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Factors contributing to development of fatty liver and ketosis in lactating dairy cows

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Iowa State University, 1989
Factors contributing to development of fatty liver and ketosis in lactating dairy cows

by

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# TABLE OF CONTENTS

**INTRODUCTION** 1

**REVIEW OF LITERATURE** 4

- Ketosis, Fatty Liver, and Fat Cow Syndrome 4
  - Lactation ketosis 4
  - Fatty liver 6
  - Fat cow syndrome 13
  - Experimental models of fatty liver and ketosis 14
- Metabolism of Glucose and Lipids in the Liver of Dairy Cows 16
  - Gluconeogenesis 16
  - Precursors and pathways 17
  - Regulation 19
  - Fatty acid metabolism 21
  - General aspects 21
  - Oxidation of fatty acids 22
  - Esterification of fatty acids 26
  - Export of triglyceride from liver 27
  - Regulation of fatty acid metabolism in ruminants 29
- Metabolism in Extrahepatic Tissues of Dairy Cows 34
  - Glucose 34
  - Lipids 35
- Summary of the Literature 38

**EXPLANATION OF DISSERTATION FORMAT** 40

**REFERENCES** 41

**SECTION I. EXPERIMENTAL FATTY LIVER AND KETOSIS ARE NOT INDUCED BY EITHER FEED RESTRICTION OR DIETARY 1,3-BUTANEDIOL AS SEPARATE TREATMENTS** 57

**ABSTRACT** 58

**INTRODUCTION** 59

**MATERIALS AND METHODS** 61

- Cows, Treatments, and Management 61
- Sampling Procedures 64
- Incubation Procedures 65
- Analytical Methods 65
- Statistical Analyses 68
V

LIST OF TABLES

SECTION ONE

TABLE 1. Composition and analysis of silage and grain mixture fed to cows 62

TABLE 2. Milk production and composition from cows either feed-restricted (FR) or fed 1,3-butanediol (BD) during the separate-treatment period, or given both during the combination-treatment period 71

TABLE 3. Average daily intakes and body weight of cows either feed-restricted (FR) or fed 1,3-butanediol (BD) during weeks 1 through 4 or given both during weeks 5 and 6 74

TABLE 4. Concentrations of metabolites and hormones in plasma of cows either feed-restricted (FR) or fed 1,3-butanediol (BD) during the separate-treatment period, or given both during the combination-treatment period 76

TABLE 5. Composition of liver and metabolism of propionate by liver slices from cows either feed-restricted (FR) or fed 1,3-butanediol (BD) during the separate-treatment period, or given both during the combination-treatment period 80

TABLE 6. Effect of nicotinic acid, carnitine, or butanediol in incubation media on conversion of propionate to glucose or carbon dioxide by liver slices in vitro 85

SECTION TWO

TABLE 1. Composition and analysis of silage and grain mixture and alfalfa hay fed to cows 104

TABLE 2. Concentrations of metabolites in blood and liver and in vitro oxidation of palmitate in cow 463, who was induced into clinical ketosis by feed restriction plus dietary 1,3-butanediol (FRBD) 134

TABLE 3. Ratio of triglyceride to glycogen in liver biopsies from ketotic and nonketotic cows in the present and earlier experiments 136
APPENDIX

TABLE A1. Analysis of variance for conversion of propionate to glucose by liver slices from cows in Section One

TABLE A2. Analysis of variance for milk production (kg/d) by cows in Section Two
LIST OF FIGURES

SECTION ONE

Figure 1. Schematic representation of dietary treatments

Figure 2. Concentrations of metabolites in plasma from cows either feed-restricted (FR) or fed butanediol (BD) during weeks 1 through 4, or given the combination during weeks 5 and 6

SECTION TWO

Figure 1. Average daily production of milk, fat content, and production of solids-corrected milk (SCM) in control cows or cows subjected to feed restriction plus dietary butanediol (FRBD) during weeks 1 through 4 (days 14 to 42 postpartum)

Figure 2. Average daily intake of dry matter (DM) as a percentage of body weight (BW), cumulative body weight change (week 0 as reference), and energy balance of control cows or cows subjected to feed restriction plus dietary butanediol (FRBD) during weeks 1 through 4 (days 14 to 42 postpartum)

Figure 3. Concentrations of metabolites in plasma from control cows or cows subjected to feed restriction plus dietary butanediol (FRBD) during weeks 1 through 4 (days 14 to 42 postpartum)

Figure 4. Concentrations of hormones in plasma from control cows or cows subjected to feed restriction plus dietary butanediol (FRBD) during weeks 1 through 4 (days 14 to 42 postpartum)

Figure 5. Concentrations of total and dextran sulfate precipitable- (DSP) cholesterol and triglyceride in serum from control cows or cows subjected to feed restriction plus dietary butanediol (FRBD) during weeks 1 through 4 (days 14 to 42 postpartum)
Figure 6. Composition of liver (percent of wet weight) from control cows or cows subjected to feed restriction plus dietary butanediol (FRBD) during weeks 1 through 4 (days 14 to 42 postpartum)

Figure 7. Conversion of palmitate to carbon dioxide or total acid-soluble metabolites (ASM) by liver slices from control cows or cows subjected to feed restriction plus dietary butanediol (FRBD) during weeks 1 through 4 (days 14 to 42 postpartum)
INTRODUCTION

High-producing dairy cows have a tremendous metabolic challenge during early lactation to provide adequate substrates for synthesis of large quantities of milk. Intake of energy-yielding nutrients from the diet usually is less than output into milk, resulting in mobilization of body tissue to supply the nutrient deficit. Most cows are able to adjust their metabolism to meet this challenge; some cows, however, are unable to make the necessary adjustments to maintain homeostasis. Metabolic diseases or disorders occur as a result.

Lactation ketosis and fatty liver are two interrelated disorders of energy metabolism. Ketosis has been studied for over a half-century, but specific biochemical causes still are unknown. Estimates of the incidence of ketosis range from 2 to 15% (Baird, 1982; Littledike et al., 1981); subclinical ketosis may occur even more frequently. Cows with clinical ketosis usually have fatty livers, but interest in fatty liver as a separate disorder has developed only in the last decade or two. British workers estimated that fatty liver occurs in up to 60% of cows during the early postpartum period (Reid, 1980); the incidence was lower, however, in the United States (Gerloff et al., 1986a,b). Ketosis results in decreased milk production, increased veterinary expenses, and possibly decreased productive lifetime (Baird, 1982; Schultz, 1988). Although detrimental effects of fatty liver on liver function remain to be demonstrated unequivocally, associations have been noted between fatty liver and increased susceptibility to disease and reproductive problems (Gerloff et al., 1986a; Reid, 1982). Decreasing the incidence of ketosis and fatty
liver should improve the health and productivity of dairy cows and increase profits for dairy farmers.

Although characteristics of ketosis and fatty liver have been documented thoroughly, finding methods of prevention seems to be dependent upon studies of the changes during development of the disorders. A major problem with such an approach is that use of field cases limits studies to cows that already have developed the disorders. Many protocols have been proposed to induce ketosis and fatty liver experimentally, but these protocols generally are unphysiological, and changes usually occur too quickly for detailed study of development.

A protocol to induce ketosis and fatty liver experimentally has been developed recently (Mills et al., 1986a,b; Veenhuizen et al., 1989a,b). The protocol involves subjecting cows in early lactation (10 to 14 d postpartum) to moderate feed restriction and a dietary source of ketone bodies (i.e., 1,3-butanediol). Fatty liver and ketosis develop gradually over a period of two to four weeks, similar to on-farm cases, which allows study of sequential changes in blood, liver, and adipose tissue during development.

The general objective of the two experiments reported in this dissertation was to further characterize and refine the feed-restriction-plus-butanediol protocol for induction of fatty liver and ketosis. Specific objectives were to:

1) Determine if either feed restriction or dietary 1,3-butanediol as separate treatments would cause development of fatty liver and/or ketosis (Section One).
2) Determine effects of either feed restriction or dietary 1,3-butanediol on in vitro hepatic gluconeogenesis and concentrations of metabolites in blood and liver (Section One).

3) Determine if a combination of feed restriction and 1,3-butanediol beginning later in lactation would induce fatty liver and/or ketosis (Section One).

4) Determine effects of the combination of feed restriction and 1,3-butanediol beginning at d 14 postpartum on energy balance, milk composition, concentrations of serum lipoproteins, and liver composition (Section Two).

5) Determine if the combination of feed restriction and 1,3-butanediol beginning at d 14 postpartum affects in vitro hepatic metabolism of palmitate (Section Two).
REVIEW OF LITERATURE

This review is intended to give an overview of ketosis and fatty liver in dairy cows and the associated metabolic processes in liver and peripheral tissues. Other recent reviews are cited wherever possible. The presentation is divided into three main sections: 1) ketosis, fatty liver, and fat cow syndrome, 2) metabolism of glucose and lipids in liver of dairy cows, and 3) metabolism in extrahepatic tissues of dairy cows.

Ketosis, Fatty Liver, and Fat Cow Syndrome

Lactation ketosis

Ketosis (i.e., acetonemia) is a metabolic disease of dairy cows in early lactation that is characterized by increased concentrations in blood of the ketone bodies, i.e., 3-hydroxybutyrate (BHBA), acetoacetate (AcAc), and acetone. Metabolic changes associated with ketosis were described as early as 1923 (Sjollema and Van Der Zande, 1923). Study of ketosis has been an active area of research by many groups for more than 30 years (Shaw, 1956), yet much still is understood poorly. Various aspects of ketosis have been reviewed extensively (Baird, 1977, 1982; Baird et al., 1974a,b; Bergman, 1971, 1973; de Boer, 1984; Fox, 1971; Hibbitt, 1979; Kronfeld, 1970, 1971, 1972; Littledike, et al., 1981; Lyle, 1983; Mills, 1982; Schultz, 1968, 1971, 1974, 1988; Shaw, 1956; Veenhuizen, 1988) and will not be reviewed in detail here.

Estimates place the incidence of clinical ketosis at between 2 and 15% of cows in the United States and western Europe, whereas incidence of subclinical ketosis may be much higher (Baird, 1982; Littledike et al.,
Cows usually are affected within the first three to four weeks postpartum, with older cows being somewhat more susceptible (Schultz, 1988). Economic losses occur through decreased milk production, treatment expenses, and possibly decreased productive lifetime (Baird, 1982).

Signs of primary ketosis (i.e., that which occurs as a primary disease, not as a secondary complication to another disease or disorder) are somewhat nonspecific. Signs may include a gaunt and dull appearance, decreased rumen activity, decreased appetite, incoordination, nervousness or deranged behavior, and decreased milk production. Ketone bodies are present in urine and milk. Presence of fever is an indication of infection and secondary ketosis.

The two major changes in blood are increased concentration of ketone bodies and decreased concentration of glucose. Other biochemical changes in blood include increased concentrations of nonesterified fatty acids (NEFA) and acetate and decreased concentrations of insulin, triglycerides, free and esterified cholesterol, and phospholipids. In liver there are increases in triglyceride and ketone bodies and decreases in glycogen, gluconeogenic amino acids, and intermediates of glycolysis and the citric acid cycle. Cows with subclinical ketosis may show no outward signs but have increased concentrations of NEFA and BHBA and decreased concentrations of glucose in blood; subclinical ketosis may either remit or progress into clinical ketosis.

The general biochemical progression toward ketosis is thought to be as follows. Production of glucose by the liver is inadequate to meet demands of increasing milk synthesis, and concentration of glucose
decreases in blood. Insulin, with the concentration already being low during early lactation, may decrease further and allow increased mobilization of NEFA and glycerol from adipose tissue. Uptake of NEFA by liver increases production of ketone bodies and deposition of triglyceride in liver. At some point, an unknown event, perhaps linked to decreased glucose or increased ketone body concentrations, triggers the onset of clinical ketosis. During early lactation, most high-producing cows will go through some degree of hypoglycemia and increased mobilization of NEFA and ketogenesis without progressing to ketosis. Indeed, Baird (1977) stated that ketosis represents one extreme of the "normal" continuum of glucose deficit and fatty acid mobilization in early lactation.

Kronfeld (1971, 1982) devised an elaborate scheme for classifying types of ketosis based on their presumed metabolic origins; the usefulness of his scheme, however, has been questioned (Schultz, 1974; Veenhuizen, 1988). In addition, Kronfeld (1982) proposed that true primary ketosis develops from an attempt by cows to supply lipogenic nutrients for milk fat synthesis when an excess of gluconeogenic nutrients from the diet stimulates increased milk synthesis. Although the theory has some merit, experimental evidence is lacking. Experimental evidence is substantial, however, for carbohydrate insufficiency being the primary causative factor (Schultz, 1988).

**Fatty liver**

Fatty liver (i.e., hepatic lipidosis) is a general term for accumulation of lipid in the hepatocytes or parenchymal cells of liver. It occurs in many species during various conditions (discussed by Mayes,
1985), including diabetes, alcoholism, deficiencies of choline or essential fatty acids, and exposure to various toxic compounds. Reviews on fatty liver in dairy cows include those of Reid (1982), Reid and Roberts (1982, 1983), and Herdt (1988).

Triglyceride is the primary type of lipid that accumulates in liver during the postpartum period in normal (Collins and Reid, 1980) or overconditioned (Fronk et al., 1980) cows. Hepatic triglyceride also accumulates during fasting in lactating (Brumby et al., 1975; Herdt et al., 1983) or nonlactating (Reid et al., 1977) cows, or during secondary disorders such as displaced abomasum (Herdt et al., 1983). Triglyceride also accumulates in liver of ewes with pregnancy toxemia (Henderson et al., 1982) and in sheep with fatty livers induced by alloxan diabetes (Henderson et al., 1982) or a combination of fasting and administration of phlorizin and epinephrine (Herdt et al., 1988). Content of cholesterol esters increased in postparturient fatty liver (Collins and Reid, 1980) and during fasting in lactating cows (Brumby et al., 1975); no increase in cholesterol ester was observed in nonlactating cows (Reid et al., 1977). Phospholipid content may be decreased (Herdt et al., 1983), unchanged (Brumby et al., 1975; Collins and Reid, 1980; Reid et al., 1977), or increased (Fronk et al., 1980).

It was noted as early as 1923 that ketotic cows had fatty livers (Sjollema and Van Der Zande, 1923), and in 1950 Saarinen and Shaw (1950) observed increased total lipid and cholesterol ester in livers of cows either fasted or with spontaneous ketosis. Cows obese at calving were found to be subject to a complex of postpartum diseases known as "fat cow
syndrome" or "fatty liver syndrome" that included development of severe fatty infiltration of the liver (Morrow, 1976). Reid (1980) reported that 66% of Friesian and 33% of Guernsey cows sampled at one week postpartum had moderate to severe fatty livers even though none were obese at calving. This finding created interest in effects of fatty liver on dairy cows during early lactation.

Degree of fatty liver has been measured in biopsy samples by using microscopic point-counting methods, chemical methods, or buoyant density (Herdt, 1988). According to Gaal et al. (1983), the three classifications of degree of fatty liver are mild (0-20% of cell volume or ≤ 5% by weight as triglyceride), moderate (20-40% of volume or 5-10% triglyceride), or severe (>40% of volume or >10% triglyceride).

Occurrence of fatty liver is related to the degree of mobilization of body tissue after calving. Roberts (1982) and Roberts et al. (1981) viewed development of fatty liver as part of a "generalized mobilization syndrome" of dairy cows in early lactation. Excessive tissue mobilization, indicated by increased concentration of NEFA in plasma and loss of body weight and condition score, also leads to deposition of fat in skeletal muscle and kidney. Other organs may be involved, including the adrenal (Saarinen and Shaw, 1950) and thyroid (Kapp and Pethes, 1981) glands. Deposition of triglyceride in tissues such as liver, which normally take up and utilize NEFA, seems to be a result of uptake of NEFA in excess of what can be oxidized or exported (Roberts et al., 1981).

Severe or clinical fatty liver is characterized by decreased responses to treatments for other diseases or disorders (Herdt, 1988).
Evidence for direct negative effects of postparturient fatty liver (i.e., "spontaneous" or "subclinical" fatty liver) on liver function is largely circumstantial (Reid and Roberts, 1983). Fatty liver was reported to decrease reproductive efficiency in some studies (Reid, 1983; Reid et al., 1979b; Reid et al., 1983a) but not in others (Gerloff et al., 1986a). Cows with fatty liver had decreased white cell counts in blood (Reid et al., 1984) and a greater incidence of infectious diseases (Gerloff et al., 1986a). Cows with fatty liver also retained bacteria in the mammary gland for longer periods after an experimental infection (Hill et al., 1985).

Clearance of dyes such as sulfobromophthalein (i.e., BSP) is generally not affected by fatty liver (Herdt et al., 1982). A propionate loading test (Bruss et al., 1986; Grohn, 1985) has been investigated as a test of liver function in ruminants. The half-life of propionate injected into blood increased in fasted or spontaneously ketotic cows, and increases in concentration of glucose were greater and occurred earlier in normal cows compared with fasted or ketotic cows. The usefulness of propionate loading to detect fatty liver, however, has been questioned (Herdt, 1988).

Study of the ultrastructure of hepatocytes from cows with severe fatty liver revealed increased cell volume, decreased volume of rough endoplasmic reticulum per cell, and evidence of mitochondrial damage (Reid and Collins, 1980). The latter two changes were reflected in vivo by decreased concentration of albumin and increases in activities of mitochondrial enzymes in blood. Qualitatively similar changes also were observed in liver from fasted cows (Reid, 1973) and from spontaneously
ketotic cows (Grohn and Lindberg, 1985). Grohn and Lindberg (1985) observed a decrease in the amount of Golgi apparatus in fatty liver from ketotic cows and an increase in peroxisomes in mildly ketotic cows, but a decrease in peroxisomes in severely ketotic cows.

In ruminants, the liver is the major source of endogenous plasma triglyceride, with intestinal synthesis being of little consequence (Pullen et al., 1988). Secretion of very-low-density lipoproteins (VLDL) by ruminant liver, however, is low when compared with that of nonruminants (Kleppe et al., 1988; Yamdagni and Schultz, 1969). It is not known whether further decreases in hepatic secretion of triglyceride occur during energy deficit in early lactation. Pullen et al. (1988) found that the turnover rate of the stored triglyceride pool in sheep liver was decreased when the size of that pool increased, suggesting that fatty liver may further decrease the ability to secrete lipoprotein. Decreased ability of hepatocytes to synthesize apoproteins necessary for secretion of triglyceride-rich lipoproteins was theorized to contribute to development of fatty liver (Herdt et al., 1983; Reid and Collins, 1980).

Fasted cows fitted with multiple catheters to study trans-liver nutrient balances changed from having a net output to having a net uptake of triglyceride from the liver as fatty liver developed (Reid et al., 1979a). In contrast, no veno-arterial difference for secretion or uptake of triglyceride or lipoprotein fractions by the liver could be detected in sheep with experimentally induced fatty livers (Herdt et al., 1988). Radioactive triglyceride was secreted, however, by the liver of sheep in the study of Herdt et al. (1988) when radioactive fatty acid was
injected into a mesenteric vein. Measurement of veno-arterial concentration differences may not be sensitive enough for detection of hepatic triglyceride secretion (Herdt et al., 1988).

Some researchers have observed changes in concentrations of lipoproteins in blood from cows with fatty liver. Concentrations of dextran sulfate precipitable (DSP) lipoproteins decreased in cows that developed severe fatty livers in association with occurrence of displaced abomasum, but they increased in cows that had fatty livers induced by fasting (Herdt et al., 1983). In a field study, cows with naturally-occurring fatty liver also had decreased DSP lipids (Gerloff et al., 1986b). Rayssiguier et al. (1988) observed decreased concentrations of low-density lipoproteins (LDL) in blood of cows with postparturient fatty liver.

