the presence and absence of BSA and DTT. Cofactor concentrations for the minus BSA plus DTT values were: ATP (as indicated), CoA (0.4 mM), α-GP (20.0 mM), $MgCl_2$ (2.0 mM), NaF (50.0 mM). Cofactor concentrations for the plus BSA plus DTT system were as above plus 5.0 mg BSA and 4.0 mM DTT.

Figure 12. Relative esterification of palmitate at six concentrations of CoA in the incubation mixture. Palmitate esterification at 0.4 mM CoA is represented as 100% to allow comparison of esterification rates in the presence and absence of BSA and DTT. Cofactor concentrations for the minus BSA plus DTT values were: ATP (7.0 mM), CoA (as indicated), α-GP (20.0 mM), $MgCl_2$ (2.0 mM) NaF (50.0 mM). Cofactor concentrations for the plus BSA plus DTT system were as above plus 5.0 mg BSA and 4.0 mM DTT.

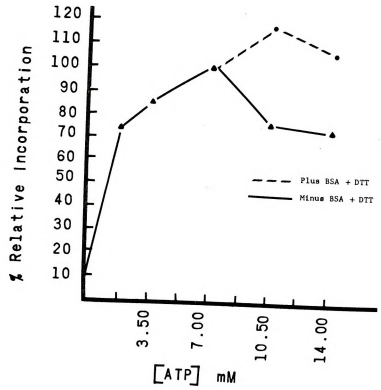

Figure 11

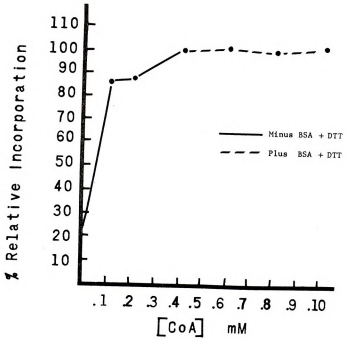

Figure 12

Figure 13. Palmitate-1-^{14}C esterification as influenced by pH of the incubation media and composition of the buffer employed. Cofactors and concentrations were: ATP (7.0 mM), CoA (0.4 mM), α-GP (20.0 mM), $MgCl_2$ (20.0 mM), NaF (50.0 mM). The buffer and pH of incubation media were varied as indicated. The two pH curves were determined with tissue from different animals. These results were supported by two similar studies conducted over a narrower pH range.

Figure 14. Palmitate esterification in the presence of sodium phosphate or Tris buffers at five different concentrations of palmitate. The pH of the incubation media was 7.2 for both buffers. Incubations were conducted simultaneously, using the same enzyme source (800 x g supernatant). Cofactors and concentrations were: ATP (7.0 mM), CoA (0.4 mM), α-GP (20.0 mM), $MgCl_2$ (2.0 mM), NaF (50.0 mM). Palmitate was varied as indicated.

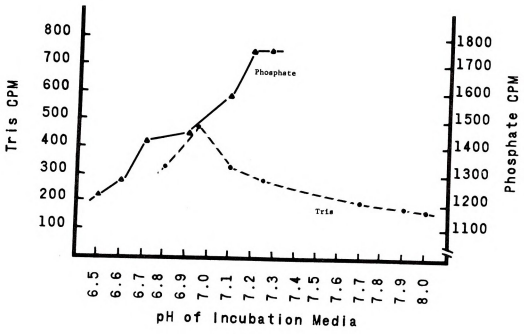

Figure 13

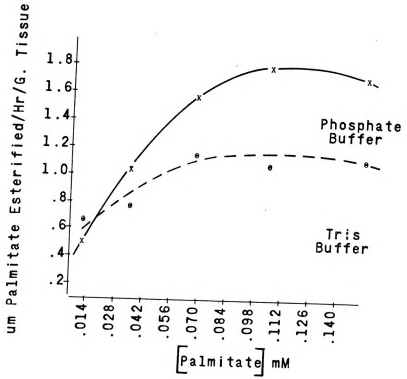

Figure 14

Figure 15. Palmitate esterification in response to increasing concentrations of homogenate in the incubation mixture. Similar results were obtained with two other tissue sources. Conditions of assay were those shown in Table 4 except the concentration of homogenate is expressed as the mg of tissue from which the homogenate was derived per ml incubation mixture.

Figure 16. Palmitate esterification as a function of incubation time. Conditions of assay were those shown in Table 4 except that incubation time was varied as indicated.

Figure 17. Palmitate esterification at six concentrations of palmitate. Each value represents the average of three identical determinations on the same homogenate. Standard errors for each value are given in Table 19. Conditions of assay were those shown in Table 4 except palmitate was varied as indicated.

Figure 18. Lineweaver Burk extrapolation of palmitate esterification by bovine mammary gland homogenate. The data transformed for this plot are those of Figure 17. An apparent Km of 0.13 mM palmitate and a Vmax of 7.89 μmoles palmitate esterified/hr./g tissue were derived from this plot.

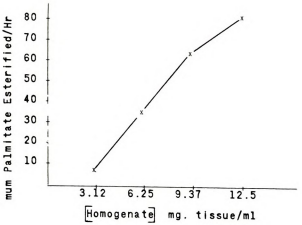

Figure 15

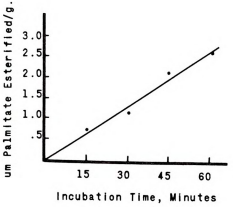

Figure 16

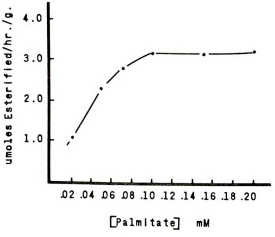

Figure 17

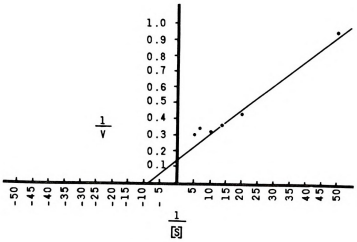

Figure 18

Figure 19. Typical thin layer chromatogram of chloroform:methanol (2:1) lipid extract of lactating bovine mammary tissue following incubation with palmitate-1-^{14}C. Solvent system was hexane:ethyl ether:acetic acid (80:20:1). Identity of numbered areas listed at the edge of the chromatogram sheet: (1) phospholipid (origin); (2) monoglyceride; (3) unknown (no visual spot); (4) 1, 2-diglyceride; (5) 1, 3-diglyceride; (6) FFA; (7) triglyceride; (8) cholesterol ester (no visual spot); (9) solvent front. Figures enclosed by representations of lipid classes are R_f values. Similar separations and identifications were obtained with four different mammary tissues. Procedures used in this separation were as described in Methods and Materials.

Figure 20. Typical thin layer chromatogram of chloroform: methanol (2:1) lipid extract of lactating bovine mammary tissue. Solvent system was chloroform:methanol:ammonium hydroxide (75:25:4). Tentative identity of numbered areas listed at the edge of the chromatogram sheet: (1) origin; (2) phosphatidic acid; (3) lyso-phosphatidyl ethanolamine or sphingomyelin; (4) phosphatidyl choline: (5) phosphatidyl ethanolamine; (6) calcium salt of phosphatidic acid; (7) FFA; (8) neutral lipids; (9) solvent front. Figures enclosed by representations of lipid classes are R_f values. The results shown are typical of four separations conducted upon different occasions from the same tissue source. Procedures used in this separation were as described in Methods and Materials.

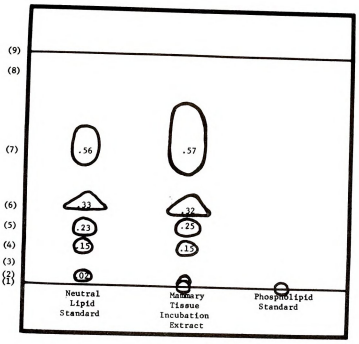

Figure 19

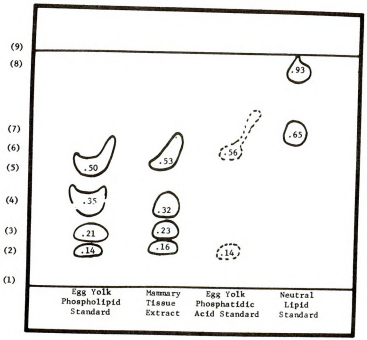

Figure 20

Figure 21. Esterification rates of several long chain fatty acids by bovine mammary homogenates. Conditions of assay were those shown in Table 4 except fatty acid substrate was varied as indicated. Each fatty acid was tested individually at the concentrations indicated. All incubations contained equal amounts of the same homogenate. Similar results were obtained under slightly different incubation conditions (i.e., substrate concentrations) in eight studies involving three animals.