Numerous investigators have attempted to identify metabolites or enzymes in blood that would predict accurately the presence and degree of fatty liver in dairy cows. These attempts have, for the most part, been unsuccessful, and studies from different groups often provide conflicting results. In general, changes in blood associated with fatty liver include increased concentrations of NEFA, BHBA, and bilirubin, and decreases in glucose, total cholesterol, albumin, magnesium, and insulin (Reid and Roberts, 1983). These changes are very similar to those that occur during ketosis. Gerloff et al. (1986c) noted a negative correlation between concentrations of the thyroid hormones and degree of fatty liver. Other changes noted in some studies include increased activities of the enzymes aspartate aminotransferase (i.e., glutamate-oxaloacetate aminotransferase)
(Bogin et al., 1988; Grohn et al., 1983; Herdt et al., 1982; Lotthammer, 1982; Reid et al., 1983b), ornithine carbamoyltransferase (Grohn et al., 1983), sorbitol dehydrogenase (Grohn et al., 1983), acid and alkaline phosphatases (Bogin et al., 1988), and glutamate dehydrogenase (Bogin et al., 1988).

No single enzyme or combination of enzymes has provided a reliable diagnostic technique. The best published prediction equation, which related liver lipid to a combination of NEFA, glucose, and aspartate aminotransferase in blood, accounted for only 53% of the variation in liver lipid (Reid et al., 1983b). By using this technique, only 60% of cows were classified correctly into either mild or moderate fatty liver groups (Reid et al., 1983b). Bogin et al. (1988) reported recently that activities of enzymes associated with production of NADPH were increased in liver and blood of cows with fatty liver. The diagnostic power of changes in activities of these enzymes in blood awaits verification. Why there would be a need for increased NADPH is unclear, because fatty acid synthesis is minimal in bovine liver (Ballard et al., 1969) and it does not increase during development of fatty liver (Ballard et al., 1968; Veenhuizen, 1988). At present, liver biopsy remains a necessary procedure for accurately determining content of lipid in liver (Herdt, 1988). It is obvious that improved diagnostic techniques for fatty liver are needed.

Reid and Roberts (1982) and Gerloff et al. (1986a,b,c) proposed that fatty liver may be an indicator of negative energy balance during early lactation rather than a separate disease. Furthermore, negative energy balance may be the causative factor in increased susceptibility to other
diseases and reproductive problems. Although the general cause of fatty liver seems to be excessive uptake of NEFA by the liver, cellular and molecular mechanisms of development remain to be determined.

Fat cow syndrome

Morrow (1976) and Morrow et al. (1979) described the fat cow syndrome as a complex of metabolic and infectious disorders occurring during the early postpartum period in cows that calve in an obese condition. Signs of the syndrome include anorexia, depression, decreased milk production, muscular weakness, and associated diseases, which may include milk fever, ketosis, displaced abomasum, retained fetal membranes, metritis, or mastitis. Response to treatment for these diseases usually is poor; rates of mortality up to 25% have been observed in affected herds (Morrow et al., 1979).

Cows with fat cow syndrome have fatty livers. Obesity at the onset of lactation may lead to rapid mobilization of NEFA, which is aggravated by the decreased appetite usually observed in such cows (Bines and Morant, 1983; Treacher et al., 1986). Biochemical changes in blood include decreased concentration of triglycerides and increased concentrations of NEFA, ketone bodies, urea, and bilirubin (Morrow, 1976). Concentration of glucose may be either decreased or increased. Activities in serum of liver-specific enzymes such as ornithine carbamoyltransferase and sorbitol dehydrogenase may increase, indicating some degree of liver damage (Morrow, 1976).

Detailed metabolic studies of fat cows are lacking. Concentration of insulin was greater prepartum in fat cows (Fronk et al., 1980; Reid et
al., 1986), and evidence for insulin resistance was noted (Reid et al., 1986). Reid et al. (1986) observed greater oxidation of acetate to CO₂ by adipocytes isolated from thin cows than from fat cows, whereas there were no differences between groups in rates of lipogenesis or lipolysis in adipocytes. Bines and Morant (1983) hypothesized that increased rates of acetate utilization by adipose tissue in thin cows would decrease the chemostatic feedback-inhibition of acetate on feed intake, allowing greater intakes. The reverse would be true in fat cows.

Heavier cows with greater body condition scores had neither significantly greater depths of subcutaneous adipose tissue nor larger adipocytes, but they displayed increased muscle mass compared with thin cows (Reid et al., 1986). Increased mobilization of body tissue by fat cows after parturition was observed to be a combination of adipose tissue and muscle mass (Reid et al., 1986). Reid et al. (1986) speculated that mobilized body protein was not adequate for synthesis of lipoproteins, contributing to development of fatty liver in the fat cows. Researchers should determine the role of quantity and quality of dietary protein in preventing or alleviating fatty liver and fat cow syndrome in early-lactating dairy cows. Comparison of development of fatty liver in fat cows and in normal cows may be useful for determining metabolic and nutritional mechanisms of the disorder.

Experimental models of fatty liver and ketosis

Much of the work discussed already in which fatty liver and ketosis were characterized was done on naturally occurring or "spontaneous" cases from the field. Although use of field cases is ideal for describing
changed metabolites or enzymes that exist in the established disorders, it is nearly impossible to characterize sequential changes during development. Numerous attempts have been made to establish valid physiological models or methods to induce fatty liver and ketosis.

Methods for experimentally inducing ketosis were reviewed by Veenhuizen (1988). These methods included fasting (Baird et al., 1972), decreased intake (Fisher et al., 1971; Kellogg and Miller, 1977), injection of thyroxine coupled with feeding high-protein diets (Hibbitt, 1966; Hibbitt and Baird, 1967), and injected thyroxine with decreased intake (Kellogg et al., 1971). Responses differed between spontaneous and fasting-induced ketosis (Ballard et al., 1968), and changes occur very quickly during fasting, which makes repeated sampling of liver difficult. Use of thyroxine artificially increases metabolic rate to much greater rates than in spontaneous ketosis (Hibbitt and Baird, 1967), which may affect measures of metabolites or enzyme activities. Changes occurring with thyroxine also occur quickly over a period of about four days, again making repeated sampling difficult.

Use of a combination of feed restriction plus dietary 1,3-butanediol as an exogenous source of ketone bodies seems to be the most promising model for study of development of lactation ketosis (Mills et al., 1986a,b; Veenhuizen, 1988). Characteristics of this induced ketosis are similar to spontaneous ketosis. Ketosis develops over a period of about 4 weeks, which allows sequential biopsy of liver or other tissues in order to study progressive metabolic changes.

The most common method previously used to study fatty liver has been
starvation (Brumby et al., 1975; Herdt et al., 1983; Reid, 1973; Reid et al., 1979a). Responses of lipids in blood and liver may be different, however, between starvation-induced and natural, postparturient fatty liver (Herdt et al., 1983). Another method used recently was to give starved sheep intramuscular injections of phlorizin and intravenous infusions of epinephrine (Herdt et al., 1988). Fatty liver developed within 48 hours after initiation of the treatment. This method seems promising as a relatively low-cost procedure for study of changes induced by development of fatty liver, but it suffers from restrictions on amounts of tissue that can be obtained for study of development.

The fatty liver induced to date by feed restriction plus dietary butanediol (Mills et al., 1986a; Veenhuizen, 1988) has not reached the severity seen with natural cases (Herdt, 1988). The technique is attractive, however, because of the ability to study progressive changes in metabolism as fatty liver develops. Further, degree of feed restriction and the amount of butanediol fed can be changed to any desired combination to study interactions of energy balance and exogenous sources of ketone bodies.

Metabolism of Glucose and Lipids in the Liver of Dairy Cows

**Gluconeogenesis**

Because of ruminal fermentation of dietary carbohydrate, little glucose is available for absorption from the small intestine of ruminants. Ruminants are dependent, therefore, on high rates of gluconeogenesis to supply their needs for glucose. Use of large quantities of glucose by the
mammary gland of high-producing cows creates a tremendous demand for
 gluconeogenesis, most of which occurs in the liver. Glucose utilization
 and gluconeogenesis have been reviewed (Amaral, 1988; Bergman, 1973; Leng,
 1970; Lindsay, 1978, 1979; Lyle, 1983; Mills, 1982; Veenhuizen, 1988;
 Young, 1977) and will be discussed only briefly.

Precursors and pathways  Precursors for gluconeogenesis include
 propionate, lactate, glycerol, and amino acids. Reynolds et al.
 (1988a,b), using multiply catheterized cows in early lactation, recently
 measured maximal contributions of propionate, lactate, and amino acids to
 be 55, 18, and 16% of net hepatic glucose production. Pyruvate and
 glycerol supplied less than 2% of glucose production in another study
 (Lomax and Baird, 1983). Propionate is the predominant gluconeogenic
 precursor in fed ruminants, and gluconeogenesis increases with increased
 propionate supply (Bergman, 1973; Veenhuizen, 1988).

About 50% of the lactate flux was derived from glucose in lactating
cows, with the remainder presumably being absorbed from the
 gastrointestinal tract (Baird et al., 1983). Recycling of glucose through
 lactate accounted for only 2% of glucose flux in lactating cows (Baird et
 al., 1983). Reynolds et al. (1988a) determined that the net amount of
 lactate taken up by the liver of early-lactating cows could provide a
 maximum of 18% of hepatic glucose production. The discrepancy between
 estimates of lactate contribution to gluconeogenesis obtained by isotopic
 versus trans-liver balance techniques suggests that most lactate taken up
 by the liver of lactating cows is oxidized or used for synthesis of
 compounds other than glucose (Baird et al., 1983).
Although glycerol is unimportant as a precursor of glucose in the fed state, it supplied up to 40% of glucose turnover in hypoglycemic pregnant ewes and 23% in fasted sheep (Bergman et al., 1968). Glycerol may become quantitatively more important for gluconeogenesis in cows in severe negative energy balance during early lactation (Peel and Bauman, 1987). The contribution of amino acids to gluconeogenesis in ruminants has been debated, with estimates ranging from 11 to 30%. Lindsay (1978) concluded that amino acids may provide about 20% of glucose in ruminants. From data reported by Reynolds et al. (1988b), however, it seems that only 10 to 15% of glucose could be derived from amino acids in cows during early lactation.

The pathways of glucose synthesis in ruminants are similar to those in nonruminants. The subcellular distribution of enzymes may vary, however. Initial metabolism of propionate occurs in the mitochondria. Propionate is activated to propionyl-CoA, carboxylated to methylmalonyl-CoA, and converted to succinyl-CoA and then oxaloacetate (OAA). Phosphoenolpyruvate carboxykinase (PEPCK), the major enzyme controlling conversion of propionate to glucose, is found in both mitochondrial and cytosolic compartments in ruminants (Ballard et al., 1969). Thus, OAA can be converted to phosphoenolpyruvate (PEP) in the mitochondria, with PEP exiting the mitochondria for conversion to glucose, or alternatively, OAA can leave the mitochondria as malate, be reconverted to OAA in the cytosol, and then be converted to glucose. Lactate, pyruvate, and amino acids that are converted to pyruvate require pyruvate carboxylase (PC) to be converted to OAA. Ruminants have PC located in both cytosolic and
mitochondrial compartments, an adaptation that evidently increases metabolic "flexibility" in ensuring formation of glucose under a variety of dietary situations (Ballard et al., 1969). Propionate, lactate, pyruvate, and amino acids all require participation of the citric acid cycle or at least are metabolized through common intermediates. Glycerol, on the other hand, enters the gluconeogenic pathway at the triose phosphate stage, and thus its metabolism is independent of the citric acid cycle.

**Regulation**

Regulation of gluconeogenesis is less well understood for ruminants than for nonruminants; this is especially true for high-producing dairy cows. Substrate availability is a primary regulator of gluconeogenesis in ruminants; gluconeogenesis increases after eating and decreases with fasting in ruminants, which are opposite the responses observed in nonruminants (Young, 1977).

Maximum fluxes through metabolic pathways can be estimated by measuring capacities of isolated cells or tissue preparations to metabolize substrates or by measuring activities of rate-limiting enzymes. Gluconeogenic capacity of liver slices was greatest in early lactation (Aiello et al., 1984), but was reported to be less in homogenates from cows in early lactation (Mathias and Elliot, 1967). Gluconeogenic capacity of liver slices was decreased dramatically by induced ketosis (Mills et al., 1986b; Veenhuizen, 1988). Effects of fatty liver or obesity on gluconeogenic capacity of bovine liver are unknown. Gluconeogenic capacity was decreased slightly in the early stages of induced fatty liver, but interpretation is complicated by concurrent
Mechanisms affecting enzyme activities in the gluconeogenic pathway are defined poorly in ruminants. Activities of PEPCK (Baird and Heitzman, 1970) and PC (Ballard et al., 1968) increased with onset of lactation. Starvation in lactating cows caused decreased activities of PEPCK, pyruvate kinase, citrate synthase (Baird et al., 1972), and malate dehydrogenase (Ballard et al., 1968). Cows with spontaneous ketosis were found to have increased activities of PC (Baird et al., 1968) and decreased activities of pyruvate kinase (Baird and Heitzman, 1971) and malate dehydrogenase (Ballard et al., 1968). A problem in interpreting many of the existing results for changes in enzyme activities is that the same animals were not used in different physiological states, and consequently variation between animals may prevent detection of true differences in enzyme activities.

Several studies have used in vitro techniques to examine regulation of gluconeogenesis by metabolites. In isolated caprine hepatocytes, butyrate decreased gluconeogenesis from propionate, whereas isobutyrate, 2-methylbutyrate, and valerate did not affect gluconeogenesis but decreased oxidation of propionate (Aiello and Armentano, 1987b). Acetoacetate decreased and BHBA increased production of glucose from propionate (Aiello and Armentano, 1987a). Chemically reducing the cytosol by adding ethanol, ammonium ions, or lactate decreased and calcium ions increased gluconeogenesis (Aiello and Armentano, 1987a). Long-chain fatty acids increased gluconeogenesis from propionate in caprine (Aiello and Armentano, 1988) and in ovine (Faulkner and Pollock, 1986) hepatocytes.
Hormonal controls of gluconeogenesis in ruminants have been summarized (Amaral, 1988; Bassett, 1975, 1978; Brockman, 1986; McDowell, 1983; Trenkle, 1981) but are understood less than for nonruminants. In general, glucagon stimulates glycogenolysis and promotes gluconeogenesis, whereas insulin largely exerts its effects on peripheral tissues to increase glucose utilization. Glucocorticoids increase gluconeogenesis by increasing delivery of amino acids to liver from skeletal muscle and increasing their incorporation into glucose. Although growth hormone increases availability of glucose for milk synthesis (Peel and Bauman, 1987), direct effects on gluconeogenesis have not been demonstrated. Catecholamines promote glycogenolysis and gluconeogenesis, and thyroxine evidently stimulates gluconeogenesis in dairy cows. The roles of other hormones and growth factors that affect glucose metabolism in nonruminants such as vasopressin and epidermal growth factor, and controls by the autonomic nervous system virtually are unknown in dairy cows.

**Fatty acid metabolism**

Lipid metabolism in the liver of ruminants has been reviewed (Bell, 1980). The present review will give an overview of metabolism of NEFA, with emphasis placed on information reported since the earlier review.

**General aspects** The liver of ruminants is a major organ of metabolism of NEFA, which is similar to the situation in nonruminants. In contrast to many nonruminant species, however, the liver is quantitatively unimportant in fatty acid synthesis (Ballard et al., 1969). Most synthesis of fatty acids in ruminants occurs in adipose tissue (Vernon, 1980). Some synthesis of fatty acids from acetate occurs in ruminant
liver, but utilization of glucose is negligible (Hanson and Ballard, 1967). Enzymes in the pathway for translocating glucose-derived acetyl units from mitochondria to cytosol ("citrate cleavage pathway") have very low activities in ruminant compared to nonruminant liver (Ballard et al., 1969). In addition, constant demand for gluconeogenesis in ruminants places use of cytosolic OAA for lipogenesis at a disadvantage; this view is supported by relative activities of enzymes in the gluconeogenic and lipogenic pathways (Ballard et al., 1969).

Fatty acids are taken up by liver in proportion to their concentration in blood reaching the liver (Bell, 1980). Rate of uptake can be modified further by changes in the NEFA to albumin ratio, with higher ratios favoring increased uptake (Bell, 1980). The fractional extraction of NEFA from blood by liver is about 10% in sheep (Katz and Bergman, 1969; Thompson et al., 1975) and early-lactating dairy cows (Reynolds et al., 1988b). Major NEFA in ruminants are palmitic, stearic, and oleic acids (Bell, 1980), with stearic acid being utilized more poorly by the liver than either palmitic or oleic acids (Thompson et al., 1975).

Fatty acids are toxic within cells (Spector and Fletcher, 1978), and so are activated quickly to acyl-CoA esters and either oxidized or esterified (Bell, 1980). Information on long-chain fatty acyl-CoA synthetase in ruminants is lacking. In nonruminants, the enzyme is located on the outer mitochondrial membrane and endoplasmic reticulum, and it does not seem to be rate-limiting for metabolism of NEFA (Bell, 1980).

Oxidation of fatty acids About 10% of the NEFA taken up by ruminant liver are oxidized to CO₂ (Jesse et al., 1986a,b; Lomax et al.,
1983). The proportion was unaffected by starvation in isolated hepatocytes from sheep (Lomax et al., 1983) but was decreased in liver slices from fasted cows (Jesse et al., 1986b). Mitochondria from ruminants seem to oxidize NEFA at slower rates than mitochondria from nonruminants (Koundakjian and Snoswell, 1970). Mitochondrial oxidation of NEFA in ruminant liver is completely dependent upon presence of carnitine, even under conditions where mitochondrial oxidation was carnitine-independent in rat liver (Koundakjian and Snoswell, 1970).

A major fate of NEFA within the liver may be conversion to the ketone bodies, AcAc and BHBA. Ketogenesis in ruminants has been reviewed recently (Heitman et al., 1987). In the fed state, ketogenesis from absorbed butyrate by portal-drained visceral tissues accounted for about 60% of total ketogenesis in ewes pregnant with twins (Katz and Bergman, 1969) and 40% in early-lactating cows (Reynolds et al., 1988b). Starvation eliminated the contribution from gut tissues in sheep (Katz and Bergman, 1969) and nonlactating cows (Baird et al., 1979), but ketogenesis from NEFA by liver increased so that total ketone body production was maintained or increased.

Hepatic ketogenesis in ruminants occurs in mitochondria via the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) pathway, similar to nonruminants (Bell, 1980). Pathways for ketogenesis were reviewed by Mills (1982). The limiting step for oxidation and ketogenesis from NEFA is catalyzed by carnitine palmitoyltransferase I (CPT I) (Butler et al., 1988; Jesse et al., 1986a), which regulates entry of NEFA into mitochondria. Ketogenesis thus depends to a great extent on delivery of NEFA to the liver, which
increases during starvation and ketosis. Alternate viewpoints describe increased ketogenesis within mitochondria as either an inhibition of activity of the citric acid cycle or as an overflow process in which citric acid cycle activity is unchanged (Bell, 1980).

Recent observations (Zammit, 1984) suggest that citric acid cycle activity is regulated by NAD/NADH ratios so that total energy production is maintained. The regulation of ketogenesis is accomplished both by entry of NEFA into mitochondria and by intramitochondrial factors. Factors affecting disposal of acety-CoA units formed within mitochondria were reviewed by Zammit (1984). These factors include regulation of the activity of citrate synthase, activity of acetoacetyl-CoA acetyltransferase, and concentration of intramitochondrial OAA. Observations that production of CO₂ in ruminant liver remains relatively constant under conditions of greatly enhanced ketogenesis (Lomax et al., 1983) are consistent with this type of control mechanism operating in ruminants as well.

Although most circulating acetate originates from fermentation in the reticulo-rumen and the lower gut, substantial production of endogenous acetate occurs in the liver of cows (Baird et al., 1975; Lomax and Baird, 1983; Reynolds et al., 1988b; Snoswell et al., 1978) and sheep (Bergman and Wolff, 1971; Costa et al., 1976; Pethick et al., 1981). The liver may also utilize substantial quantities of acetate (Pethick et al. 1981). It has been suggested that hepatic production of acetate is an alternative to ketogenesis for disposal of excessive accumulations of acetyl-CoA (Costa et al., 1976). Snoswell (cited by Bell, 1980) hypothesized that the
balance between the two processes may be controlled by the concentration of carnitine, with adequate carnitine favoring acetate production and deficiencies favoring ketogenesis. Regulation and metabolic significance of hepatic acetate production still are understood poorly, and further research is needed on interrelationships between ketogenesis and acetate production in ruminants.