Figure 22. Esterification of several long chain fatty acids by the 800 x g supernatant and particulate fraction of bovine mammary tissue. The esterification rate reported is the maximum value achieved over the substrate concentrations assayed (0 to 0.3 mM). Conditions of assay were those shown in Table 4 except fatty acid and enzyme source were varied as indicated. The results reported are for a single determination but are supported by similar studies conducted under slightly different conditions (Appendix Table 3).

Figure 23. Esterification of Palmitate-1-^{14}C in the presence of several unlabelled fatty acids. Each of five unlabelled fatty acids was tested at the concentrations indicated for its effect on the esterification of 0.10 mM Palmitate-1-^{14}C into neutral lipids. The conditions of assay were those shown in Table 4 except fatty acids were added as indicated. The data presented here represent one trial. Similar experiments conducted under slightly different conditions support these findings (Table 38, 39, 40, Appendix Table 8).

Figure 24. Esterification of several combinations of fatty acids by the mammary gland. The cofactors and concentrations used were those shown in Table 4. All fatty acids used were 1-^{14}C fatty acids of equal specific activity. The results are expressed as a percent of the esterification observed when Palmitate-1-^{14}C at 0.20 mM was the sole substrate. The data presented are from one trial (Table 36). Similar results were obtained under slightly different experimental conditions (Appendix Tables 8 and 10).

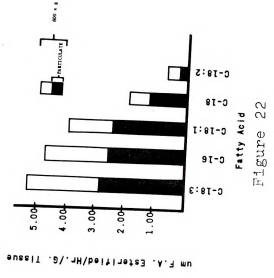

Figure 22

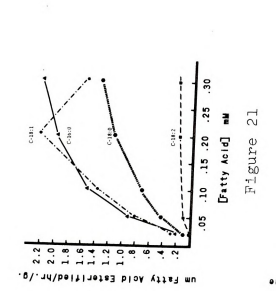

Figure 21

Figure 23

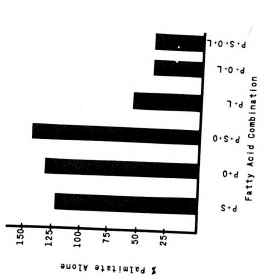

Figure 24

Figure 25. Fatty acid esterification in the presence of increasing concentrations of linoleate. Cofactors and concentrations were those shown in Table 4. Palmitate-1-^{14}C was present at 0.10 mM at all concentrations of linoleate-1-^{14}C. The control value was the esterification rate of palmitate-1-^{14}C at 0.10 mM in the absence of linoleate. The data presented are the results from trials with tissue from four cows, 32169, 333, 3669, 642.

Figure 26. Fatty acid esterification in the presence of several combinations of fatty acids. Cofactors and concentrations were those shown in Table 4. The esterification of 1-^{14}C-palmitate at 0.20 mM in the absence of the other acids is expressed as 100%. Each fatty acid was of equal specific activity. These values are the result of one trial but are supported by a similar study conducted at 0.10 mM fatty acid concentrations (Table 36). The combined esterification rate for palmitate-1-^{14}C and oleate-1-^{14}C (upper curve) is less than the sum of the esterification rates of either acid incubated separately (approximately 200% on this scale).

Figure 27. Linoleic acid inhibition of fatty acid esterification expressed by 1/V vs [i] plots. Conditions of assay are the same as those expressed for Figure 25. The inhibitor (i) in this case is linoleate.

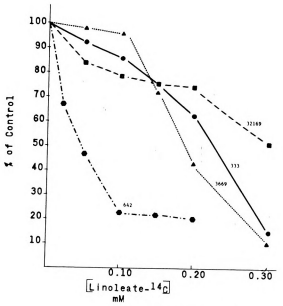

Figure 25

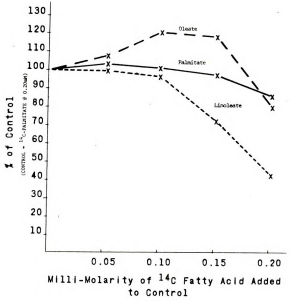

Figure 26

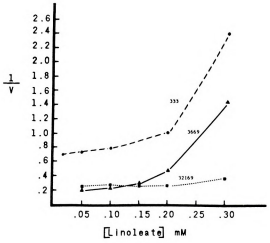

Figure 27

Figure 28. Comparison of lipoprotein lipase activities in mammary and adipose tissues of the same cows fed three rations. Enzyme activity is expressed as percent of normal. Enzyme activity exhibited by tissues fron animals receiving the normal ration was designated 100%. Experimental design was as described in Methods and Materials. N = normal ration, RR = restricted roughage-high grain, RR + MgO = restricted roughage-high grain plus MgO. Adipose lipoprotein lipase activity was determined by Benson (1969).

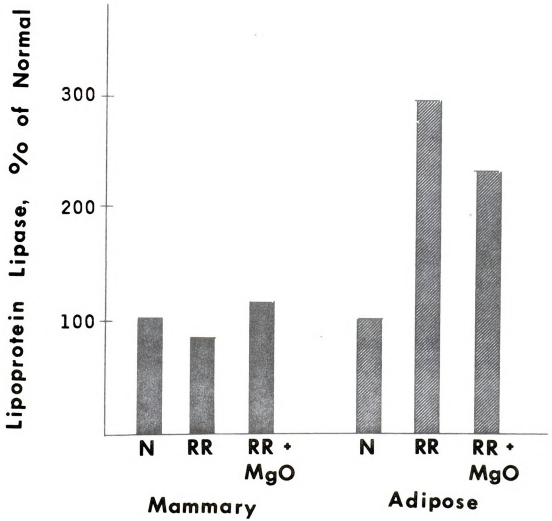
Lipoprotein Lipase, % of Normal
300
200
100
N
RR
RR + MgO
N
RR
RR + MgO
Mammary
Adipose

Figure 28

Figure 29. Comparison of glyceride synthetase activities in mammary, liver, and adipose tissues of the same cows fed three rations. Enzyme activity is expressed as percent of normal. Enzyme activity exhibited by tissues from animals receiving the normal ration was designated 100%. Experimental design was as described in Methods and Materials. N = normal ration, RR = restricted roughage-high grain, MgO = restricted roughage-high grain plus MgO. Liver and adipose glyceride synthesis was determined by Benson (1969).

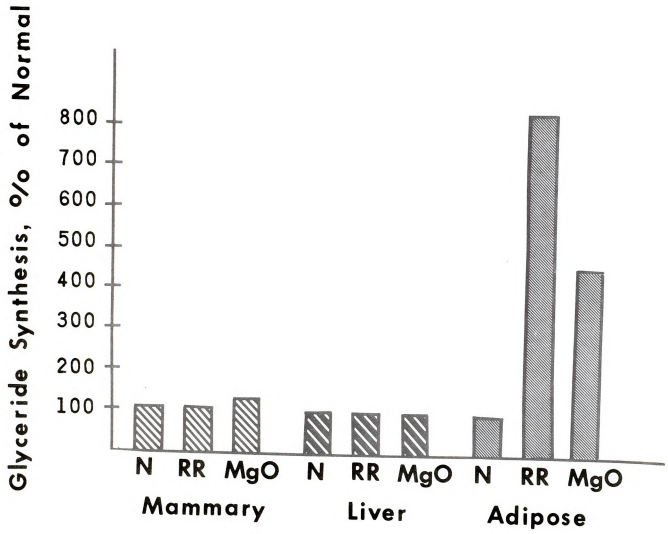
Glyceride Synthesis, % of Normal
800
700
600
500
400
300
200
100
N RR MgO N RR MgO N RR MgO
Mammary
Liver
Adipose

Figure 29

APPENDIX B

TABLES

Appendix Table 1

Fat Test, Milk Production and Lipolytic Activity of Cow's Milk[1]

Cow. No.	Fat Test[2] %	Milk Production[3] (Kg)	Lipolytic Activity per ml Milk[4] (μeq./hr./ml)
771	3.3	25.2	304.6
832	2.6	22.5	243.2
880	3.6	17.3	229.6
891	3.0	26.8	313.6
896	3.1	25.9	271.0
908	3.2	20.9	205.9
950	3.3	23.6	217.3
968	3.5	18.4	119.0
969	2.4	42.5	206.4
972	3.2	23.4	248.1
976	2.7	30.5	390.7

[1] Assay conditions: Each flask contained 100 mg BSA, 0.4 ml "activated" Ediol, 0.5 ml diluted skim milk (1.0 part fresh milk centrifuged 800 x g for 10 minutes:9 parts .15 M KCl) in a total volume of 3.0 ml. Flasks were incubated 30 minutes at 37°C. Values for identical flasks containing "non-activated" Ediol were substracted from flasks containing "activated" Ediol prior to calculation of results.