Another factor related to oxidation of NEFA that has not been studied in ruminants is the possible involvement of peroxisomes. Peroxisomes are cellular organelles that possess capacity for beta-oxidation of fatty acids. Reducing equivalents from the first reduction step in the oxidative pathway are transferred directly to molecular oxygen, thus producing hydrogen peroxide that is degraded by catalase (Mannaerts and Debeer, 1982; Masters and Crane, 1984). Consequently, production of ATP from peroxisomal oxidation is much less than from mitochondrial oxidation (Mannaerts and Debeer, 1982). Peroxisomes can be induced to proliferate within cells of nonruminants by starvation, administration of certain drugs, or feeding fish oils rich in very-long-chain fatty acids (Bergseth et al., 1986; Veerkamp and van Moerkerk, 1986).

Studies with inhibitors of the respiratory chain have provided estimates that the contribution of peroxisomes to total cellular fatty acid oxidation is around 5% (Mannaerts and Debeer, 1982). In contrast, Kondrup and Lazarow (1985), using an elegant isotopic technique without metabolic inhibitors, estimated that as much as 30% of palmitate oxidation by rat hepatocytes was initiated in peroxisomes. The possibility of a significant contribution of peroxisomal oxidation in liver of dairy cows
during times of increased uptake of NEFA by liver should be investigated. 

**Esterification of fatty acids**  Little is known about the processes of esterification of NEFA to form triglycerides and phospholipids in ruminant liver. Enzymes for esterification are located on the endoplasmic reticulum and the outer mitochondrial membrane as in other species (Daae, 1973). Alternate pathways in addition to esterification of glycerol-3-phosphate have been proposed (Benson and Emery, 1971).

Much more is known about the processes of esterification in nonruminants, and control of triglyceride synthesis has been reviewed (Hems, 1975; Mayes, 1976). Evidence, often conflicting, exists for regulation by various factors at several steps in the synthetic pathway, including glycerol-phosphate acyltransferase (Bates and Saggerson, 1979; Pollard and Brindley, 1984; Zammit, 1983), phosphatidate phosphohydrolase (Cascales et al., 1984; Pittner et al., 1986; Pollard and Brindley, 1984; Sturton et al., 1981), and diacylglycerol acyltransferase (Haagsman et al., 1982). For ruminants, Herdt et al. (1988) reported large increases in activity of phosphatidate phosphohydrolase early during development of induced fatty liver in sheep, whereas activities of glycerol-phosphate acyltransferase and diacylglycerol acyltransferase increased more slowly and to a lesser degree. Vernon et al. (1987) found no difference between nonlactating and lactating sheep in hepatic activity of glycerol phosphate acyltransferase. In a study with lactating cows, capacity of liver homogenates to esterify palmitate was correlated with the degree of milk fat depression caused by a high-grain, restricted-roughage diet (Benson et al., 1972).
Export of triglyceride from liver  In nonruminant animals, NEFA are esterified in the liver to form triglycerides, which then are packaged into VLDL and secreted (Havel, 1985, 1987; Mayes, 1985). The capacity of ruminant liver to esterify fatty acids, however, evidently is greater than is its capacity to export triglyceride as VLDL. Hepatocytes isolated from lactating goats esterified NEFA at rates similar to those of rats, but hepatocytes from rats secreted about 25 times more VLDL than did hepatocytes from goats (Kleppe et al., 1988). Studies using sheep (Bergman et al., 1971), lactating cows (Pullen et al., 1989), or lactating goats (Yamdagni and Schultz, 1969) have shown little incorporation of $^{14}C$-NEFA into triglycerides in plasma when $^{14}C$-NEFA were infused intravenously; in contrast, specific radioactivity of liver triglyceride was increased greatly (Yamdagni and Schultz, 1969). In a study using appropriately catheterized nonlactating cows, however, Reid et al. (1979a) measured significant net production of triglyceride (375 g/d) across the liver in vivo.

Reasons for the apparently low capacity of ruminant liver to synthesize and/or secrete VLDL are not known. At least three hypotheses have been proposed. First, sheep hepatocytes have a continuous basal lamina that may physically limit secretion of large particles such as VLDL (Grubb and Jones, 1971; Mamo et al., 1983). Second, insufficient synthesis of apoproteins may limit rate of formation of VLDL (Herdt et al., 1983; Reid and Collins, 1980). Third, low rates of synthesis of phospholipid or cholesterol may limit formation of VLDL (Brumby et al., 1975; Fronk et al., 1980; Herdt et al., 1988). The first hypothesis was
disproven recently by Herdt et al. (1988), who directly measured secretion of labelled triglyceride from the liver after injection of a tracer fatty acid into a mesenteric vein. Evidence supporting the other two hypotheses is circumstantial.

The rate of synthesis of triglyceride in liver determines the rate of VLDL secretion in nonruminants (Beynen et al., 1981), and the supply of other components of VLDL is thought to be regulated by the rate of triglyceride synthesis (Mayes, 1985). It seems that regulation of VLDL secretion in ruminant liver may be different than in nonruminants. A recent report demonstrated a requirement for active synthesis of phosphatidylcholine for secretion of VLDL from rat hepatocytes (Yao and Vance, 1988). This is interesting in light of the low rate of hepatic synthesis of phosphatidylcholine in ruminant liver (Snoswell and Xue, 1987).

Triglyceride synthesized in excess of the amount that can be exported as VLDL accumulates as lipid droplets and can result in fatty liver. Pullen et al. (1988) found that hepatic triglyceride in sheep existed in two pools: 1) a microsomal pool presumably associated with secretion of lipoprotein and 2) a lipid droplet pool that is relatively inert. Turnover of the inert droplet pool was slower than the microsomal pool, and 80 to 90% of the hepatic triglyceride was in the inert droplet pool. Pathways for removal of the accumulated lipid droplets are unknown in ruminants, but in nonruminants very little transfer of droplet triglyceride to VLDL occurs. Rather, stored triglyceride in lipid droplets must first be hydrolyzed by lysosomal acid lipase (Debeer et al.,
1982). The fatty acids that are liberated then can be oxidized or reesterified and secreted as VLDL.

Characteristics and metabolism of lipoproteins in ruminants have been reviewed (Christie, 1978; Emery, 1979; Grummer and Carroll, 1988; Palmquist, 1976; Puppione, 1978, 1983). Bovine lipoproteins are isolated predominantly in the high-density lipoprotein (HDL) range (90%), with lesser amounts of LDL (10%) and VLDL or chyomicrons (<1%). Apoprotein composition of bovine lipoproteins differs from that of nonruminants (Grummer et al., 1987).

Metabolically, the VLDL and chyomicron fractions are the most active in providing triglyceride-fatty acids to peripheral tissues, most of which will be taken up by the mammary gland in lactating cows (Palmquist and Mattos, 1978). Half-lives reported for VLDL triglyceride in lactating cows range from 4.5 minutes (Glascock and Welch, 1974) to less than 2 minutes (Palmquist and Mattos, 1978). Turnover of LDL is much slower (half-life of 59 to 370 min), which helps explain the low concentration of triglyceride and high concentrations of cholesterol and phospholipid in ruminants (Palmquist and Mattos, 1978). Estimates of turnover rates for HDL in lactating cows are unavailable.

Regulation of fatty acid metabolism in ruminants

The primary point of control of fatty acid metabolism is the branch between synthesis of glycerides and the synthesis of acylcarnitine derivatives for transport of NEFA into mitochondria (Zammit, 1983). As alluded to already, factors controlling activity of the initial enzyme in the pathway of esterification (glycerol-phosphate acyltransferase) are virtually unknown
in ruminants. In ruminants as well as in nonruminants, CPT I seems to be the rate-limiting enzyme for entry into mitochondria, and some factors modifying its activity in ruminants have been identified (Aiello et al., 1984; Jesse et al., 1986a).

In rats, the transition from fed to starved states results in decreased esterification and increased oxidation of NEFA, with no change in total utilization of NEFA (McGarry and Foster, 1980). In hepatocytes isolated from starved sheep, however, total utilization of NEFA was increased, primarily as a result of increased ketogenesis (Lomax et al., 1983). These observations suggest that different control mechanisms for disposal of NEFA exist between ruminants and nonruminants.

In nonruminants, conditions such as fasting that promote ketogenic states result in increases in the absolute amount of CPT I activity (Zammit, 1984). Total activities of CPT I in sheep, however, did not change in response to fasting despite greatly increased rates of ketogenesis (Butler et al., 1988). It was speculated that effectors that modulate activity of CPT I were more important in ruminants for controlling fatty acid oxidation than were changes in amount of enzyme (Butler et al., 1988). In lactating cows, however, activities of CPT I decreased with stage of lactation in parallel with the ketogenic capacity of liver slices from the same cows (Aiello et al., 1984).

Concentration of malonyl-CoA, the first intermediate of fatty acid synthesis, is the major effector of CPT I activity in nonruminants (McGarry and Foster, 1980). Concentration of malonyl-CoA changes in parallel with fatty acid synthesis and thus can regulate the balance
between fatty acid synthesis and oxidation. In addition, sensitivity of CPT I to inhibition by malonyl-CoA is enhanced by high malonyl-CoA concentrations (Zammit, 1984), which serves to amplify the inhibitory effect.

Because of the low rate of fatty acid synthesis in ruminant liver, the potential role of malonyl-CoA in controlling fatty acid oxidation has been ignored. Recently, however, it was shown that concentrations of malonyl-CoA in sheep liver were similar to those in rat liver, and concentrations were correlated with changes in the low rates of fatty acid synthesis (Brindle et al., 1985). Concentrations of malonyl-CoA in sheep can be similar to those in nonruminants because the ratio of activity of acetyl-CoA carboxylase to that of fatty acid synthetase is similar between species, even though absolute activities are much lower in sheep. Bovine CPT I was reported to be exceptionally sensitive to the inhibitory effects of malonyl-CoA (Jesse et al., 1986a). The role of malonyl-CoA in regulation of oxidation of NEFA should be explored further.

In addition to synthesis of malonyl-CoA, the same enzyme (acetyl-CoA carboxylase) also catalyzes the cytosolic synthesis of methylmalonyl-CoA from propionate (Brindle et al., 1985). Methylmalonyl-CoA was shown to be a potent inhibitor of CPT I from sheep but not from rats or guinea pigs (Brindle et al., 1985). This mechanism may provide another link between carbohydrate availability and ketogenesis in ruminants. That the link between fatty acid oxidation and carbohydrate status observed in vitro may be important in vivo is suggested by decreased oxidation of NEFA by liver slices from cows fed high-grain, restricted-roughage diets (Jesse et al.,
In contrast to nonruminants, glucagon had no effect on fatty acid metabolism in isolated hepatocytes from sheep (Lomax et al., 1983). Insulin slightly decreased oxidation of palmitate in bovine liver slices (Jesse et al., 1986b), similar to effects in nonruminants. Reports in the literature are few concerning effects of other hormones on fatty acid metabolism in ruminants.

Carnitine greatly increased rates of fatty acid oxidation in ovine liver cells (Lomax et al., 1983) and bovine liver slices (Jesse et al., 1986a). Carnitine content of sheep liver increases about 5-fold during fasting (Snoswell and Henderson, 1970) and over 40-fold during pregnancy toxemia (Snoswell and Henderson, 1980), but it decreases with onset of lactation in cows (Snoswell et al., 1978). Infusions of carnitine into ketotic cows decreased concentration of NEFA in plasma but had variable effects on concentrations of glucose and ketone bodies (Erfle et al., 1971). These observations suggest a possible physiological role of carnitine in regulating ketogenesis in ruminants.

In addition to formation of long-chain acylcarnitine derivatives for transport into mitochondria, carnitine also forms short-chain acyl derivatives, such as acetylcarnitine, and branched-chain alpha-keto acid derivatives. These reactions are catalyzed by carnitine acetyltransferase, which is found in high activity in liver of ruminants (Snoswell and Henderson, 1970). The function of this process is not entirely clear, but it may serve as a means to relieve "acetyl pressure" in mitochondria during times of increased beta-oxidation (Bieber, 1988).
Snoswell et al. (1978), however, questioned the role of carnitine as an "acetyl buffer" in lactating cows. Other functions of carnitine in mammals have been proposed, including involvement in peroxisomal beta-oxidation of fatty acids, and these have been reviewed recently (Bieber, 1988; Feller and Rudman, 1988).

Propionate is a potent inhibitor of long-chain fatty acid oxidation in ovine liver cells (Lomax et al., 1983) and in bovine liver slices (Bush and Milligan, 1971; Jesse et al., 1986a). As discussed already, propionate may decrease oxidation of NEFA via cytosolic carboxylation to methylmalonyl-CoA (Brindle et al., 1985). Bush and Milligan (1971) concluded that propionate decreased ketogenesis by inhibiting formation of acetoacetate from acetoacetyl-CoA. This conclusion was discounted in later studies, which suggested an effect on reactions within the beta-oxidation pathway (Lomax et al., 1983). Propionate decreased oxidation of octanoate, which does not require CPT I for oxidation within mitochondria. Propionate, lactate, glycerol, and fructose increased esterification and decreased oxidation of NEFA in ovine hepatocytes (Lomax et al., 1983), and acetate and glucose decreased oxidation of palmitate in bovine liver slices (Jesse et al., 1986b).

An additional mechanism that may influence metabolism of many compounds would be the "zonal heterogeneity" of liver. Liver cells may function differently depending on their location within the lobules of liver, i.e., whether located near oxygen- and nutrient-rich portal/arterial blood versus near relatively depleted central-venous blood. Veenhuizen (1988) discussed implications of zonal heterogeneity
for ruminant metabolism, but the topic has received little research emphasis in ruminants. An excellent discussion of this active area of research in nonruminants has been published (Thurman et al., 1986).

Metabolism in Extrahepatic Tissues of Dairy Cows

Production and utilization of metabolites by peripheral tissues was reviewed recently (VandeHaar, 1988). VandeHaar (1988) also presented calculations for flux of energy-yielding metabolites in the lactating cow. Only a brief review of utilization of glucose and lipid metabolites during early lactation is presented in the current review.

Glucose

Utilization of glucose by nonmammary tissues has been reviewed (Lindsay, 1979). The most significant adaptation of glucose metabolism during onset of lactation is a decreased utilization by peripheral tissues to spare glucose for mammary utilization (Bauman and Elliot, 1983). As discussed by Lindsay (1979), other glucogenic compounds may be utilized directly rather than being converted to glucose and hence spare glucose indirectly.

Mechanisms by which changes in glucose metabolism occur with onset of lactation are not known, although growth hormone, prolactin, and progesterone have been implicated (Bauman and Currie, 1980; Bauman and Elliot, 1983; Bauman and McCutcheon, 1986). Administration of exogenous growth hormone to cows in early lactation decreased oxidation of glucose but increased the irreversible loss of glucose, which is consistent with the proposed role of growth hormone as a "repartitioning agent" (Bauman et
With onset of lactation, metabolism of adipose tissue is redirected toward providing fatty acids to other organs of the body (Vernon, 1980). In vitro studies showed that adipose tissue is less responsive to insulin and glucose in early lactation and that reesterification ceases (Metz and van den Bergh, 1977). Lipolysis increases and lipogenesis virtually ceases (McNamara and Hillers, 1986; Pike and Roberts, 1980; Smith and Walsh, 1988). These changes were shown to begin during the last month before parturition (McNamara and Hillers, 1986). Mechanisms that are responsible may include changes in prolactin, progesterone, and growth hormone (Bauman and Currie, 1980; Bauman and McCutcheon, 1986; McNamara and Hillers, 1986). Growth hormone increases responsiveness of bovine adipose tissue to catecholamines and decreases sensitivity to insulin (Bauman and McCutcheon, 1986). It is not known whether cows that are susceptible to fatty liver and ketosis have either enhanced sensitivity to lipolytic stimuli or decreased sensitivity to suppressive factors.

The end result of changes in metabolism of adipose tissue during early lactation is a tremendous increase in the ability of adipose tissue to mobilize NEFA. As discussed already, NEFA are taken up in large quantities by liver and also by kidney, heart, and skeletal muscle (Lindsay, 1975). It is likely that the mammary gland also takes up NEFA directly when concentrations of NEFA are increased in blood (Pullen et al., 1989). Utilization of NEFA by peripheral tissues has not been studied extensively in dairy cows but was reported to be dependent on
arterial concentration in sheep (Pethick et al., 1983, 1987). Bauman et al. (1988) reported that oxidation of NEFA accounted for 3 to 6% of total CO₂ in dairy cows.

Peripheral utilization of ketone bodies by ruminants has been reviewed recently (Heitman et al., 1987). Although most production of ketones is by gut mucosa and liver as discussed already, there is evidence for production in skeletal muscle (Pethick et al., 1983). Unlike nonruminants, brain and nervous tissue of ruminant animals evidently do not use ketone bodies, even during starvation (Lindsay and Setchell, 1976; Pell and Bergman, 1983). Ketones are used, however, by heart, skeletal muscle, kidney, and the lactating mammary gland (Heitman et al., 1987). Utilization of ketones is proportional to arterial concentration up to about 4 mM. At this point, rate of utilization does not increase further and concentrations of ketone bodies may increase rapidly if rapid production continues (Pethick and Lindsay, 1982). Measurements of ketone body utilization in lactating cows are not available, but in sheep oxidation of ketone bodies accounted for 10 to 30% of CO₂ (Pethick and Lindsay, 1982).

It has been assumed, on the basis of early evidence (Bergman, 1971), that bovine ketosis is a problem of overproduction of ketone bodies and that ketone body utilization is not limiting. Recent evidence, however, suggests that utilization may indeed be impaired at high concentrations (Herdt et al., 1988; Pethick and Lindsay, 1982). Utilization also may be decreased during deficiency of insulin (Balasse and Havel, 1971) such as is seen in bovine ketosis. In addition, increased concentrations of NEFA
and triglycerides decreased utilization of ketone bodies by skeletal muscle of humans (Rett et al., 1988). Future research should re-examine the role of ketone body utilization during early lactation and in the etiology of bovine ketosis.

Ketone bodies exert several effects on metabolism in addition to serving as energy sources, and these effects have been reviewed (Robinson and Williamson, 1980). Ketone bodies decrease peripheral utilization of glucose, decrease proteolysis in skeletal muscle, and serve as primers for fatty acid synthesis. In addition, ketone bodies may decrease gluconeogenesis in ruminants (Radcliffe et al., 1983). Ketone bodies directly inhibit lipolysis in bovine adipose tissue (Metz and van den Bergh, 1972; Metz et al., 1974) and stimulate secretion of insulin, which also acts to decrease lipolysis (Heitman et al., 1987). It has been proposed recently that ketone bodies may autoregulate their own production in ruminant liver, perhaps by direct inhibition of CPT I (Heitman et al., 1987). This could be important to dairy cows in early lactation because insulin is low during that time.

Turnover of plasma acetate in lactating cows was reported to be from 2500 g/d (Konig et al., 1984) to 3700 g/d (Bickerstaffe and Annison, 1974). Oxidation of acetate accounts for 20 to 30% of CO₂ production in sheep (King et al., 1985; Pethick et al., 1981). Acetate seems to be utilized by most peripheral tissues, including mammary gland, skeletal muscle, gut, heart, kidney, and brain. Liver used 17% of the acetate turnover in fed sheep, even though the liver is also the major site of production of endogenous acetate (Pethick et al., 1981). Many tissues
simultaneously take up and release acetate (Pethick et al., 1981); the physiological significance of this phenomenon is not understood.

Summary of the Literature

Clinical and metabolic characteristics of fatty liver and ketosis have been described by many researchers. The underlying biochemistry of the disorders has been much more difficult to establish. Many discrepancies exist in the literature between studies and between laboratories, especially with respect to activities of enzymes. A large source of variation in these observations likely can be attributed to variation between animals, in that most studies compared changes between cows with ketosis or fatty liver and normal controls rather than using the same animals before and during the disorders. Techniques that allow sequential study of development of fatty liver and ketosis seem essential for determining biochemical mechanisms.