[2] Average of three determinations.

[3] Milk production on day of sampling.

[4] Mean value ± SE = 244 ± 23.

Appendix Table 2

Time Course Glyceride Synthesis by Bovine Mammary Tissue[1]

Glyceride Class		Minutes 15	30	45	60	75	120	150
Mono	mμ moles	0.34	0.71	1.39	2.97	3.60	4.46	7.46
	%	18	19	21	24	24	18	21
Di	mμ moles	0.87	1.63	2.68	3.96	3.52	8.56	11.47
	%	47	44	41	32	23	35	33
Tri	mμ moles	0.66	1.39	2.55	5.41	8.19	11.81	15.99
	%	35	37	39	44	54	48	46
Total mμ moles		1.86	3.73	6.62	12.34	15.28	24.83	34.91

[1] Conditions of assay were those described in Table 27.

Appendix Table 3

Enzyme Velocity Measurements Used in Determining Km and Vm Estimates in Table 32[1]

Fatty Acid Concentration	Cow											
	μmoles fatty acid esterified/hour/gram tissue											
	Palmitate				Stearate			Oleate		Linoleate		
	330	332	333	3669	330	332	3669	332	3669	332	333	3669
.02	1.21	0.23*	0.25*	--	0.41	0.09*	--	0.27*	--	0.00*	0.02	--
.05	2.73	0.92	0.64	1.77	0.96	0.42	0.50	0.81	1.02*	0.09	0.07	0.08
.10	3.77	1.52	1.13	2.88	1.63	0.71	0.99	1.39	2.52	0.07	--	0.14
.15	--	--	--	3.50	--	--	1.28	--	3.17	--	--	0.20
.20	--	1.96	1.45	3.92	--	1.14	1.82*	2.21	3.82	0.19	0.12	0.28
.30	--	2.20	1.69	4.70	--	1.35	1.74	1.53*	3.83*	0.21	0.15	0.43

* These values were omitted from calculating Lineweaver Burk regression equation because they were either past the enzyme saturation point or were at low substrate level.

[1] Conditions of assay were those of Table 4, except fatty acid was varied as indicated.

Appendix Table 4

Liberation of Endogenous FFA by Mammary Gland Homogenate[1]

Cow	0-Time[2]	60-Minutes[3]	FFA released per hour
	- - µmoles - -		
329	0.10	0.12	0.02
330	0.07	0.11	0.04
642	0.08	0.14	0.06
Average	0.08	0.12	0.04

[1] FFA were measured before and after a 1 hour, 37°C, incubation of 0.2 ml of a 1:8 mammary homogenate in a 2.0 ml assay volume in the absence of cofactors

[2] Endogenous FFA present at 0-Time.

[3] Endogenous FFA present after 60 minutes incubation time.

Appendix Table 5

Free Fatty Acid Concentrations in Cellular Components of Bovine Mammary Tissue[1]

Cellular Fraction	FFA	% Distribution
	- - µeq./g tissue - -	
Crude Homogenate	29	100
800 x g Supernatant	31	107
100,000 x g Supernatant	4	14
Particulate[2]	21	72

[1] Values reported here for FFA concentrations are higher than normally found in fresh tissue. Since this sample was several months old lipolysis had probably occurred during storage.

[2] Fatty acids found in the particulate fraction constituted 84% (21 ÷ 25) of the fatty acids recovered in the combined 100,000 x g supernatant and particulate fractions.

Appendix Table 6

Esterification of Endogenously Released Free Fatty Acids[1]

Cofactors[2]	0-Time[3]	60-Minutes[4]	FFA released (+) or esterified (-)
	- - µmoles - -		
-	0.104	0.124	+ 0.020
+	0.065	0.046	- 0.019

[1] FFA were measured before and after a 1 hour 37°C incubation with and without cofactors. A 2.0 ml incubation volume was used containing 0.2 ml of a 1:8 homogenate.

[2] Cofactors and concentrations were those shown in Table 4.

[3] Endogenous FFA present at 0-Time.

[4] Endogenous FFA present after 60 minutes incubation time.

Appendix Table 7

Palmitate-1-^{14}C Esterification in the Presence of *cis* or *trans* Isomers of Octadecenoic Acid[1]

Fatty Acid	mM	µmoles Palmitate esterified/hr./g tissue
Oleic (*cis*-9-octadecenoic)	0.02	1.98
	0.05	1.73
	0.10	1.25
Vaccenic (*trans*-11-octadecenoic)	0.02	2.26
	0.05	2.15
	0.10	1.80

[1] The values presented are the results of a trial using the same tissue homogenate for both acids. Conditions of assay were as shown in Table 4, except palmitate-1-^{14}C was present in all incubations at 0.10 mM and unlabelled oleic or vaccenic acids were added as indicated. Palmitate-1-^{14}C esterification in the absence of unlabelled acids was 2.10 µmoles/hr./g.

Appendix Table 8

Palmitate-1-^{14}C Esterification in the Presence of Various Unlabelled Fatty Acids[1]

Labelled Acid	mM	Unlabelled Acid Additions	mM	µmoles Palmitate/hr./g
Palmitate-1-^{14}C	0.10	Stearate	.02	
		Oleate	.02	
		Linoleate	.02	1.32
		Butyrate	.02	
Palmitate-1-^{14}C	0.10	Stearate	.02	
		Oleate	.02	1.31
		Linoleate	.02	
Palmitate-1-^{14}C	0.10	Stearate	.02	
		Oleate	.02	1.73
		Butyrate	.02	
Palmitate-1-^{14}C	0.10	Stearate	.02	
		Linoleate	.02	1.40
		Butyrate	.02	
Palmitate-1-^{14}C	0.10	Oleate	.02	1.74
		Linoleate	.02	
		Butyrate	.02	

[1] As a reference value, palmitate-1-^{14}C at 0.10 mM incubated alone exhibited an esterification rate of 2.08 µmoles/hr/g. Conditions of assay were those described in table 4, except fatty acid was varied as indicated.

Appendix Table 9

Linoleate Inhibition of Palmitate Esterification[1]

Palmitate-1-^{14}C mM	642[2]		333[3]		3669[4]	
	V_P	V_{P+L}[6]	V_P	V_{P+L}	V_P	V_{P+L}
	- - μmoles/hr./g - -					
.02	0.35	0.28	0.25	0.29	0.59	0.45
.05	0.72	0.25	0.64	0.59	--	--
.07	0.82	0.27	--	--	--	--
.10	0.91	0.24	1.13	0.99	1.83	1.65
.15	1.00	0.20	--	--	--	--
.20	1.04	0.26	1.45	1.31	2.38	1.91
.30	--	--	1.69	1.43	--	--

[1] Linoleate-1-^{14}C present at 0.10 mM at all concentrations of palmitate-1-^{14}C.

[2] Cow 642 (800 x g supernatant) linoleate source Hormel (Hormel Institute, Austin, Minn.).

[3] Cow 333 (800 x g supernatant) linoleate source Sigma (Sigma Chem. Co., St. Louis, Mo.).

[4] Cow 3669 (particulate) linoleate source Applied Sciences (The Anspec Co., Ann Arbor, Mich.).

[5] V_P = Velocity of reaction, esterification palmitate alone.

[6] V_{P+L} = Velocity of reaction, esterification palmitate + linoleate.

Appendix Table 10

Inhibition of Palmitate Esterification by Various Tissue Sources - Linoleate Sources

Cow	Linoleate Source[1]	Palmitate-1-^{14}C mM	Linoleate-1-^{14}C mM	% Control[2]
330	H	.05	.02*	80
330	H	.10	.10*	35
330	H	.10	.10	52
642	H	.10	.10	26
332	S	.10	.10	79
332	S	.30	.10	75
333	S	.10	.10	88
333	S	.30	.10	85
333	S	.20	.10	90
3669	S	.20	.10	88
3669	A	.20	.10	95
3219	A	.20	.10	78
642	H	.20	.10	23
444	A	.20	.10	78
445	A	.20	.10	65

[1] Linoleate sources: H = Hormel (Hormel Institute, Austin, Minn.)
S = Sigma (Sigma Chem. Co., St. Louis, Mo.)
A = Applied Sciences (The Anspec Co., Ann Arbor, Mich.).

[2] % Control: Control = Palmitate-1-^{14}C without linoleate-1-^{14}C.

* Represents determinations when linoleate was not isotopically labelled.