Knowledge of carbohydrate and lipid metabolism in liver of ruminants is very limited in comparison with what is known in nonruminants. Areas of understanding that are particularly limited include regulation of gluconeogenesis, regulation of fatty acid metabolism, and mechanisms of hepatic secretion of triglycerides. Interactions between metabolism in peripheral tissues and in liver during early lactation also deserve further study.

Although ketosis has been characterized, the sequential changes in metabolism that cause a cow to go from subclinical to clinical ketosis still are not known. Likewise, metabolic controls whereby fatty liver may
develop in some cows but not in others under seemingly identical management remain undefined. Further research addressing these questions is justified to improve health and productive efficiency of dairy cows.
This dissertation is presented in the alternate format, as outlined in the Iowa State University Graduate College Thesis Manual. Use of the alternate format allows preparation of independent sections, each of which is in a form suitable for submission for publication in a scientific journal.

Two separate papers have been prepared from research performed to partly fulfill requirements for the Ph.D. degree. Each paper is complete in itself and includes an abstract, introduction, methods, results and discussion, general discussion, and references. The papers report independent research from two different experiments, but the similar focus of subject matter allows presentation of a general summary.
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SECTION I. EXPERIMENTAL FATTY LIVER AND KETOSIS ARE NOT INDUCED BY EITHER FEED RESTRICTION OR DIETARY 1,3-BUTANEDIOL AS SEPARATE TREATMENTS
ABSTRACT

Sixteen multiparous Holstein cows were assigned to one of three groups during d 14 to 42 postpartum: 1) control (fed ad libitum throughout), 2) 20% feed restriction, or 3) ad libitum plus dietary 1,3-butanediol (5.5% of diet dry matter). From d 43 to 56 postpartum, cows assigned to both the feed-restricted and butanediol treatments received a combination of feed restriction and dietary butanediol. One cow quickly developed clinical ketosis but not fatty liver after only 4 d of feed restriction. No other cows subjected to the separate treatments of either feed restriction or dietary butanediol developed fatty liver or ketosis, nor did any cows subjected to the combination treatment from d 43 to 56 postpartum. Treatments decreased milk production. Concentrations of β-hydroxybutyrate and insulin in plasma were increased by dietary 1,3-butanediol. Feed restriction caused transient increases in concentrations of nonesterified fatty acids, acetate, and β-hydroxybutyrate in plasma. Concentration of glycogen in liver was less in feed-restricted cows, whereas glycogen and total lipid in liver were greater from cows given dietary 1,3-butanediol separately. Gluconeogenic capacity of liver slices was not different among groups. Addition of L-carnitine to in vitro incubation media decreased conversion of propionate to glucose, whereas addition of either L-carnitine or 1,3-butanediol decreased oxidation of propionate to CO₂. Neither feed restriction nor dietary 1,3-butanediol as separate treatments during the early-postpartum period induced the fatty liver and ketosis seen in earlier experiments where the two treatments were given in combination starting at d 14 postpartum.
Fatty liver and lactation ketosis are interrelated metabolic disorders that affect dairy cows very early in lactation. Fatty liver has been associated with reproductive problems and increased susceptibility to other postpartum diseases (Reid, 1982), although specific physiological mechanisms for such a link have not been determined. Incidence of clinical ketosis is estimated to be between 2 and 15% (Baird, 1982), but the incidence of subclinical ketosis may be much higher. Efforts to increase understanding of biochemical and physiological mechanisms of fatty liver and ketosis are justified, therefore, to ensure productive efficiency and health of high-producing cows.

A major obstacle in studying development of fatty liver and ketosis has been the lack of suitable experimental models. Use of naturally occurring cases limits study to conditions existing at diagnosis and excludes metabolic changes during development. Recently, a protocol to induce fatty liver and ketosis was developed (Mills et al., 1986a; Veenhuizen et al., 1989a) that allowed study of sequential changes in blood, liver, and adipose tissue during development of fatty liver and ketosis. The protocol involves moderate restriction of feed intake, beginning at about d 14 postpartum, coupled with inclusion of a ketone body precursor (1,3-butanediol). Fatty liver developed by about d 28 postpartum and a clinical ketosis by about d 42. A major finding was that gluconeogenesis in vitro was decreased at clinical ketosis (Mills et al., 1986b; Veenhuizen et al., 1989b).

To explain metabolic events occurring in development of induced fatty
liver and ketosis, it is necessary to ascertain separate metabolic effects of feed restriction and dietary 1,3-butanediol (butanediol). Subjecting cows to up to 50% feed restriction produced ketonemia but not clinical ketosis (de Boer et al., 1985; 1986); however, changes in liver composition and function were not determined. Effects of dietary butanediol on liver function and metabolism in cows during early lactation are unknown.

Measures to prevent the decrease in gluconeogenic capacity that occurs at clinical ketosis should be explored. Nicotinic acid, through its antilipolytic action, is effective in decreasing incidence and severity of ketosis (Waterman et al., 1972). Effects of nicotinic acid on carbohydrate metabolism in liver have been proposed (Thornton et al., 1975), but few experimental data are available. Carnitine increases oxidation of long-chain fatty acids by hepatocytes (Lomax et al., 1983), and could, therefore, increase gluconeogenesis because oxidation of long-chain fatty acids stimulated gluconeogenesis in ruminant hepatocytes (Aiello and Armentano, 1988).

Our objective was to answer four questions. First, would either feed restriction or dietary butanediol alone, starting at 14 d postpartum, induce fatty liver or ketosis in dairy cows? Second, could fatty liver and ketosis be induced by combining feed restriction and butanediol at d 43 of lactation? Third, would feed restriction or butanediol alone cause a decrease of in vitro hepatic gluconeogenesis? Fourth, does nicotinic acid, carnitine, or butanediol affect metabolism of propionate by liver slices from normal or ketonemic cows?
MATERIALS AND METHODS
Cows, Treatments, and Management

Seventeen multiparous Holstein cows were assigned randomly at calving to three groups as control, FR (feed restriction), or BD (ad libitum-fed plus butanediol). Production of milk in the previous lactation (305-d mature equivalent) was 9465 kg (+676 kg over herdmates). From parturition through d 13 postpartum, all cows were fed a forage and grain mixture (Table 1) for ad libitum intake, which continued throughout for control cows. The experimental protocol is depicted schematically in Figure 1. The pretreatment period was d 7 to 13 postpartum, or wk 0. For FR cows, intake was restricted at d 14 to 80% of the average ad libitum intake of the previous 5 d. Cows in group BD continued to be fed for ad libitum intake, but butanediol\(^1\) was included at 5.5% of DM. Cows in both groups FR and BD continued their respective separate treatments from wk 1 through wk 4 (d 14-42 postpartum). On d 43, butanediol was added to the ration at 7% of DM for cows in group FR, and feed for group BD was restricted to 80% of ad libitum intake with the amount of butanediol remaining constant. Both groups were maintained on the combined treatment for wk 5 and 6 (d 43-56 postpartum).

Amounts of butanediol fed and degree of feed restriction were similar to a previous experiment (Veenhuizen et al., 1989a) in which cows developed fatty livers and ketosis from the combination. Butanediol was introduced by feeding one-half of the final amount for 3 d and then

\(^1\)The 1,3-butanediol (butylene glycol) was a gift from Celanese Chemical Co., Dallas TX, through the courtesy of Mr. Ken Jones.
TABLE 1. Composition and analysis of silage and grain mixture fed to cows

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Forage and grain mixture&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Composition:</strong></td>
<td></td>
</tr>
<tr>
<td>Alfalfa haylage</td>
<td>18.4</td>
</tr>
<tr>
<td>Cracked corn</td>
<td>51.3</td>
</tr>
<tr>
<td>Corn silage</td>
<td>19.6</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>9.2</td>
</tr>
<tr>
<td>Vitamin-mineral supplement&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1.5</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>100.0</td>
</tr>
</tbody>
</table>

| Analysis                        |                                      |
| Acid detergent fiber            | 16.4                                 |
| Crude protein                   | 14.9                                 |

<sup>1</sup>Cows also were fed separately an estimated 4 kg (DM) of alfalfa hay daily.

<sup>2</sup>Silage and grain mixture was a combination of the ISU herd silage mix (79%, as fed) and concentrate mix (21%, as fed), and was 64.3% DM.

<sup>3</sup>Consisted of sodium bicarbonate, magnesium oxide, dicalcium phosphate, calcium carbonate, sodium chloride, trace mineral premix, and vitamins A and D in amounts to meet requirements for vitamins and minerals.
Figure 1. Schematic representation of dietary treatments increasing to the full amount on d 4. Average daily intakes of butanediol were 885 g for group BD during wk 1 to 4, and 783 and 1000 g for groups FR and BD, respectively, during wk 5 to 6, which reflects the change from ad libitum to restricted feeding with butanediol being held constant.

Cows had access as a group to a limited amount of alfalfa hay before each milking and individually after the evening milking. Estimated consumption of hay was 4 kg of DM daily. Cows were housed in individual
tie stalls and fed the forage and grain mixture twice daily, with ors weighed once daily. Cows were milked twice daily, and all milk weights were recorded. Cows were weighed every second day. Development of ketonemia during the experiment was monitored by use of urine ketone test strips².

Sampling Procedures

Puncture biopsies (Hughes, 1962) of about 5 g of liver were obtained under local lidocaine anesthesia (Veenhuizen et al., 1989b) from all cows at four times: on d 13 postpartum (end of pretreatment), d 28, d 42 (1 d before butanediol and feed restriction were combined for groups FR and BD), and d 56. A portion of liver was frozen and stored at -20° C. Another portion was placed into buffered 10% formalin until prepared for histological examination³. The remaining portion of liver was placed immediately into an ice-cold solution of phosphate-buffered (.015 M) .9% NaCl (pH 7.4) and used within 1.5 h for in vitro metabolic studies.

Samples of jugular venous blood were obtained weekly from all cows before the AM feeding and immediately before liver biopsy if both were on the same day. Blood (10 ml) was collected in heparinized tubes containing 150 ul of 4% NaF. Plasma was obtained by centrifugation, aliquoted, and frozen at -20° C until analyzed.

²Keto-Diastix®, Ames Division, Miles Laboratories, Inc., Elkhart, IN.

³Histological analyses were conducted by Drs. L. D. Miller and T. P. Sanderson, Department of Veterinary Pathology, ISU.
Incubation Procedures

Incubations were essentially as described earlier (Veenhuizen et al., 1989b). Liver tissue was sliced (Stadie and Riggs, 1944), then blotted, weighed, and placed into 25-ml flasks that contained 3 ml of Krebs-Henseleit bicarbonate buffer (Lasser, 1961) at pH 7.4. Media contained .03% bovine serum albumin, 10 mM Na-propionate with 1 uCi Na-[2-14C]-propionate*, and 2 mM Ca**, because previous work (Lyle et al., 1984a; Veenhuizen et al., 1989b) showed that gluconeogenic rates were maximized by physiological concentrations of Ca**. To determine the effects of carnitine, butanediol, and nicotinic acid on in vitro metabolism of propionate, flasks also contained either 1 mM L-carnitine5, 10 mM 1,3-butanediol6, .8 mM nicotinic acid7, or no addition.

Flasks were gassed with a mixture of O2:CO2 (95:5) and incubated in triplicate for 2 h at 37° C. Incubations were terminated at the end of 2 h by injecting .5 ml of 1.5 N sulfuric acid into each flask. Radiolabelled CO2 was collected as described earlier (Lyle et al., 1984a).

Analytical Methods

Acidified media were processed after addition of 3H-glucose as a recovery marker as described earlier (Veenhuizen et al., 1989b).

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4ICN Biomedicals, Inc., Costa Mesa, CA.

5L-carnitine was a gift from Lonza, Inc., Fair Lawn, NJ, through the courtesy of Mr. Stephen Blum.

6Aldrich Chemical Co., Inc., Milwaukee, WI.

7Sigma Chemical Co., St. Louis, MO.
Radioactive glucose was isolated by ion-exchange chromatography (Mills et al., 1981). Scintillation cocktail\(^8\) was added to total radioactive glucose, and radioactivity was determined by dual-isotope counting. Rate of synthesis of glucose was corrected for recovery of \(^3\)H-glucose marker. Background contamination of radioactive glucose and CO\(_2\) by unremoved or volatilized \(^14\)C-propionate was measured in each incubation in media that were acidified immediately before adding liver slices. Conversions of propionate to glucose and CO\(_2\) were calculated (Mills et al., 1984) and are reported as umoles of propionate converted to product per h per g tissue wet weight.

Frozen samples of liver were powdered under liquid N\(_2\), and a portion used for determination of glycogen and free glucose (Keppler and Decker, 1974). Lipid was extracted from remaining liver by shaking overnight in 2:1 (v:v) chloroform-methanol. The chloroform layer was filtered through glass wool and evaporated under moving air to determine total lipid gravimetrically. The dry lipid extract was dissolved in 3:2 (v:v) hexane-isopropanol, and concentration of triglyceride was determined by using a modification of a commercial kit\(^9\). Sample plus reagent were incubated for 45 min at 37\(^\circ\) C to evaporate the hexane and allow the reaction to go to completion.

Concentration of BHBA (Williamson and Mellanby, 1974) was determined in protein-free filtrates prepared from plasma (Somogyi, 1945).

\(^8\)Ready Solv EP, Beckman Instruments, Inc., Fullerton, CA.

\(^9\)Sigma triglyceride kit no. 338, Sigma Chemical Co., St. Louis, MO.
Concentration of glucose in plasma was determined by using glucose oxidase\textsuperscript{10}, and acetate was determined enzymatically (Knowles et al., 1974). Concentration of nonesterified fatty acids (NEFA) was determined by using a modification (McCutcheon and Bauman, 1986) of a commercial kit\textsuperscript{11} with the following additional modifications: Color reagent A and its diluent were combined with 16 ml of water, and color reagent B and its diluent were combined with 33 ml water. To 25 \( \mu l \) of sample or standard were added .4 ml reagent A and .8 ml reagent B. The reaction was linear through 2000 uequiv/l.

Concentrations of immunoreactive insulin and glucagon in plasma were measured by validated radioimmunoassays (Amaral, 1988). Plasma for glucagon contained 2500 IU of aprotinin\textsuperscript{12} per ml. All samples for each hormone were assayed together. Intraassay coefficients of variation were 8\% and 12\% for glucagon and insulin, respectively.

Contents of fat and protein in milk samples taken at four consecutive milkings weekly were determined by using an infrared analyzer\textsuperscript{13}, and fat-corrected milk (FCM) and solids-corrected milk (SCM) were calculated (Tyrrell and Reid, 1965). The mixed ration was sampled monthly, and DM was determined by drying at 60\(^\circ\) C for 2 d in a forced-air oven. Samples then were composited for the experiment, and contents of crude protein

\textsuperscript{10}Sigma glucose (Trinder) kit no. 315, Sigma Chemical Co., St. Louis, MO.

\textsuperscript{11}NEFA-C kit, WAKO Chemical Co. USA, Dallas, TX.

\textsuperscript{12}Trasylol\textsuperscript{\textregistered}, Mobay Chemical Corp., FBA Pharmaceuticals, New York, NY.

\textsuperscript{13}Milk-O-Scan 203, Foss Food Technology, Eden Prairie, MN.
Statistical Analyses

Two single values for each dependent variable were generated for each cow by averaging repeated measurements from wk 1 through 4 (separate-treatment period) and from wk 5 and 6 (combination-treatment period). Separate analyses of covariance were conducted for each period by using the General Linear Models (GLM) package (SAS Institute, 1982), with the pretreatment measurement (wk 0) as the covariate. Covariate-adjusted means within each time period then were compared by using orthogonal contrasts of control vs. FR plus BD, and FR vs. BD.

To evaluate effects of time, all data involving repeated measurements also were subjected to analysis of variance for a split-plot design (Gill, 1986) by using GLM. Treatment groups were the main plot and were tested using the error term of cow within group. Times of sampling (weeks) were subplots and were tested against residual error, as was the treatment by week interaction. Conservative degrees of freedom were used to evaluate all F-tests containing week as a factor (Geisser and Greenhouse, 1958). Conditional comparisons between treatments at each sampling time were evaluated by using Bonferroni t-tests (Gill, 1986).

Analysis of variance for in vitro propionate metabolism contained a third factor of type of addition (nicotinic acid, carnitine, butanediol, or none) to incubation media. Appendix Table A2 shows the analysis of variance for conversion of propionate to glucose as an example. Because
interactions of incubation additions with treatment or week were not significant, mean rates of propionate metabolism by type of addition were separated by using the Tukey test (SAS Institute, 1982). Least-squares means are presented throughout. Tests of significance with P ≤ .05 are considered statistically significant.
RESULTS

General Health of Cows

One cow assigned to FR developed a serious ketosis 4 d after initiation of feed restriction. A blood sample was obtained and she then was treated by administering 500 g dextrose intravenously, 250 g dextrose subcutaneously, and 20 mg dexamethasone\(^\text{14}\) intramuscularly. Recovery was rapid, as judged by return of milk production to preketotic amounts and decreased content of ketone bodies in urine, and she was retained on experiment. Data for liver function and metabolites in plasma of this cow will be discussed later.

During wk 3, one cow in group BD briefly went off-feed, which increased concentrations of NEFA and BHBA and decreased glucose in plasma. The cow recovered quickly and was retained on experiment, but data for that week were not used in statistical analyses. Another cow in group BD developed severe peritonitis following biopsy of liver at wk 2. She was removed from the experiment because of high fever and lack of feed intake for 3 d and was not replaced. All other cows were healthy throughout the experiment. Thus, total cows per group used in statistical analyses were 5, 6, and 5 for control, FR, and BD, respectively.

Milk Production and Composition

Production of milk (Table 2) by FR and BD cows was less than that of controls during both wk 1 to 4 and wk 5 to 6 (P<.003 and .03,

\(^\text{14}\)Azium\(^R\), Schering Veterinary, Kenilworth, NJ.
<table>
<thead>
<tr>
<th>Item</th>
<th>Separate-treatment Period</th>
<th>Combination-treatment Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treatment Group</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control       FR  BD    SE</td>
<td>Control     FR  BD    SE</td>
</tr>
<tr>
<td>Milk'^, kg/d</td>
<td>36.5          33.8   33.3   .6  A, .003</td>
<td>35.8        32.1   30.6   1.5  A, .03</td>
</tr>
<tr>
<td>Protein®, kg/d</td>
<td>1.08          1.03   1.06   .02</td>
<td>1.05        1.02   1.01   .99  .04</td>
</tr>
<tr>
<td>Fat®, kg/d</td>
<td>1.24          1.24   1.22   .06</td>
<td>1.18        1.16   1.25   1.16 B, .09</td>
</tr>
<tr>
<td>Protein®, %</td>
<td>2.9           3.0    3.2    .09 A, .12</td>
<td>3.0         3.2    3.2    .12 A, .02</td>
</tr>
<tr>
<td>Fat®, %</td>
<td>3.3           3.6    3.6    .19</td>
<td>3.1         3.2    3.2    .12 A, .02</td>
</tr>
<tr>
<td>SCM®'^, kg/d</td>
<td>33.4          32.0   32.1   .8</td>
<td>32.6        31.6   30.0   .8  A, .10; B, .19</td>
</tr>
<tr>
<td>FCM®'^, kg/d</td>
<td>33.5          32.3   31.9   .8</td>
<td>32.8        31.8   29.9   .7  A, .05; B, .10</td>
</tr>
</tbody>
</table>

1 Weeks 1 through 4 of experiment (days 14 through 42 postpartum). Cows in group FR were restricted to 80% of ad libitum intake, and cows in group BD were fed ad libitum plus 1,3-butanediol (5.5% of DM). Means were corrected for covariate of week 0 (pretreatment).

2 Weeks 5 and 6 of experiment (days 43 through 56 postpartum). Cows in both groups FR and BD received a combination of feed restriction and dietary 1,3-butanediol. Means were corrected for covariate of week 0 (pretreatment). Separate analyses of covariance were conducted for the individual-treatment and combination-treatment periods.