Appendix Table 11

Investigations of Butyrate Esterification by Mammary Homogenates

Theory	Conditions of Assay[1]	Esterification[2]
1. Butyrate esterified similar to LCFA	0.10 mM butyrate-1-^{14}C	0.04
2. Butyrate esterified as β-hydroxybutyrate, then reduced to butyrate	0.10 mM β-hydroxybutyrate-1, 3-^{14}C	0.02
3. Freezing tissue may destroy butyrate specific enzyme	0.2 ml fresh tissue homogenates	0.03
4. Butyrate esterifying activity associated with 800 x g pellet	0.2 ml crude homogenate	0.03
5. Cellular unity necessary for butyrate esterification	0.1 gram tissue slice	0.01
6. Butyrate esterification takes place in freshly secreted milk	1.0 ml fresh milk	--
7. A cofactor may inhibit butyrate esterification	ATP, CoA, $MgCl_2$, BSA, NaF, and DTT omitted singly (6 assays)	0.01-0.02
8. Butyrate may not be activated to its CoA derivative	0.1 g freeze-thawed bovine liver mitochondria (known to activate butyrate) added to incubation mixture	0.03
9. Carnitine necessary for transport of butyrate into mitochondria for activation	3.0-6.0 mM DL-carnitine	0.01

Continued

Appendix Table 11 Cont.

Theory	Conditions of Assay[1]	Esterification[2]
10. Guanosine-triphosphate not Adenosine-triphosphate activates butyrate	10.0 mM GTP	0.01
11. Monoglyceride serves as acyl acceptor for butyrate	10.0 mM α-Monopalmitin	0.01
12. Specific endogenous acyl acceptor required for butyrate	lipid extract from 0.5 g mammary tissue[3]	--
13. Reducing equilavents may be required for un-defined pathways of butyrate esterification	6.0 mM G-6-P, 6.0 mM TPN+, 1.0 mg glucose-6-phosphate dehydrogenase	0.02
14. Esterification of butyrate sensitive to pH	pH 6.5 pH 7.4 pH 8.0	0.02 0.04 0.04
15. Buffer composition may influence butyrate esterification.	(0.1 mM phosphate buffer) (0.05 mM Tris buffer)	0.04 0.02

[1] All assays contained the cofactors and concentrations of the standard assay system shown in Table 4, except for the desired alterations.

[2] μmoles butyrate esterified/hr./g tissue

[3] Prepared by extracting 0.5 g mammary tissue with chloroform: methanol (2:1), reducing lipid extract to dryness, and resuspending the lipid extract in 0.25 ml 10% BSA + 0.25 ml phosphate buffer + 0.05 ml triton x - 100.

Appendix Table 12

Mammary Gland Parameters Measured in Experiment I[1]

Cow	Ration	GST[2]	GSP[3]	LPLT[4]	LPLP[5]	OHP[6]	Protein[7]	Milk fat[8] test
329	MgO	1.86	22.56	271.1	3.28	2.60	82.59	2.6
	N	1.89	19.59	321.1	3.33	1.37	96.46	3.1
	RR-HG	2.45	34.80	458.0	6.50	2.31	70.48	3.2
330	N	1.88	15.34	541.6	6.03	1.79	89.88	2.9
	RR-HG	2.31	26.04	282.1	3.18	1.71	88.84	1.3
	MgO	2.21	29.33	797.7	10.59	4.07	75.32	3.3
642	RR-HG	0.59	8.42	54.1	0.77	8.14	70.13	2.8
	MgO	1.46	20.26	234.2	3.26	8.92	71.86	3.0
	N	1.77	29.46	384.5	5.80	1.75	60.08	2.7
331	N	1.81	26.24	274.6	3.98	2.11	69.05	2.7
	RR-HG	2.74	31.26	467.0	5.32	7.79	87.76	2.7
	MgO	2.22	34.05	489.6	7.50	6.72	65.24	2.9
332	MgO	2.28	28.19	480.7	5.95	7.93	80.84	2.6
	N	1.04	16.19	287.6	4.48	8.28	64.21	2.9
	RR-HG	2.19	32.12	752.5	11.06	2.93	68.02	2.7
333	RR-HG	1.71	21.56	306.7	3.86	9.43	79.45	1.9
	MgO	1.89	25.32	374.5	5.24	9.98	74.60	2.7
	N	2.12	33.16	451.2	6.74	5.61	66.97	3.3
334	N	1.53	21.87	553.9	7.92	6.85	69.90	3.1
	RR-HG	1.77	24.83	670.3	9.41	2.77	71.23	2.5
	MgO	2.02	31.12	398.5	6.14	6.55	64.89	3.3
341	MgO	2.51	29.15	401.9	4.68	2.40	85.92	3.3
	N	3.12	35.34	588.2	6.67	2.14	88.25	3.6
	RR-HG	0.85	8.10	12.3	0.18	4.25	68.23	2.7
340	RR-HG	2.13	29.43	398.5	5.52	4.54	72.23	3.1
	MgO	2.08	27.83	440.9	5.91	3.94	74.58	3.2