3 Probabilities of greater F for orthogonal contrasts: A = control vs. FR plus BD; B = FR vs. BD.

4 Means of 5, 6, and 5 cows for control, FR, and BD, respectively.
5Means of 4, 5, and 4 cows for control, FR, and BD, respectively.

6Solids-corrected milk.

74% fat-corrected milk.
respectively). Production of milk was similar for cows in FR and BD, even though BD cows were fed ad libitum. Yields of fat and protein were similar among groups, whereas contents of fat and protein in milk from cows in FR and BD tended to increase (Table 2).

Yields of SCM and FCM were similar among treatment groups during wk 1 to 4, but during wk 5 to 6 yield of SCM tended (P<.10) to be less and yield of FCM was significantly less (P<.05) for cows in FR and BD compared with control cows (Table 2). Cows in group BD tended to produce the least SCM and FCM.

Intake and Body Weight Change

Intake of DM from the silage-grain mixture (Table 3) was not different for cows in control and BD groups during wk 1 to 4 when both groups were fed for ad libitum intake, but was less for FR as designed. During wk 5 to 6, restricted intakes were similar for cows in FR and BD. Intake averaged 84% of ad libitum for FR, which was slightly more than the desired 80% because of two cows that had very low pretreatment intakes and were restricted only to about 90% of pretreatment intake. Intake of ME by cows in group FR was estimated to be 83% of requirements at wk 0 (National Research Council, 1978).

Intake of hay was only estimated and caution must be exercised in interpretation of intake data, but it seems that cows in group BD consumed less DM and ME as a percentage of body weight than did control cows (Table 3). The estimated decrease of ME intake by BD cows was sufficient to account for the decrease in milk production discussed already.
### TABLE 3. Average daily intakes and body weight of cows either feed-restricted (FR) or fed 1,3-butanediol (BD) during weeks 1 through 4 or given both during weeks 5 and 6.

<table>
<thead>
<tr>
<th>Item and time</th>
<th>Treatment</th>
<th>Control</th>
<th>FR</th>
<th>BD</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intake, silage-grain mixture, kg DM/d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 0²</td>
<td>16.7</td>
<td>14.6</td>
<td>15.8</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>Weeks 1 to 4</td>
<td>19.6</td>
<td>12.3</td>
<td>19.2</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Weeks 5 and 6</td>
<td>18.5</td>
<td>12.5</td>
<td>15.1</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Estimated total DM intake, % of body weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 0</td>
<td>3.6</td>
<td>3.4</td>
<td>3.2</td>
<td>.2</td>
<td></td>
</tr>
<tr>
<td>Weeks 1 to 4</td>
<td>4.2</td>
<td>3.1</td>
<td>3.8</td>
<td>.1</td>
<td></td>
</tr>
<tr>
<td>Weeks 5 and 6</td>
<td>4.0</td>
<td>3.1</td>
<td>3.2</td>
<td>.2</td>
<td></td>
</tr>
<tr>
<td>Estimated total ME intake³, Mcal/100 kg body weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 0</td>
<td>9.6</td>
<td>9.0</td>
<td>8.4</td>
<td>.6</td>
<td></td>
</tr>
<tr>
<td>Weeks 1 to 4</td>
<td>11.1</td>
<td>8.0</td>
<td>10.5</td>
<td>.4</td>
<td></td>
</tr>
<tr>
<td>Weeks 5 and 6</td>
<td>10.6</td>
<td>8.6</td>
<td>8.8</td>
<td>.6</td>
<td></td>
</tr>
<tr>
<td>Body weight, kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 0</td>
<td>573</td>
<td>546</td>
<td>625</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Week 4</td>
<td>567</td>
<td>529</td>
<td>614</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Week 6</td>
<td>564</td>
<td>530</td>
<td>608</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Body weight loss, kg/d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weeks 1 to 4</td>
<td>.22</td>
<td>.62</td>
<td>.36</td>
<td>.47</td>
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<tr>
<td>Weeks 5 and 6</td>
<td>.30</td>
<td>.12</td>
<td>.74</td>
<td>.89</td>
<td></td>
</tr>
</tbody>
</table>

¹During weeks 1 through 4, cows in groups FR were feed-restricted, and cows in group BD were fed ad libitum plus dietary 1,3-butanediol. During weeks 5 and 6, cows in both groups FR and BD were given a combination of feed restriction and dietary 1,3-butanediol. Values are means resulting from separate analyses of variance conducted for each period.

²Week 0 = days 7 to 13 postpartum and was the pretreatment period.

³Actual intake of dry matter (DM) from forage-grain mixture plus estimated consumption of 4 kg DM from alfalfa hay. Metabolizable energy (ME) of 1,3-butanediol was assumed to be 6.0 Mcal/kg.
Cows in group BD were heavier at the start of the experiment (Table 3). Cows in group FR tended (P<.10) to have greater loss of weight than control cows during wk 1.

Metabolites and Hormones in Plasma

Average concentrations of glucose, NEFA, and acetate in plasma (Table 4) during wk 1 to 4 were not different among groups. Concentration of BHBA tended (P<.12) to be higher for cows in groups FR and BD than in control cows. During wk 5 to 6, glucose tended to be lower (P<.12) and BHBA higher (P<.06) in plasma of cows in groups FR and BD than in control cows. Dietary butanediol increased BHBA and decreased glucose in plasma of rats (Mehlman et al., 1971), steers (Young, 1975), and goats (Drackley et al., 1989b).

Figure 2 shows concentrations of NEFA, acetate, and BHBA by week. Concentrations of NEFA (Figure 2a) and acetate (Figure 2b) were greater at wk 1 in FR cows than in control cows (P<.05). Concentration of BHBA (Figure 2c) tended to be greater for FR cows compared with controls at wk 1 (P<.10) and wk 5 (P<.10), and greater for BD cows compared with controls at wk 1 (P<.10) and wk 6 (P<.10). Changes in concentrations of metabolites in FR cows indicate adaptive responses to decrease in energy intake. Concentrations of BHBA increased in response to initiation of treatments in both groups FR and BD.

Insulin in plasma (Table 4) was greater (P<.05) for group BD than group FR during wk 1 to 4 but was not different among groups during wk 5 to 6. It is uncertain whether the difference during wk 1 to 4 is a result
TABLE 4. Concentrations of metabolites and hormones in plasma of cows either feed-restricted (FR) or fed 1,3-butanediol (BD) during the separate-treatment period, or given both during the combination-treatment period

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Separate-treatment Period</th>
<th>Combination-treatment Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treatment Group</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>FR</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>60.2</td>
<td>60.2</td>
</tr>
<tr>
<td>NEFA&lt;sup&gt;4&lt;/sup&gt;, uequiv/l</td>
<td>389</td>
<td>494</td>
</tr>
<tr>
<td>Acetate, umol/l</td>
<td>891</td>
<td>941</td>
</tr>
<tr>
<td>BHBA&lt;sup&gt;5&lt;/sup&gt;, mg/dl</td>
<td>4.7</td>
<td>6.5</td>
</tr>
<tr>
<td>Insulin, pg/ml</td>
<td>311</td>
<td>286</td>
</tr>
<tr>
<td>Glucagon, pg/ml</td>
<td>152</td>
<td>154</td>
</tr>
<tr>
<td>Insulin:glucagon, molar</td>
<td>1.3</td>
<td>1.2</td>
</tr>
</tbody>
</table>

<sup>1</sup>Weeks 1 through 4 of experiment (days 14 through 42 postpartum). Cows in group FR were restricted to 80% of ad libitum intake, and cows in group BD were fed ad libitum plus 1,3-butanediol (5.5% of DM). Means were corrected for covariate of week 0 (pretreatment).

<sup>2</sup>Weeks 5 and 6 of experiment (days 43 through 56 postpartum). Cows in both groups FR and BD received a combination of feed restriction and dietary 1,3-butanediol. Means were corrected for covariate of week 0 (pretreatment). Separate analyses of covariance were conducted for the individual-treatment and combination-treatment periods.

<sup>3</sup>Probability of greater F for orthogonal contrasts: A = control vs. FR plus BD; B = FR vs. BD.

<sup>4</sup>Nonesterified fatty acids.

<sup>5</sup>&#8212;Hydroxybutyrate.
Figure 2. Concentrations of metabolites in plasma from cows either feed-restricted (FR) or fed butanediol (BD) during weeks 1 through 4, or given the combination during weeks 5 and 6

At any week, treatment means with the same letter are not different (P>.1). Week 0 is d 7 to 13 postpartum.

A. Nonesterified fatty acids (standard error of the difference (SED) between any two treatment means at any week = 158). Probabilities of greater F for effects in model: treatment, P=.68; week, P=.11; treatment x week, P=.18.

B. Acetate (SED = 143). Treatment, P=.64; week, P=.18; treatment x week, P=.14.

C. B-Hydroxybutyrate (SED = 2.0). Treatment, P=.31; week, P=.31; treatment x week, P=.25.
of decreased insulin in group FR or of increased insulin in group BD; however, insulin tended (P<.2) to be greater in group BD than in controls. Acute doses of butanediol did not increase concentration of insulin in lactating goats (Drackley et al., 1989b).

Concentration of glucagon was similar among groups during both periods, resulting in a slightly higher, although nonsignificant, ratio of insulin to glucagon for cows in group BD during wk 1 to 4 (Table 4). Changes in hormones and metabolites in plasma in FR cows were similar to those caused by feed restriction in earlier studies (de Boer et al., 1985; 1986).

Liver Composition

When averaged over all treatment groups, concentration of glycogen in liver increased significantly (P<.05) from wk 0 to wk 6 (data not shown). Average concentration of glycogen (Table 5) was less (P<.05) in cows in group FR compared with those in group BD during wk 1 to 4, but was similar among groups and essentially normal at wk 6. Concentration of free glucose in liver was not affected by treatment (Table 5). Contents of glycogen and glucose were decreased in cows with spontaneous (Baird and Heitzman, 1971) or starvation ketosis (Baird et al., 1972), and glycogen content of liver was decreased markedly in cows given a combination of feed restriction and butanediol (Mills et al., 1986a; Veenhuizen et al., 1989a).

Concentration of total lipid in liver decreased (P<.004) from wk 0 to wk 6 when averaged over all treatments (data not shown). Average total
TABLE 5. Composition of liver and metabolism of propionate by liver slices from cows either feed restricted (FR) or fed 1,3-butanediol (BD) during the separate-treatment period, or given both during the combination-treatment period

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Separate-treatment Period</th>
<th>Combination-treatment Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>FR</td>
</tr>
<tr>
<td>Glycogen, %</td>
<td>3.6</td>
<td>2.8</td>
</tr>
<tr>
<td>Glucose, %</td>
<td>.5</td>
<td>.4</td>
</tr>
<tr>
<td>Total lipid, %</td>
<td>3.7</td>
<td>3.6</td>
</tr>
<tr>
<td>Triglyceride, %</td>
<td>.8</td>
<td>.7</td>
</tr>
<tr>
<td><strong>Product from propionate</strong></td>
<td>**---(umol·h⁻¹·g⁻¹ wet wt.)**⁵---</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>10.2</td>
<td>9.8</td>
</tr>
<tr>
<td>CO₂</td>
<td>.6</td>
<td>.6</td>
</tr>
<tr>
<td>Glucose/CO₂</td>
<td>18.1</td>
<td>17.5</td>
</tr>
</tbody>
</table>

¹Weeks 1 through 4 of experiment (days 14 through 42 postpartum). Cows in group FR were restricted to 80% of ad libitum intake, and cows in group BD were fed ad libitum plus 1,3-butanediol (5.5% of DM). Means were corrected for covariate of week 0 (pretreatment).

²Weeks 5 and 6 of experiment (days 43 through 56 postpartum). Cows in both groups FR and BD received a combination of feed restriction and dietary 1,3-butanediol. Means were corrected for covariate of week 0 (pretreatment). Separate analyses of covariance were conducted for the individual-treatment and combination-treatment periods.

³Probabilities of greater F for orthogonal contrasts: A = control vs. FR plus BD; B = FR vs. BD.

⁴Percent of wet weight of liver.

⁵Micromoles propionate converted to product per h per g tissue (wet weight).
lipid in liver (Table 5) from cows in group BD tended to be greater (P<.12) during wk 1 to 4 and was significantly greater (P<.005) at wk 6 than for cows in group FR. This increase was not triglyceride (Table 5); other lipid constituents were not determined. In earlier studies (Mills et al., 1986a; Veenhuizen et al., 1989a), triglyceride in liver was increased markedly by a combination of feed restriction and butanediol beginning at d 14. Triglyceride is the primary lipid type that accumulates in cows with either postparturient (Collins and Reid, 1980) or starvation-induced (Brumby et al., 1975) fatty livers, or with spontaneous ketosis (Baird, 1982). Absence of marked accumulation of lipid in liver of cows from all treatments was verified also by histological examination15.

Contents of total lipid and triglyceride in liver of the ketotic cow were decreased slightly 2 d after ketosis compared with wk 0 (total lipid: 5.5 vs. 4.1% of wet weight; triglyceride: 2.2 vs. 1.1% of wet weight, respectively). It is unlikely that administration of dexamethasone decreased content of lipid in liver, because content of liver lipid in cows in the study of Mills et al. (1986a) remained greatly elevated 1 wk after administration of dexamethasone during treatment for ketosis.

---

In Vitro Metabolism of Propionate

Rates of conversion of propionate to glucose and CO$_2$ were linear through 3 h of incubation (data not shown). Conversion of propionate to glucose and CO$_2$ by liver slices decreased ($P<.02$ and $0.01$, respectively) from wk 0 to wk 6 but was not significantly different among treatment groups (not shown). The decrease with time is in agreement with Aiello et al. (1984), in which rates of conversion of propionate to glucose decreased from d 30 to d 60. The capacity for synthesis of glucose may be increased during early lactation, when feed intake is low relative to glucose requirements, to maximize production of glucose from available substrates.

When adjusted for pretreatment rates (Table 5), conversion of propionate to glucose at wk 6 tended ($P<.11$) to be lower for cows in group BD compared with those in group FR, whereas conversion to CO$_2$ and the ratio of glucose to CO$_2$ produced were not different among groups. Mills et al. (1984) observed tendencies for decreased conversion of several substrates to glucose by liver slices from steers fed butanediol. Decreases, however, were much less than when ketosis was induced by feed restriction plus butanediol (Mills et al., 1986b; Veenhuizen et al., 1989b).

Rates of conversion of propionate to glucose by liver slices from the ketotic cow in group FR were decreased by 38% compared with pretreatment rates observed one week earlier (8.3 vs. 5.2 umol·h$^{-1}·g^{-1}$). Rather than being an effect of ketosis, rates may have been decreased by treatment with dexamethasone 2 d before the liver biopsy was obtained, because Baird
and Heitzman (1971) observed decreased activity of phosphoenolpyruvate carboxykinase in liver of ketotic cows 48 h after treatment with dexamethasone. Decreased gluconeogenic capacity in the ketotic cow was observed despite concentration of glucose in plasma of 99.0 mg/dl (compared with 51.6 mg/dl when ketosis was diagnosed), and content of liver glycogen of 6% of wet weight (compared with 2.5% 1 wk before ketosis).

Effects of Nicotinic Acid, Carnitine, and 1,3-Butanediol

Effects of adding three compounds (nicotinic acid, carnitine, and butanediol) to incubation media on rates of propionate metabolism were evaluated (Table 6). The only compound to affect conversion of propionate to glucose was carnitine, which slightly decreased the conversion (P<.05). The mechanism whereby carnitine might exert this effect is unknown. Carnitine stimulated oxidation of long-chain fatty acids in bovine liver slices (Drackley et al., 1989a) and ovine hepatocytes (Lomax et al., 1983), which in turn stimulated gluconeogenesis in ruminant hepatocytes in vitro (Aiello and Armentano, 1988). Carnitine slightly stimulated conversion of propionate to glucose in slices of bovine kidney in vitro (Weidemann and Krebs, 1969).

A tendency (P<.10) for interaction of week and type of addition was observed for conversion of propionate to glucose (Appendix Table A2), and was attributable to addition of butanediol. At wk 0, butanediol tended to stimulate the conversion, whereas by wk 4, it tended to decrease the conversion. Although the mechanism for the effect is unknown, it may be
TABLE 6. Effect of nicotinic acid, carnitine, or butanediol in incubation media on conversion of propionate to glucose or carbon dioxide by liver slices in vitro

<table>
<thead>
<tr>
<th>Product</th>
<th>Addition to media&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Nicotinic Acid</th>
<th>Carnitine</th>
<th>Butanediol</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>10.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>.25</td>
</tr>
<tr>
<td>CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>.02</td>
</tr>
<tr>
<td>Ratio glucose to CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>14.6</td>
<td>13.2</td>
<td>21.5</td>
<td>25.2</td>
<td>---</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup> Means within a row with a common superscript are not different, P>.05.

<sup>1</sup>Rates from all treatment groups and biopsy times averaged by type of addition to media (n=63).

<sup>2</sup>Nicotinic acid (.8 mM), l-carnitine (1 mM), 1,3-butanediol (10 mM).

<sup>3</sup>Micromoles propionate converted to product per h per g tissue (wet weight).
related to the tendency for the conversion to be decreased at wk 6 in cows in group BD, as discussed already.

Addition of either carnitine or butanediol to incubation media decreased oxidation of propionate to CO₂ (Table 6), which increased the ratios of glucose to CO₂ produced. Carnitine, by increasing oxidation of endogenous long-chain fatty acids (Aiello and Armentano, 1988; Drackley et al., 1989a) and cytosolic oxidation of butanediol to BHBA (Mehlman et al., 1971) could spare oxidation of propionate by increasing the supply of acetyl-CoA or reducing equivalents, respectively.

Nicotinic acid did not affect conversion of propionate to glucose but slightly increased oxidation of propionate to CO₂ (Table 6). Lyle (1983) observed that niacin increased incorporation of propionate to glucose and tended to increase oxidation of propionate by liver slices from steers. The small effects observed may be pharmacological and of no significance in vivo.
The primary significance of this experiment is that neither moderate feed restriction nor dietary butanediol individually caused the gradual development of fatty liver and lactation ketosis that was seen in earlier studies (Mills et al., 1986a; Veenhuizen et al., 1989a). Combining feed restriction and butanediol beginning at d 43 postpartum also did not cause fatty liver or ketosis, in contrast to studies where the combination was begun at about d 14 postpartum (Mills et al., 1986a; Veenhuizen et al., 1989a). These findings are interpreted to indicate that both feed restriction and an exogenous ketone source are necessary, beginning before d 42 postpartum, to induce fatty liver and ketosis.

Inability of the combined protocol to induce ketosis after d 43 postpartum agrees with the greater incidence of spontaneous ketosis during the first 3 to 4 wk postpartum (Baird, 1982). Others also produced ketosis most effectively very early in lactation. Their methods included starvation (Baird, 1982), thyroxine with high-protein diets (Hibbitt, 1966), and thyroxine with decreased intake (Kellog et al., 1971). Reasons for the apparent loss of susceptibility to fatty liver and ketosis between d 14 and d 42 are unclear but are probably related to loss of the initial homeorhetic "drive" to produce milk that exists after parturition (Bauman and Currie, 1980). From wk 0 to wk 4 (d 13 to d 42 postpartum) in the present experiment, concentration of glucose in plasma increased and NEFA decreased, content of liver glycogen increased, and content of total lipid and triglyceride decreased; these changes indicate a lessening of energy deficit and occurred regardless of treatment, which seems to rule out
increases in feed intake as the primary cause. Whatever the cause, these changes may have resulted in greater resistance to induction of ketosis and fatty liver. Future research should attempt to delineate biochemical causes of the loss in susceptibility to induction of fatty liver and ketosis.

Neither feed restriction nor dietary butanediol alone, nor combined feed restriction and butanediol at d 43 postpartum, markedly decreased in vitro hepatic gluconeogenesis from propionate, in contrast to studies with induced ketosis (Mills et al., 1986b; Veenhuizen et al., 1989b). These findings point to the decrease in gluconeogenic capacity as a specific aberration related to onset of clinical ketosis. Cause and effect, however, has not been determined. That the decreased capacity for in vitro gluconeogenesis also may be reflected in vivo was suggested by limited in vivo measurements on the kinetics of conversion of propionate to glucose (Veenhuizen et al., 1989b).