[1] Each ration is listed in the order that it was fed.
[2] GST = glyceride synthetase activity, µm palmitate esterified/ hr/g tissue.
[3] GSP = glyceride synthetase activity, µm palmitate esterified/ hr/µg extractable protein.
[4] LPLT = lipoprotein lipase activity, µeq FFA released/hr/g tissue.
[5] LPLP = lipoprotein lipase activity, µeq FFA released/hr/mg extractable protein.
[6] OHP = hydroxyproline, mg/g tissue.
[7] Protein = extractable protein, 800 x g supernatant, mg/g tissue.
[8] Milk fat test = percent fat in milk, average of three fat tests determined in the week prior to biopsy.

Appendix Table 13

Milk Production and Composition, Experiment I[1]

Cow	Ration[2] N Milk	N % Fat	RR Milk	RR % Fat	RR + MgO Milk	RR + MgO % Fat
	kg		kg		kg	
329	25.3	3.1	27.0	3.2	27.2	2.6
330	16.4	2.9	14.5	1.3	13.8	3.3
642	12.5	2.7	12.9	2.8	10.9	3.0
331	22.8	2.7	25.5	2.7	23.0	2.9
332	16.9	2.9	20.4	2.7	21.1	2.6
333	18.4	3.3	22.5	1.9	20.4	2.7
334	20.8	3.1	22.5	2.5	13.1	3.3
340	--	--	24.3	3.1	23.5	3.2
341	15.1	3.6	12.8	2.7	16.0	3.3
Mean	18.5± 1.5	3.0± 0.1	20.3± 1.8	2.5± 0.2	18.8± 1.8	3.0± 0.1

[1] Milk production values are means of the last 7 days of each period. Fat % values are the mean of three fat tests determined in the week prior to biopsy.

[2] Rations: N = normal ration, RR = restricted roughage-high grain, RR + MgO = restricted roughage-high grain + MgO.

Appendix Table 14

Feed Consumption, Experiment I[1]

Cow	N			Ration[2] RR			RR + MgO		
	Hay	Grain	Silage	Hay	Grain	Silage	Hay	Grain	Silage
					- - - kg - - -				
329	15.0	6.8	4.5	1.4	16.4	4.5	1.4	14.1	4.5
330	11.4	5.9	4.5	1.4	10.0	4.5	1.4	10.9	3.6
642	5.9	4.5	4.5	1.4	8.2	4.5	1.4	5.0	4.5
331	14.1	6.8	4.5	1.4	15.0	4.5	1.4	12.7	4.5
332	12.7	4.5	4.5	1.4	15.0	4.5	1.4	15.7	4.5
333	11.4	5.5	4.5	1.4	14.1	4.5	1.4	11.8	4.5
334	15.5	3.6	4.5	1.4	15.0	4.5	1.4	4.5	4.5
340	--	--	--	1.4	13.6	4.5	1.4	13.6	4.5
341	14.1	4.5	4.5	0.0	15.0	4.5	1.4	14.1	4.5

[1] Weights are the mean of last 7 days of each period.

[2] Rations: N = normal ration, RR = restricted roughage-high grain, RR + MgO = restricted roughage + MgO.

Appendix Table 15

Feed Consumption and Milk Production Data from Experiment II[1]

Parameter	Cow 445 N	Cow 445 RR	Cow 444 N	Cow 444 RR
Grain consumption, kg/day	7.3	14.5	7.3	12.7
Hay consumption, kg/day	7.3	0	9.5	0
Milk production, kg/day	29.6	25.1	26.4	20.6
Fat test, %	3.9	3.4	3.4	1.2

[1] Values reported are the mean values for the last 7 days of each period.

N = normal ration.
RR = restricted roughage-high grain.