The early case of ketosis in one cow in the feed restriction group is the first to occur in three experiments in our laboratory (de Boer et al., 1985; 1986; present study). Ketosis in this cow seemingly was induced by feed restriction, but her case does not seem typical of either "spontaneous" ketosis or our induced ketosis for three reasons. First, up to 50% feed restriction alone did not cause clinical ketosis in 10 cows in two previous studies from our laboratory (de Boer et al., 1985; 1986) or in a study from another laboratory (Kellogg et al., 1971), although fasting cows in early lactation will induce a ketosis (Baird et al., 1972). Second, the rapid onset of ketosis after initiating feed
restriction contrasts with the gradual development of ketosis induced by feed restriction plus butanediol (Mills et al., 1986a; Veenhuizen et al., 1989a). Finally, the cow did not have the significantly increased lipid in liver that is observed in cows having either spontaneous (Baird, 1982) or induced ketosis (Mills et al., 1986a; Veenhuizen et al., 1989a).

Although the nature and significance of this isolated case are unclear, it remains valid to conclude that feed restriction alone is not a reliable technique for experimentally producing ketosis.

Results of this study provide further insight on development of fatty liver and ketosis in dairy cows during early lactation. During the first week of experiment, cows subjected to feed restriction were mobilizing greater amounts of lipid, as indicated by increases in concentrations of NEFA, acetate, and BHBA in plasma and increased loss of body weight. Changes in concentrations of metabolites were qualitatively similar to those reported previously (Mills et al., 1986a; Veenhuizen et al., 1989a) for cows that eventually developed fatty liver. By the second week of experiment, however, concentrations of metabolites in feed-restricted cows were returning to values similar to those in control cows. It seems, therefore, that cows were able to adjust metabolically to a moderate degree of feed restriction, even though they already were in negative energy balance. Amaral (1988) reached similar conclusions on the basis of experiments with cows subjected to short-term decreases in glucose availability. Fasted, early-lactation cows develop severe ketonemia and fatty liver (Baird et al., 1972; Brumby et al., 1975), but it is unknown to what degree intake can be restricted before cows develop fatty liver.
and/or ketosis because they are unable to maintain homeostasis.

Cows given dietary butanediol alone also maintained normal concentrations of metabolites in plasma and liver, with the exception of a slightly elevated concentration of BHBA in plasma. With the diet fed ad libitum in the present study, dietary butanediol consumed in amounts similar to that in earlier studies (Mills et al., 1986a; Veenhuizen et al., 1989a) did not cause fatty liver, which seems to exclude butanediol toxicity as a cause of fatty liver in the earlier experiments. On the basis of response to dietary butanediol, it seems that increased intake of dietary sources of ketone bodies, such as hay silages high in butyric acid, usually will be of minor significance in causing ketosis in cows fed adequately.

On the basis of our observations, therefore, it is suggested that when individual effects of feed restriction and butanediol are combined in cows early in lactation and in substantial negative energy balance, a synergism leads first to development of fatty liver and eventually to clinical ketosis. The nature of this synergism is not clear, but a possible sequence of events can be suggested.

The events probably start with the decreased availability of gluconeogenic precursors caused by feed restriction and the increased mobilization of NEFA from adipose tissue that is stimulated (Baird, 1982). Heitmann et al. (1987) suggested that ketone bodies may directly regulate their own production in ruminant liver via an inhibition of carnitine palmitoyltransferase I, and BHBA was shown to decrease oxidation of palmitate by bovine liver slices (Drackley et al., 1989a). Conversion of
butanediol to BHBA, therefore, could decrease oxidation of NEFA. Alternatively, oxidation of butanediol to BHBA in hepatic cytosol (Mehlman et al., 1971) could decrease oxidation of NEFA by increasing transfer of reducing equivalents from cytosol to mitochondria, a mechanism similar to the decrease in oxidation of long-chain fatty acids caused by ethanol (Mayes, 1985). The cytosol of liver from ketotic cows was more reduced chemically (Baird and Heitzman, 1971), suggesting a similarity between induced and naturally occurring ketosis. Regardless of mechanism, butanediol may mimic the effects of increased ketogenesis caused by natural depletion of citric acid cycle intermediates during energy deficit (Baird and Heitzman, 1971; Baird et al., 1972). Metabolism of butanediol in feed-restricted cows may decrease hepatic oxidation of NEFA during an increased mobilization of NEFA from adipose tissue and uptake by liver. Decreased hepatic oxidation of NEFA could result in an increase in esterification at the same time that metabolism of butanediol provides an increase in ketogenesis.

Besides decreasing oxidation and increasing esterification of NEFA, butanediol or ketone bodies may decrease production of glucose by the liver. Radcliffe et al. (1983) observed decreased production of glucose in sheep infused with ketone bodies, whereas acetoacetate decreased but BHBA increased gluconeogenesis from propionate by hepatocytes isolated from lactating goats (Aiello and Armentano, 1987). Gluconeogenesis from propionate in isolated hepatocytes is decreased when the cytosol is reduced chemically by ethanol, ammonium ions, or lactate (Aiello and Armentano, 1987); if metabolism of butanediol chemically reduces cytosol
similar to metabolism of ethanol (Mayes, 1985), gluconeogenesis could be decreased. Mehlman et al. (1971) concluded that butanediol decreased gluconeogenesis by inhibiting conversion of malate to oxaloacetate, which is consistent with a more reduced cytosol. In our experiment, effects of butanediol on gluconeogenesis in vitro were modest, but the presence of Ca** in vitro may counteract the effects of cytosolic reduction (Aiello and Armentano, 1987).

Decreased availability of glucose would lead to continued and possibly excessive mobilization of fatty acids from adipose tissue. Because hepatic uptake of fatty acids is concentration-dependent, uptake might exceed the capacity to oxidize or export fatty acids, thereby resulting in accumulation of triglyceride. Effects of butanediol or ketone bodies on hepatic glucose production have not been determined in lactating cows, but butanediol did not affect kinetics of glucose metabolism in steers (Lyle et al., 1984b). The tendency observed in our experiment for butanediol to decrease gluconeogenesis in vitro plus data already published suggest that direct measurements of the effect of butanediol and ketone bodies on glucose metabolism should be made in lactating cows.

The scheme already outlined could result in fatty liver. Veenhuizen et al. (1989a) suggested that lactation ketosis is preceded by development of fatty liver, at least in the case of induced ketosis. Kleppe et al. (1988) observed that ketogenesis in vitro increased only after the rate of esterification began to decrease. Veenhuizen et al. (1989b) suggested that increased hepatic lipid and/or decreased hepatic glycogen contents
were responsible for the eventual impairment of gluconeogenesis and development of clinical ketosis. Results of the current study provide indirect support for their conclusion because liver composition and function were not altered by feed restriction or butanediol alone, and ketosis did not develop.

In conclusion, our results support the hypothesis that normally neither feed restriction nor dietary butanediol as individual treatments will induce fatty liver and/or ketosis in high-producing dairy cows. It seems that the combination of feed restriction and a source of ketone bodies (butanediol) in early lactation may act by providing the two factors stated by Bergman (1971) to be prerequisites for hyperketonemia: a "primary factor" of increased mobilization of NEFA and an "hepatic factor" of increased degree of ketogenesis. Future experiments using the feed restriction plus butanediol model should help to define the nature of these "hepatic factors" and the interactions among mobilization of NEFA, carbohydrate insufficiency, and increased ketogenesis.
ACKNOWLEDGMENTS

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REFERENCES


SECTION II. METABOLIC CHANGES IN LIVER AND BLOOD DURING AN INDUCED SUBCLINICAL KETOSIS IN DAIRY COWS DURING EARLY LACTATION
ABSTRACT

The objective was to characterize changes in lipid metabolism in cows subjected to a protocol shown to induce a fatty liver and ketosis. From d 14 to 42 postpartum, 13 Holstein cows were in either a control group or a group restricted to 80% of ad libitum intake and fed 1,3-butanediol at 7% of dry matter. Six ketosis-induction cows developed a subclinical ketosis, but the other one developed clinical ketosis after only 9 d, and her data are not included. Milk and solids-corrected milk were less, but fat content was greater for ketosis-induction cows. Energy balance reached a low of -6.6 Mcal/d during week 1, whereas control cows reached equilibrium at week 2. Concentrations of nonesterified fatty acids in plasma and 3-hydroxybutyrate in whole blood were increased. Glucose, acetate, insulin, and glucagon in plasma and cholesterol and triglyceride in serum were similar between groups. Concentrations of dextran sulfate-precipitable cholesterol and triglyceride in serum were increased during week 1 of ketosis induction but were similar to controls at other times. Glycogen, total lipid, triglyceride, and total cholesterol in liver increased slightly during treatment. Oxidation of palmitate to CO₂ was greater at week 1 in liver slices from ketosis-induced cows, whereas conversion to total acid-soluble metabolites remained constant but decreased for controls. Our subclinically ketotic cows had lower ratios of triglyceride to glycogen in liver during pretreatment compared to cows that became clinically ketotic in earlier studies (.5 vs. >2.3). Therefore, susceptibility to fatty liver and clinical ketosis may be indicated by ratios of hepatic triglyceride to glycogen greater than 2.0.
INTRODUCTION

Fatty liver and ketosis are two related metabolic disorders that affect dairy cows during early lactation. Although many studies have characterized metabolic changes in spontaneously ketotic and fasted cows (Schultz, 1988), underlying biochemical causes still are unknown. A major problem in using field cases to study these disorders is that changes during development are missed because cows are referred for study at diagnosis. By this time, the disorders are established and possibly complicated by decreased feed intake or other postpartum diseases. Fasting has been used to produce a model for fatty liver and ketosis (Baird et al., 1979; Brumby et al., 1975; Herdt et al., 1983; Reid et al., 1979), but differences exist between fasted cows and those with spontaneous ketosis or fatty liver (Ballard et al., 1968; Herdt et al., 1983). Changes occur over such a short time in fasted cows that limitations on frequency of repeated sampling of liver or adipose tissue may hamper detection of transient metabolic changes.

A protocol developed and modified in our laboratory produced a fatty liver and ketosis over a period of about 4 wk (Mills et al. 1986a; Veenhuizen et al., 1989a), which allowed study of progressive development of the disorders by use of repeated samples of blood, liver, and adipose tissue from the same animals. The protocol involves moderate feed restriction plus feeding a ketone precursor (1,3-butanediol). Some metabolic changes occurring in liver (Mills et al., 1986a,b; Veenhuizen et al., 1989a,b) and adipose tissue (Mills et al., 1986b) during development of fatty liver and ketosis have been quantitated.
Hepatic metabolism of butyrate and stearate during development of induced ketosis was investigated (Mills et al., 1986b). Ketogenesis tended to decrease, whereas oxidation of stearate to CO₂ increased at ketosis. Stearate, however, is poorly utilized by ruminant liver (Thompson et al., 1975). This fact, coupled with the lack of control animals in the original experiment (Mills et al., 1986b), made it pertinent to re-examine in vitro fatty acid oxidation during development of induced fatty liver and ketosis by using palmitate, which is utilized to a much greater extent by ruminant liver (Jesse et al., 1986a,b; Thompson et al., 1975).

The objectives of our experiment were, first, to characterize changes in milk composition, energy balance, concentrations of plasma insulin and glucagon, concentrations of serum lipoproteins, and hepatic lipid composition that occur during the feed restriction-plus-dietary 1,3-butanediol (FRBD) protocol for inducing fatty liver and ketosis, and second, to measure in vitro oxidation of palmitate by liver slices from cows subjected to the FRBD protocol.
MATERIALS AND METHODS

Treatments, Design of Experiment, and Management of Cows

Thirteen multiparous Holstein cows were assigned alternately at calving to two treatment groups, designated "control" or "FRBD". Milk production in the previous lactation (305-d mature equivalent) was 8,763 kg (+358 kg over herdmates). All cows were fed a mixed silage and grain diet (Table 1) for ad libitum intake from parturition through d 13 postpartum, and control cows continued to be fed ad libitum through d 56 postpartum. Intake for FRBD cows was restricted at d 14 to 80% of the average intake from d 9 to 13; 1,3-butanediol¹ (butanediol) was included at 7% of ration DM. Average daily intake of butanediol was 890 ± 175 g. Cows in group FRBD continued the feed restriction-plus-butanediol protocol through d 42 postpartum, at which time butanediol was discontinued and cows were returned to ad libitum intake. The period from d 9 through d 13 postpartum (wk 0) is referred to as the pretreatment period, d 14 through 42 (wk 1 to 4) as the treatment period, and d 43 through d 56 (wk 5 to 6) as the recovery period.

Cows were housed in a tie-stall barn and milked twice daily. The mixed ration and 1.6 kg of alfalfa hay (Table 1) were fed individually to each cow twice daily, with ors removed and weighed once daily. Butanediol was introduced by feeding one-half of the final daily amount for 3 d and the full amount starting on d 4. Cows were weighed three times weekly. Development of ketonemia was monitored by use of urine

¹The 1,3-butanediol was a gift from Celanese Chemical Co., Dallas, TX, through the courtesy of Mr. Ken Jones.
TABLE 1. Composition and analysis of silage and grain mixture and alfalfa hay fed to cows

<table>
<thead>
<tr>
<th>Component</th>
<th>Percentage of DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silage and grain mixture¹</td>
<td></td>
</tr>
<tr>
<td>Alfalfa haylage</td>
<td>5.1</td>
</tr>
<tr>
<td>Corn silage</td>
<td>33.2</td>
</tr>
<tr>
<td>Cracked corn</td>
<td>51.0</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>9.2</td>
</tr>
<tr>
<td>Vitamin-mineral supplement²</td>
<td>1.5</td>
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<tr>
<td>TOTAL</td>
<td>100.0</td>
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Analysis:

<table>
<thead>
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<th>Silage and grain mixture</th>
<th></th>
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<tbody>
<tr>
<td>Acid detergent fiber</td>
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<td>Crude protein</td>
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</table>

<table>
<thead>
<tr>
<th>Alfalfa hay³</th>
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</thead>
<tbody>
<tr>
<td>Acid detergent fiber</td>
<td>38.0</td>
</tr>
<tr>
<td>Crude protein</td>
<td>19.8</td>
</tr>
</tbody>
</table>

¹Silage and grain mixture was a combination of the ISU herd silage mixture (79%, as fed) and concentrate mixture (21%, as fed) and was 63.7% DM.

²Consisted of sodium bicarbonate (20.9%), magnesium oxide (10.5%), dicalcium phosphate (26.2%), calcium carbonate (15.7%), sodium chloride (20.9%), trace mineral premix (5.2%) and Vitamin A and D premix (.6%).

³Fed separately from silage and grain mixture.
ketone test strips

**Sampling Procedures**

Puncture biopsies (Hughes, 1962) of 5 to 6 g of liver were obtained under local lidocaine anesthesia (Veenhuizen et al., 1989a) before the AM feeding on each of six days for each cow: d 13 (end of pretreatment), 21, 28, 35, 42, and 56 (recovery). A 1 g piece of liver was frozen and stored at -20° C until used for determination of liver composition. Another piece (.2 g) was stored in 10% buffered formalin until prepared for histological observation (Dr. L. D. Miller, ISU Department of Veterinary Pathology). The remaining liver was placed into ice-cold phosphate-buffered saline and used within 1.5 h for in vitro metabolic studies.

Weekly samples of jugular venous blood (32 ml) were obtained from each cow on the day before liver biopsy. Serum obtained from 12 ml of blood by using commercial serum separator tubes was frozen at -20° C. The remaining 20 ml of blood was divided between two heparinized tubes, one of which contained 150 ul 4% NaF to inhibit glycolysis. Two ml of whole blood without NaF were deproteinized immediately by adding 2 ml of ice-cold 10% (w/v) perchloric acid while vortexing the tube. After centrifugation, the supernatant was frozen at -20° C until analyzed. Plasma harvested by centrifugation from the remaining blood was aliquoted and stored at -20° C.

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2Keto-Diastix®, Ames Division, Miles Laboratories, Inc., Elkhart, IN.

3Corvac®, Monoject Scientific, Sherwood Medical, St. Louis, MO.
Incubation Procedures

Incubations were essentially as described by Mills et al. (1986b). Liver slices (Stadie and Riggs, 1944) were blotted, weighed, and placed into 25-ml flasks that contained 3 ml of calcium-free Krebs-Henseleit bicarbonate buffer at pH 7.4 (Lasser, 1961). Sodium palmitate (2 mM) and .5 uCi of [1-14C]-palmitic acid4 were complexed with fatty acid-poor bovine serum albumin (BSA)5. Final concentration of BSA was 4% (w/v), giving a molar ratio of palmitate to BSA of 4:1.

Flasks were gassed for 15 sec with 95:5 O2:CO2 and incubated in triplicate for 2 h at 37° C with constant shaking in a Dubnoff metabolic shaker. Incubations were terminated by injecting 3 ml of 10% (w/v) perchloric acid into the incubation medium. Radioactive CO2 was trapped by using NaOH and quantified (Mills et al., 1986b; Veenhuizen et al., 1989b). Carryover of radioactive substrate through the analytical procedures was measured in zero-time flasks acidified immediately after addition of the liver slice.

Analytical Procedures

After CO2 was trapped, flasks were allowed to cool, and contents were transferred to polyethylene tubes. Tubes were centrifuged at 2000 x g for 10 min, and 2 ml of supernatant were transferred to glass tubes and neutralized with 2 M K2CO3. After centrifugation at 2500 x g for 10 min,

4ICN Biomedicals, Inc., Costa Mesa, CA.

5Item no. 7030, Sigma Chemical Co., St. Louis, MO.
.5 ml of the second supernatant was transferred to a scintillation vial, 10 ml of scintillation cocktail were added, and $^{14}$C incorporated into total acid-soluble metabolites (ASM) was determined. The ASM fraction consists mainly of ketone bodies and acetate, which are products of incomplete oxidation of long-chain fatty acids, but it also includes dicarboxylic acids and amino acids that become labeled in the citric acid cycle (Jesse et al., 1986a). Thus, ASM provides a more complete estimate of total fatty acid oxidation than measuring only $\text{CO}_2$ production or ketogenesis.

Concentrations of glycogen and glucose in liver were determined (Keppler and Decker, 1974). Lipid was extracted from liver tissue with 2:1 (v:v) chloroform-methanol, and concentration of total lipid was determined gravimetrically after drying under moving air, which was determined to be equivalent to drying under $\text{N}_2$ for subsequent analyses. The lipid extract was redissolved in 3:2 (v:v) hexane-isopropanol, and concentrations of triglyceride (Drackley et al., 1989a) and total cholesterol were determined. A portion of the extract was dried and redissolved in chloroform, and concentration of total phospholipid was determined (Stewart, 1980) from a standard curve prepared from a combination of bovine phosphatidyl choline and phosphatidyl ethanolamine.

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6Ready Solv EP, Beckman Instruments, Inc., Fullerton, CA.

7Sigma total cholesterol kit no. 352, Sigma Chemical Co., St. Louis, MO.

8Phosphatidyl choline (no. 7763) and phosphatidyl ethanolamine (no. 2018), both prepared from bovine liver, from Sigma Chemical Co., St. Louis, MO.
Concentration of BHBA (Williamson and Mellanby, 1974) was determined in perchloric acid filtrates, which were neutralized with 2 M K₂CO₃. Plasma was analyzed for concentrations of glucose⁹, acetate (Knowles et al., 1974), and nonesterified fatty acids (NEFA) (Drackley et al., 1989a). Concentrations of insulin and glucagon in plasma were determined by validated radioimmunoassays (Amaral, 1988). Plasma for glucagon was stored with 2500 IU of aprotinin¹⁰ per ml. All samples for each hormone were assayed together, with intraassay coefficients of variation of 11 and 5% for insulin and glucagon, respectively.

To estimate concentration of triglyceride-rich lipoproteins, serum was thawed and subjected to dextran sulfate¹¹ precipitation (Herdt et al., 1983), with amounts of serum and reagents halved (Gerloff et al., 1986). Concentrations of cholesterol⁸ and triglyceride¹² were determined in the precipitate, supernatant, and total serum. Preliminary experiments showed that freezing serum did not change distribution of either triglyceride or cholesterol among the fractions analyzed.

Samples of feeds obtained monthly were dried for 48 h at 60°C in a forced-air oven for determination of DM, and then were ground, composited

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⁹Sigma glucose (Trinder) kit no. 315-500, Sigma Chemical Co., St. Louis, MO.

¹⁰Trasylol®, Mobay Chemical Corp., FBA Pharmaceuticals, New York, NY.

¹¹Dextran sulfate, molecular weight 5000, Sigma Chemical Co., St. Louis, MO.

¹²Sigma triglyceride kit no. 339, Sigma Chemical Co., St. Louis, MO.
for the whole experiment, and analyzed for contents of crude protein (AOAC, 1980) and acid detergent fiber (Goering and Van Soest, 1970). Milk samples taken from four consecutive milkings each week were analyzed for concentrations of fat, protein, and total solids by using an infrared analyzer\(^{13}\).

Presentation of Data

Metabolic rates and composition of liver were expressed as a function of wet weight of liver. This is justified because changes in metabolic rates observed in liver slices from ketotic cows with fatty liver were similar when expressed either on the basis of wet weight or per unit of DNA (Mills et al. 1986b). Other workers (Collins and Reid, 1980; Jesse et al., 1986b; Mehnert, 1986) showed that protein content of liver changed only slightly in comparison with changes in lipid over the range of hepatic lipid concentrations observed in our experiment.

Statistical Analyses

One cow assigned to FRBD quickly developed a severe clinical ketosis only 9 d after initiating FRBD, which is much sooner than expected from earlier studies (Mills et al., 1986a; Veenhuizen et al., 1989a). Because response to FRBD by this cow differed markedly from that of the other six cows, her data were excluded from statistical analysis but are discussed later for comparison. Statistical analyses, therefore, were conducted by

\(^{13}\)Milk-O-Scan 203, Foss Food Technology, Eden Prairie, MN.
using 6 cows per treatment.

All repeated measurements data were subjected to analysis of variance for a split-plot design (Gill, 1986) by using the General Linear Models (GLM) package of SAS (1982). Dietary treatment (control vs. FRBD) was the main plot, and time (week of sampling) was the subplot. Conservative degrees of freedom (Geisser and Greenhouse, 1958) were used to evaluate F-tests containing time as a factor. Use of conservative degrees of freedom, which are obtained by dividing degrees of freedom for both terms of the F-test by degrees of freedom for time, compensates for lack of randomization of the subplots when using a split-plot design to analyze repeated-measures data (Geisser and Greenhouse, 1958). Conditional comparisons of treatments at each week were made by using t-tests, with approximate degrees of freedom calculated by Satterwaite's procedure (Gill, 1986). Standard errors were calculated by dividing the appropriate error term by the harmonic mean of number of observations per treatment group.

Because some cows were not sampled every week, least squares means are presented throughout. Tests of significance with $P \leq 0.05$ were considered statistically significant.
RESULTS AND DISCUSSION

To our initial surprise, only one cow out of seven assigned to FRBD developed fatty liver and clinical ketosis, which is a much lower rate of induction of fatty liver and ketosis than in two earlier studies (Mills et al., 1986a; Veenhuizen et al., 1989a). The other 6 cows of the present study did, however, develop ketonemia and symptoms of subclinical ketosis. Careful analysis and interpretation of the data, in our opinion, still provides important information on why some cows are susceptible to clinical ketosis whereas others are not. It is in this light that we present and discuss the results.

Milk Production and Composition

Average production of milk by FRBD cows was less than that of control cows throughout the experiment (P<.03). Milk production by FRBD cows began to decrease during wk 1 (Figure 1a), and was significantly less than that of controls by wk 2. Pretreatment milk production by FRBD cows was less than that of cows induced into ketosis by FRBD in earlier studies (Mills et al., 1986a; Veenhuizen et al., 1989a). Production of milk fat was not different between groups, but productions of protein and total solids were less for FRBD during the treatment period (not shown).

The tendency of FRBD cows to maintain fat yield, despite a decreased milk yield, was attributable to a greater (P<.05) content of fat in milk compared with that of control cows (Figure 1b). Milk fat content is usually increased during subclinical and clinical ketosis (Schultz, 1988). Contents of protein and total solids were not different between groups.
Figure 1. Average daily production of milk, fat content, and production of solids-corrected milk (SCM) in control cows or cows subjected to feed restriction plus dietary butanediol (FRBD) during weeks 1 through 4 (d 14 to 42 postpartum)

Asterisks denote significant differences (P<.05) between treatments at that week.

A. Daily milk production (standard error of the difference (SED) between treatment means at any week = 2.3). Probabilities of greater F for effects in model: treatment, P=.03; week, P=.17; treatment x week, P=.03.

B. Milk fat content (SED = .34). Treatment, P=.22; week, P=.13; treatment x week, P=.20.

C. Daily SCM production (SED = 2.6). Treatment, P=.18; week, P=.66; treatment x week, P=.09.
Production of SCM (Figure 1c) was less from FRBD cows than from control cows during treatment and recovery periods.

Intakes, Change in Body Weight, and Energy Balance

Average daily intake of DM by FRBD cows during the treatment period was 69% of that of controls (15.3 vs. 22.2 kg). As seen in Figure 2a, DM consumption by FRBD cows averaged about 2.8% of body weight during wk 1 to 4, whereas control cows increased intake of DM from 3.4 to nearly 4.6% of body weight. By the second week of recovery, FRBD cows increased their intakes to values similar to controls.

Cows in group FRBD lost body weight at an average rate of 1.8 kg/d during treatment, reaching a cumulative weight loss of 52 kg by wk 4 (Figure 2b). Control cows, however, began regaining body weight by wk 3, coinciding with attainment of energy balance and had returned to pretreatment body weights by wk 5 of experiment. Weight loss by our FRBD cows was not as extreme as the 3.1 kg/d observed in cows that were induced into ketosis by FRBD in an earlier study (Mills et al., 1986a), but was similar to the 1.6 kg/d for a cow that resisted induction into ketosis (Mills et al., 1986a) and to the weight loss of 2.0 kg/d for cows induced into ketosis by FRBD in another experiment (Veenhuizen et al., 1989a).

Calculated intake of net energy of lactation ($NE^L$) by FRBD cows averaged 73% of that of control cows during the treatment period (26.2 vs. 35.9 Mcal/d). For FRBD cows, intake was 80% of the $NE^L$ requirement calculated at wk 0 (National Research Council, 1978); the comparable value for control cows was 93% of $NE^L$ requirement. Calculated net energy
Figure 2. Average daily intake of dry matter (DM) as a percentage of body weight (BW), cumulative body weight change (week 0 as reference), and energy balance of control cows or cows subjected to feed restriction plus dietary butanediol (FRBD) during weeks 1 through 4 (d 14 to 42 postpartum)

Asterisks denote significant differences (P<.05) between treatments at that week.

A. DM intake, % of BW (standard error of the difference (SED) between treatment means at any week = .3).
   Probabilities of greater F for effects in model: treatment, P=.02; week, P=.66; treatment x week, P=.09.
B. Cumulative weight change (SED = 13.7). Treatment, P=.02; week, P=.04; treatment x week, P=.08.
C. Energy balance (SED = 2.3). Treatment, P=.02; week, P=.006; treatment x week, P=.04.
balance (Figure 2c) was less for FRBD cows than control cows from wk 1 through 5, and was most negative during wk 1 (-6.6 Mcal/d). Control cows reached energy balance during wk 2 (4 wk postpartum), which is similar to results of another recent study (R. O. Harrison, J. W. Young, and A. E. Freeman, Department of Animal Science, Iowa State University, unpublished data) but earlier in lactation than summarized elsewhere (Bauman and Currie, 1980). Crude protein content of the total diet (14.5% of DM) was quite low for cows in early lactation (National Research Council, 1978), and may have decreased the extent of negative energy balance by limiting milk production. Orskov et al. (1977) reported increases in degree of energy deficit when cows that were fed a protein-deficient diet were supplemented with abomasal infusions of casein.

Feed intakes and milk composition were not measured earlier when ketosis was induced (Mills et al., 1986a; Veenhuizen et al., 1989a), and energy balances cannot be calculated. It can be estimated that FRBD cows in one study (Veenhuizen et al., 1989a) reached a net energy deficit of at least 9.7 Mcal/d. It seems, therefore, that earlier FRBD cows were in greater energy deficit during treatment than were our FRBD cows. Fisher et al. (1971) concluded that cows must be in negative energy balance in the range of 7.2 to 10.8 Mcal/d before signs of clinical ketosis are seen. Our results and estimations of energy balance in the study of Veenhuizen et al. (1989a) tend to support this conclusion. Other factors evidently are involved, however, because two of our FRBD cows with energy balances of -8 and -13 Mcal/d became ketonemic but did not develop fatty livers or clinical ketosis.
Figure 3. Concentrations of metabolites in plasma from control cows or cows subjected to feed restriction plus dietary butanediol (FRBD) during weeks 1 through 4 (d 14 to 42 postpartum)

Asterisks denote significant differences (P<.05) between treatments at that week.

A. Glucose (standard error of the difference (SED) between treatment means at any week = 6.8). Probabilities of greater F for effects in model: treatment, P=.50; week, P=.13; treatment x week, P=.56.

B. Nonesterified fatty acids (SED = 165). Treatment, P=.27; week, P=.12; treatment x week, P=.21.

C. Acetate (SED = 85). Treatment, P=.12; week, P=.22; treatment x week, P=.47.

D. B-Hydroxybutyrate (SED = 2.3). Treatment, P=.07; week, P=.08; treatment x week, P=.05.
Metabolites and Hormones in Blood

Concentration of glucose in plasma was not significantly different between treatment groups (Figure 3a), whereas NEFA were greater (P<.05) at wk 2 in FRBD cows than in controls (Figure 3b). Acetate in plasma (Figure 3c) was similar between groups during the treatment period but was higher (P<.05) in controls at wk 5 during recovery, which was attributable to a consistent but unexplainable increase in that group. Concentration of BHBA in whole blood was increased (P<.05) throughout the 4 wk treatment period (Figure 3d). Metabolite concentrations in FRBD cows were typical of subclinical ketosis rather than of the more dramatic changes characteristic of clinical ketosis that were observed previously (Mills et al., 1986a; Veenhuizen et al., 1989b).

Insulin in plasma was similar between groups during the treatment period (Figure 4a), but was increased (P<.03) in FRBD cows compared with controls during the recovery period (wk 5-6), during which time FRBD cows seemed to assign a higher metabolic priority to replenishment of body stores than to milk production. Concentration of glucagon in plasma was similar between groups throughout the experiment (Figure 4b). The changes in insulin also caused the insulin-to-glucagon ratio to be increased during recovery (Figure 4c).

For FRBD cows to maintain a concentration of insulin similar to that of controls during the treatment period was unexpected because of the greater negative energy balance of FRBD cows (Figure 2c). Tendencies for an increased concentration of insulin in cows fed diets containing butanediol at ad libitum intake have been observed (Drackley et al.,
Figure 4. Concentrations of hormones in plasma from control cows or cows subjected to feed restriction plus dietary butanediol (FRBD) during weeks 1 through 4 (d 14 to 42 postpartum)

Asterisks denote significant differences (P<.05) between treatments at that week.

A. Insulin (standard error of the difference (SED) between treatment means at any week = 61). Probabilities of greater F for effects in model: treatment, P=.24; week, P=.10; treatment x week, P=.31.

B. Glucagon (SED = 13). Treatment, P=.71; week, P=.23; treatment x week, P=.30.

C. Molar ratio of insulin to glucagon (SED = .35). Treatment, P=.39; week, P=.20; treatment x week, P=.32.
1989a), but acute administration of butanediol to lactating goats did not stimulate increases in insulin (Drackley et al. 1989b). Earlier, acetate concentration increased greatly in cows made ketotic by FRBD (Veenhuizen et al., 1989a). Because acetate utilization is insulin-dependent, (Schultz, 1988), concentration of insulin or the insulin-to-glucagon ratio may have been lower in the earlier cows (Veenhuizen et al., 1989a).

Concentrations of total cholesterol and triglyceride in serum were unaffected by treatment (Figures 5a and b), although triglyceride tended to be higher in FRBD cows early in the treatment period and then again during recovery. Circulating triglycerides usually are decreased in cows with spontaneous ketosis (Schultz, 1988). Dextran sulfate-precipitable (DSP)-cholesterol and DSP-triglyceride (Figures 5c and d) were greater (P<.05) in FRBD cows during wk 1 but were similar to controls from wk 2 through 6.

In nonruminant species, the DSP fraction consists of the triglyceride-rich lipoproteins (very-low-density and low-density lipoproteins) (Lee, 1976), and the procedure has been used to quantify bovine lipoproteins (Gerloff et al.; 1986: Herdt et al., 1983). Polyacrylamide gel electrophoresis (Naito et al., 1973) of the DSP fraction in the present study showed no contamination with high-density lipoproteins (data not shown). The supernatant contained predominantly high-density lipoproteins, but also contained significant amounts of unprecipitated very-low-density lipoproteins (VLDL). This agrees with a recent report (Simo et al., 1988) of incomplete precipitation of
Figure 5. Concentrations of total and dextran sulfate precipitable-(DSP-) cholesterol and triglyceride in serum from control cows or cows subjected to feed restriction plus dietary butanediol (FRBD) during weeks 1 through 4 (d 14 to 42 postpartum)

Asterisks denote significant differences (P<.05) between treatments at that week.

A. Total cholesterol (standard error of the difference (SED) between treatment means at any week = 21).
Probabilities of greater F for effects in model: treatment, P=.54; week, P=.003; treatment x week, P=.67.

B. Total triglyceride (SED = 3.4). Treatment, P=.43; week, P=.22; treatment x week, P=.25.

C. DSP-cholesterol (SED = 3.4). Treatment, P=.42; week, P=.02; treatment x week, P=.33.

D. DSP-triglyceride (SED = 2.3). Treatment, P=.56; week, P=.29; treatment x week, P=.18.
triglyceride-rich lipoproteins, which results in underestimation of triglyceride-rich lipoproteins and overestimation of triglyceride in non-DSP fractions. Because cholesterol is in relatively small quantities in VLDL compared with triglyceride, errors in determination of cholesterol distribution are much less. Nevertheless, the procedure is fast and in the bovine yields a fraction that correlates with changes in physiological state (Gerloff et al., 1986; Herdt et al., 1983).

Incorporation of plasma NEFA into plasma triglycerides is less in cows than nonruminants (Pullen et al., 1989), and secretion of VLDL from sheep liver (Herdt et al., 1988) and goat hepatocytes (Kleppe et al., 1988) was shown to be minimal. Hepatic secretion of VLDL may decrease further during development of fatty liver (Reid et al., 1979), although few data are available. Gerloff et al. (1986) reported that serum DSP-cholesterol was decreased early postpartum and was correlated negatively with hepatic triglyceride. Cows with fatty liver had decreased DSP-triglyceride and DSP-cholesterol compared with normal cows (Herdt et al., 1983), whereas fasted lactating cows had increased DSP-lipids. In contrast, Brumby et al. (1975) reported decreased DSP-cholesterol esters in fasted lactating cows.

Increases in DSP-cholesterol and DSP-triglyceride in our FRBD cows during wk 1 could indicate 1) increased synthesis and secretion of VLDL by the liver, 2) decreased clearance of VLDL by peripheral tissues, 3) decreased clearance of low-density lipoprotein (LDL) by liver, or 4) altered physiochemical characteristics of the lipoproteins resulting in different precipitation characteristics. The mechanism(s) responsible for
and the physiological significance of the short-lived increases in DSP-lipids cannot be determined from available data. Detailed study of lipoprotein metabolism in cows subjected to FRBD is needed.

Composition of Liver

Concentration of glycogen in liver (Figure 6a) was less (P<.05) in FRBD cows during the pretreatment period but increased steadily during the experiment, whereas concentration of liver glycogen in control cows changed much less. The increasing concentration of glycogen during FRBD treatment contrasts with results of previous experiments (Mills et al., 1986a; Veenhuizen et al., 1989a) in which glycogen content of liver decreased steadily during FRBD until becoming almost depleted at least 1 wk before diagnosis of clinical ketosis.

Concentrations of total lipid (not shown) and triglyceride (Figure 6b) were greater in control cows during pretreatment and decreased steadily throughout the experiment. Liver triglyceride content in FRBD cows increased during treatment and then decreased to control values during recovery, but increases in liver triglyceride in FRBD cows were much less than those observed in earlier experiments (Mills et al., 1986a; Veenhuizen et al., 1989a).

Concentrations of total cholesterol and phospholipid in liver were not different between groups during either treatment or recovery periods, but concentration of total cholesterol was greater (P<.05) in controls during pretreatment (Figure 6c). Concentration of total cholesterol paralleled concentrations of total lipid and triglyceride in both groups,
Figure 6. Composition of liver (percent of wet weight) from control cows or cows subjected to feed restriction plus dietary butanediol (FRBD) during weeks 1 through 4 (d 14 to 42 postpartum)

Asterisks denote significant differences (P<.05) between treatments at that week.

A. Glycogen (standard error of the difference (SED) between treatment means at any week = .8). Probabilities of greater F for effects in model: treatment, P=.46; week, P=.31; treatment x week, P=.29.

B. Triglyceride (SED = .9). Treatment, P=.57; week, P=.15; treatment x week, P=.15.

C. Total cholesterol (SED = .14). Treatment, P=.25; week, P=.56; treatment x week, P=.25.

D. Phospholipid (SED = .2). Treatment, P=.67; week, P=.04; treatment x week, P=.38.
whereas concentration of phospholipid increased in both groups during the experiment (Figure 6d). Modest increases in cholesterol esters in fatty liver have been noted in some studies (Brumby et al., 1975; Collins and Reid, 1980) but not in others (Fronk et al., 1980), whereas content of phospholipid was unchanged (Brumby et al., 1975; Collins and Reid, 1980) or decreased (Fronk et al., 1980).

In Vitro Hepatic Oxidation of Palmitate

Rates of oxidation of palmitate to CO2 were linear for 3 h, whereas rates to ASM were somewhat greater during the first 30 min of incubation; they then remained linear but at a slightly lower rate for the next 2.5 h (data not shown). Similar results have been reported (Jesse et al., 1986a).

Oxidation of palmitate to CO2 was significantly greater (P<.05) in liver slices from FRBD cows at wk 1 (Figure 7a). Explanation of the early increase in CO2 production in FRBD cows is complicated by variability in rates from both groups but could indicate increased oxidation of palmitate to meet energy demands of the liver when feed restriction decreased supply of other fuels such as propionate or acetate. Oxidation of palmitate to ASM by liver slices from control cows tended to decrease throughout the experiment (Figure 7b), whereas oxidation to ASM remained relatively constant for FRBD cows. Rates were significantly greater (P<.05) for FRBD cows at wk 4. The decrease in control cows is similar to results of an earlier study (Aiello et al., 1984) in which hepatic ketogenesis in vitro decreased from d 30 to d 60 of lactation.
Figure 7. Conversion of palmitate to carbon dioxide or total acid-soluble metabolites (ASM) by liver slices from control cows or cows subjected to feed restriction plus dietary butanediol (FRBD) during weeks 1 through 4 (d 14 to 42 postpartum).

Asterisks denote significant differences (P<.05) between treatments at that week.

A. Palmitate to carbon dioxide (standard error of the difference (SED) between treatment means at any week = 6.4). Probabilities of greater F for effects in model: treatment, P=.24; week, P=.39; treatment x week, P=.34.

B. Palmitate to ASM (SED = 35). Treatment, P=.28; week, P=.39; treatment x week, P=.34.
The tendency of FRBD cows to maintain rates of palmitate oxidation to ASM rather than to decrease oxidation as in controls may reflect intrahepatic changes in response to greater negative energy balance and increased availability of NEFA. Oxidation of NEFA in ruminants evidently is controlled by carnitine palmitoyltransferase I (CPT I), which is similar to nonruminants (Aiello et al., 1984; Jesse et al., 1986a). It has been suggested (Butler et al., 1988) that in ruminants, concentrations of effectors of CPT I activity such as carnitine, malonyl-Coenzyme A (malonyl-CoA), cytosolic methylmalonyl-CoA, or other controlling metabolites (Jesse et al., 1986a,b) are more important than is induction of CPT I enzyme activity in controlling oxidation of NEFA. Few data are available concerning regulation of CPT I in lactating cows during negative energy balance or development of ketonemia.

Comparison with the Clinically Ketotic Cow

As already mentioned, one cow (No. 463) assigned to FRBD became clinically ketotic very quickly, which was a different response than the other six cows assigned to FRBD. It seems instructive to compare results from No.463 with the other six FRBD cows and with ketotic cows in earlier experiments (Mills et al., 1986a,b; Veenhuizen et al., 1989a,b).

Cow No. 463 had developed milk fever at calving and was off-feed for 3 d. By the time pretreatment samples of blood and liver were taken at d 13, she had a moderate fatty liver (Table 2) but seemed healthy, was producing 34.5 kg/d of milk, and had concentrations of BHBA, glucose, and NEFA in blood that were within normal ranges (Table 2). When clinically
TABLE 2. Concentrations of metabolites in blood and liver and in vitro oxidation of palmitate in cow 463, who was induced into clinical ketosis by feed restriction plus dietary 1,3-butanediol (FRBD)

<table>
<thead>
<tr>
<th>Item</th>
<th>Pretreatment (d 13)</th>
<th>Ketosis (d 22)</th>
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</thead>
<tbody>
<tr>
<td>Whole blood</td>
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<tr>
<td>BHBA$^1$, mg/dl</td>
<td>2.7</td>
<td>38.7</td>
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<tr>
<td>Plasma</td>
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<tr>
<td>Glucose, mg/dl</td>
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<td>NEFA$^2$, uequiv/l</td>
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<tr>
<td>Liver</td>
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<tr>
<td>Glycogen, %$^3$</td>
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</tr>
<tr>
<td>Glucose, %</td>
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</tr>
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<td>Total lipid, %</td>
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<tr>
<td>Total cholesterol, %</td>
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<td>Phospholipid, %</td>
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<td>1.3</td>
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<tr>
<td>In vitro metabolism of propionate$^4$</td>
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<td>Glucose, umol/h x g</td>
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<td>In vitro oxidation of palmitate$^5$</td>
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<td>$CO_2$, nmol/h x g</td>
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<tr>
<td>ASM, nmol/h x g</td>
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<td>238</td>
</tr>
</tbody>
</table>

$^1$β-Hydroxybutyrate.

$^2$Nonesterified fatty acids.

$^3$Percent of wet weight.

$^4$Production of $CO_2$ and glucose during 2 h incubations (Drackley et al., 1989a), expressed as micromoles of propionate converted to product per h per g tissue wet weight.

$^5$Production of $CO_2$ and acid-soluble metabolites (ASM) during 2 h incubations, expressed as nanomoles of palmitate converted to product per h per g tissue wet weight.
ketotic 9 d later, she had concentrations of metabolites in blood and liver similar to those of ketotic cows reported earlier (Mills et al., 1986a; Veenhuizen et al., 1989a). Gluconeogenic capacity of liver slices was decreased by 84% at ketosis (Table 2), similar to previous results (Mills et al., 1986b; Veenhuizen et al., 1989b). In contrast, limited measurement of in vitro gluconeogenic capacity of liver slices from two of the subclinically-ketotic cows showed no differences from controls (not shown). Oxidation of palmitate to CO₂ by liver slices (Table 2) was increased over five fold, and oxidation to ASM was increased by 26%. Mills et al. (1986b) observed decreased ketogenesis and increased production of CO₂ from stearate by liver slices from ketotic cows, but utilization of stearate by ruminant liver is less than that of palmitate or oleate (Thompson et al., 1975).

The other six FRBD cows showed changes in metabolites in blood and liver that were characteristic of subclinical ketosis. Changes were much more modest than changes in No. 463 and in cows induced into clinical ketosis in earlier experiments (Mills et al., 1986a; Veenhuizen et al., 1989a).

**Ratio of Triglyceride to Glycogen in Liver**

Mehnert (1986) proposed that the ratio of lipid to glycogen in liver be used as an indicator of metabolic stress in dairy cows during early lactation, with greater ratios being associated with increased metabolic disorders. Table 3 shows calculated ratios of triglyceride to glycogen in liver from cows of the present and earlier experiments. The average liver
TABLE 3. Ratio of triglyceride to glycogen in liver biopsies from ketotic and nonketotic cows in the present and earlier experiments

<table>
<thead>
<tr>
<th>Experiment and group</th>
<th>n</th>
<th>Pretreatment (mean ratio)</th>
<th>Ketosis (mean ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mills et al. (1986a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ketotic</td>
<td>4</td>
<td>4.1</td>
<td>18.3</td>
</tr>
<tr>
<td>Nonketotic</td>
<td>1</td>
<td>.4</td>
<td>1.2</td>
</tr>
<tr>
<td>Veenhuizen et al. (1989a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ketotic</td>
<td>6</td>
<td>2.3</td>
<td>12.0</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>1.2</td>
<td>.7</td>
</tr>
<tr>
<td>Present study</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ketotic</td>
<td>1</td>
<td>3.0</td>
<td>25.4</td>
</tr>
<tr>
<td>Subclinically ketotic</td>
<td>6</td>
<td>.5</td>
<td>.4</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>.9</td>
<td>.3</td>
</tr>
</tbody>
</table>

1Biopsy of liver obtained before start of FRBD protocol, from 10 to 14 d postpartum.

2Biopsy of liver obtained at time of clinical ketosis or from 35 to 46 d postpartum.
triglyceride-to-glycogen ratio in the ketotic cows of Mills et al. (1986a) was 4.1 before initiating FRBD, and increased during ketosis induction to 18.3 at clinical ketosis. Ketotic cows of Veenhuizen et al. (1989a) had an average ratio of 2.3 before starting FRBD, which increased to over 90.0 1 wk before ketosis and was 12.0 at diagnosis of clinical ketosis. The ratio in their control cows decreased from 1.2 to .7 during the same time. Our subclinically-ketotic FRBD cows had an average triglyceride-to-glycogen ratio of only .5 before initiation of FRBD, and the ratio increased only to .6 during treatment before decreasing to less than .4 at wk 4 (d 42 postpartum). By that time, cows in the study of Veenhuizen et al. (1989a) were ketotic. The one cow in the present study that quickly developed fatty liver and ketosis had a pretreatment ratio of 3.0 and a ratio of 25.4 at ketosis. A weight ratio of triglyceride-to-glycogen in liver of greater than about 2.0 during the early postpartum period (before initiation of FRBD at d 14) seems to be a reasonable indicator of susceptibility to induction of fatty liver and clinical ketosis.
GENERAL DISCUSSION

The original objective of this experiment was to investigate metabolic changes in cows induced into fatty liver and ketosis by a protocol previously shown to generate such disorders (Mills et al., 1986a; Veenhuizen et al., 1989a). As discussed already, fatty liver and ketosis were not induced as expected. Much valuable information was gained, however, and our discussion will center on that information and the picture that is emerging about why cows do or do not become susceptible to fatty liver and ketosis in early lactation.

Cows that eventually became clinically ketotic in earlier studies showed greater negative energy balance, greater concentration of NEFA and lower concentration of glucose in plasma, greater concentration of triglyceride and lower concentration of glycogen in liver, and greater hepatic triglyceride-to-glycogen ratios in early lactation before initiation of FRBD. Such changes indicate that cows that were susceptible to fatty liver and ketosis were mobilizing more body tissue before FRBD was started than were cows that resisted induction of fatty liver and ketosis. Mills et al. (1986a,b) indicated a similar conclusion from the original experiment, but it seemed from results of Veenhuizen et al. (1989b) that normal cows in early lactation could be induced into fatty liver and ketosis by using FRBD.

A hypothesis for the sequence of metabolic changes that occur during development of fatty liver and ketosis has been presented (Veenhuizen et al., 1989b). Our experiment does not refute the hypothesis, but requires that it be modified to explain why susceptibility to induction of fatty
liver and ketosis is dependent on cows being in a greater state of tissue mobilization than the average healthy cow in early lactation. Greater mobilization of body tissue probably is a response to greater "homeorhetic" stimulus of milk production, which creates a demand for more nutrients than can be consumed in the diet (Bauman and Currie, 1980). In turn, homeorhetic signals probably are determined by interactions of genetics and nutrition during very early lactation (Bauman and Currie, 1980).

Although the protocol of the present experiment was designed to be similar to that of Veenhuizen et al. (1989a), several differences did occur that may have contributed to greater mobilization of body stores in the earlier study. First, as discussed already, protein content of the diet was less than the 16% protein diet fed earlier (Veenhuizen et al., 1989a). Greater dietary protein may have stimulated greater milk production and mobilization of body stores, in agreement with Orskov et al. (1977). Second, hay intake was estimated earlier (Veenhuizen et al., 1989a) and may have been overestimated, in which case cows would have been in even greater energy deficit. Third, average age of the cows was less in our study (42 months vs. 53 months at calving) than previously (Veenhuizen et al., 1989a). Older cows usually are higher producers and have a greater incidence of fatty liver and ketosis (Schultz, 1988). Fourth, cows in the earlier experiment (Veenhuizen et al., 1989a) may have had greater genetic potential for milk production. Ketotic cows in the earlier study produced 10,030 kg of milk (305-d mature equivalent) in the lactation preceding the experiment (+893 kg over herdmates), whereas our
FRBD cows averaged 8,665 kg (+218 kg). Fifth, cows in the earlier experiment (Veenhuizen et al., 1989a) had an additional liver biopsy taken 6 d postpartum. On the basis of our unpublished data (J. K. Drackley, R. O. Harrison, and J. W. Young, Department of Animal Science, Iowa State University), it seems that cows react more adversely to liver biopsy within the first week postpartum, often leading to brief decreases in feed intake and increases in concentrations of NEFA and BHBA in blood. It seems unlikely that any of the five differences per se would have rendered the FRBD protocol unsuccessful; rather, they may have contributed to a lesser reliance on body tissue to support milk production.

A greater triglyceride-to-glycogen ratio in liver of ketotic cows represents the combined effects of increased uptake of fatty acids and decreased content of glycogen, and may provide a better indicator of overall energy status than glycogen content alone. We observed significant Pearson correlation coefficients between that ratio and energy balance ($r = -0.39, P<0.002$), concentrations of glucose ($r = -0.34, P<0.005$), NEFA ($r = 0.51, P<0.0001$), and BHBA ($r = 0.61, P<0.0001$), and in vitro oxidation of palmitate to $\text{CO}_2$ ($r = 0.36, P<0.003$) and ASM ($r = 0.24, P<0.05$). Correlations were weaker when using either hepatic triglyceride or glycogen contents. Maintenance of hepatic glycogen in nonketotic FRBD cows in the present study was associated with normal plasma glucose concentrations, which in turn would prevent excessive mobilization of NEFA from adipose tissue. Because uptake of NEFA by liver is concentration-dependent (Schultz, 1988), less NEFA would be available for hepatic esterification and ketogenesis, which may be a primary factor determining
differences in response between studies.

Bergman (1971) stated that two factors were necessary for development of hyperketonemia: first, increased delivery of NEFA to liver ("primary factor"), and second, intrahepatic changes that increase the amount of NEFA directed to ketogenesis ("hepatic factor"). As already discussed, concentrations of NEFA in our FRBD cows generally were less than in ketotic cows of earlier studies (Mills et al., 1986a; Veenhuizen et al., 1989a) and may have been less than the "threshold" concentration above which ketogenesis increases. The threshold concentration has not been determined for lactating cows, but it is about 1.5 mM in sheep (Bergman, 1971). In two of our cows, however, NEFA reached concentrations similar to earlier ketotic cows (Mills et al., 1986a; Veenhuizen et al., 1989a) but they did not develop fatty livers or ketosis. This suggests that "hepatic factors" that increase ketogenesis may not have changed in our study; furthermore, these "hepatic factors" must somehow be dependent upon increased tissue mobilization early postpartum before FRBD is started.

The nature of the intrahepatic changes cannot be determined from available data, but there are at least four possibilities. The first is that butanediol or ketone bodies may potentiate increased mobilization and hepatic esterification of NEFA, which has been discussed (Drackley et al., 1989a). Second, hepatic capacity for triglyceride synthesis may increase. Although few data are available in lactating cows, activity of phosphatidate phosphohydrolase (PPase), which regulates the branch point between synthesis of phospholipids and triglycerides, is increased in rats by long-chain fatty acids (Cascales et al., 1984). Herdt et al. (1988)
measured an increase in hepatic activity of PPase during development of induced fatty liver in sheep. Cows with high rates of tissue mobilization may develop increased activity of PPase, which could allow greater rates of triglyceride development when NEFA are increased further during FRBD. Limited information suggests that increased deposition of triglyceride in liver then may lead to increased ketogenesis (Kleppe et al., 1988).

The third possibility is that concentrations of intermediates in the citric acid cycle are insufficient to maintain activity of the cycle. Depletion of hepatic glycogen in bovine liver is associated with depletion of glycolytic and citric acid cycle intermediates and is related inversely to ketogenesis (Baird et al., 1979). Ketogenesis may increase if insufficient intermediates are available to sustain a high rate of flux through the citric acid cycle (Baird et al., 1979).

Fourth, controls linking carbohydrate status with ketogenesis may activate ketogenesis to a greater degree in cows mobilizing body tissue at high rates. In nonruminants, content of glycogen in liver and ketogenesis are linked by concentration of malonyl-CoA, an intermediate in fatty acid synthesis that is a potent inhibitor of CPT I (McGarry and Foster, 1980). Malonyl-CoA was shown recently to be a regulator of fatty acid oxidation in sheep liver as well (Brindle et al., 1985). Although some controls of fatty acid oxidation and ketogenesis in ruminants have been studied (Butler et al., 1988; Jesse et al., 1986a,b), little is known about regulation in cows in negative energy balance or during development of fatty liver or ketosis. A major advantage provided by our FRBD model is that these possible intrahepatic changes can be studied in the same cow as
she moves through development of fatty liver and ketosis. With the information provided by the present study, additional comparisons may now be made with cows developing subclinical ketosis.

The triglyceride-to-glycogen ratio shows promise as a tool for predicting susceptibility to induced fatty liver and ketosis. Cows could be screened at 10 to 14 days postpartum for elevated triglyceride-to-glycogen ratios (perhaps above 2.0) to detect cows most likely to be induced successfully into fatty liver and ketosis. Subjecting cows with low triglyceride-to-glycogen ratios to FRBD, as in the present experiment, may provide a model of subclinical ketosis. Future experiments must verify the utility of the ratio and define more closely the critical value above which cows are susceptible to induction of fatty liver and ketosis. The triglyceride-to-glycogen ratio in a sample of liver from biopsy, however, is not likely to become a practical diagnostic test in the field.

We believe that the present experiment strengthens the utility of the FRBD model as a tool to determine sequential metabolic events in development of fatty liver and ketosis. Fatty liver and ketosis will not be induced indiscriminantly in all cows given the protocol; susceptibility evidently depends on a predisposition that develops in early lactation. We have identified the triglyceride-to-glycogen ratio in liver as a possible indicator of this susceptibility, which should aid in selecting cows for use in future studies. Even in "normal" early lactation cows, however, the protocol can be used to induce a model of subclinical ketosis. The challenge now is to use the FRBD protocol and other experimental techniques to elucidate the metabolic processes involved in
fatty liver and clinical and subclinical ketosis. Although many questions about metabolic origins of the disorders have remained unanswered after years of study, it seems that tools are now available to make considerable progress in increasing the understanding of fatty liver and ketosis in dairy cows.

Our experiment points to changes in the early-postpartum period as being critical in determining susceptibility to fatty liver and ketosis. From an experimental view, mobilization of body tissue must be maximized during this time to induce fatty liver and ketosis. From a producer's standpoint, our results reemphasize the importance of efforts to prevent excessive loss of body tissue during the early-postpartum period to decrease incidence of fatty liver and ketosis. As discussed by Schultz (1988) and Gerloff et al. (1986), such efforts should include prevention of other peripartum and postpartum diseases, maximizing intake of high-quality rations, and minimizing stresses that lead to catecholamine-induced increases in release of NEFA from adipose tissue.
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GENERAL SUMMARY

The two experiments reported in this dissertation were designed to answer questions raised at the conclusion of experiments by Mills (1982) and Veenhuizen (1988) in which fatty liver and lactation ketosis were induced by a combination of feed restriction plus dietary 1,3-butanediol. Some questions were: 1) Would either feed restriction or 1,3-butanediol as separate treatments produce any of the effects observed when the two treatments were administered together? 2) Would either feed restriction or dietary 1,3-butanediol as separate treatments affect gluconeogenic capacity of liver slices in vitro? 3) Are there compounds or agents that would prevent or alleviate the decrease in hepatic gluconeogenic capacity that occurs at clinical ketosis? 4) Is the fatty liver observed earlier a result of toxic effects of 1,3-butanediol? 5) Is induction of fatty liver and ketosis specific to early lactation or will the induction protocol produce fatty liver and ketosis later in lactation? 6) Is oxidation of long-chain fatty acids altered in liver during development of fatty liver and/or ketosis? 7) Are there changes in lipid transport associated with development of fatty liver or ketosis?

Results of the first experiment (Section I) indicate that neither moderate feed restriction nor dietary 1,3-butanediol administered as separate treatments will induce experimental fatty liver or ketosis. Furthermore, combining the two treatments at d 43 postpartum also did not cause fatty liver or ketosis. It seems that feed restriction and an exogenous source of ketone bodies work synergistically to cause experimental ketosis and fatty liver. This synergism evidently is
effective only during the very early-postpartum period. The nature of the synergism and the exact timing of the period of susceptibility remain to be determined.

Dietary 1,3-butanediol did not cause either decreased in vitro gluconeogenic capacity or fatty liver. In addition, butanediol added to incubation media did not decrease gluconeogenic capacity of liver slices. Thus, no evidence for a toxic effect of butanediol was observed.

No major decreases of in vitro gluconeogenic capacity were observed in experiment 1, so evaluations of effects of carnitine and nicotinic acid on gluconeogenic capacity were made over a limited range of capacities. Nevertheless, neither carnitine nor nicotinic acid increased in vitro gluconeogenic capacity. Beneficial effects of nicotinic acid in prevention and treatment of ketosis likely are restricted to decreasing lipolysis in adipose tissue. Carnitine actually decreased gluconeogenic capacity. This unexpected finding deserves further research in light of the interrelationships between carnitine, fatty acid metabolism, and gluconeogenesis.

Results of the second experiment (Section II) initially seemed to contradict the conclusion of Veenhuizen (1988) that "normal herd cows" can be induced into fatty liver and clinical ketosis by using the FRBD protocol. By chance the cows selected for induction into ketosis in Veenhuizen's study were relying more on body tissue to support milk production and probably were more susceptible, therefore, to induction of clinical ketosis. The seemingly "normal" cows used in our second experiment were not relying on tissue reserves to the extent that
Veenhuizen's cows were. As a result, cows of experiment 2 responded to FRBD with a subclinical ketosis. Future success with induction of fatty liver and clinical ketosis in "normal" cows might be improved by implementing measures to increase mobilization of NEFA during the first week or two postpartum. Examples of such measures could include: 1) more drastic restriction of feed intake for 1 to 3 d during the first week postpartum, before beginning FRBD; 2) injection of phlorizin for 2 to 3 d to increase the demand for glucose; or 3) increasing the degree of feed restriction and/or altering the ratio of feed restriction to dietary butanediol used in the FRBD protocol.

The second experiment seemed at first to contradict the conclusion from experiment 1 that feed restriction and butanediol act synergistically to induce fatty liver and clinical ketosis. Because fatty liver and clinical ketosis were not induced by the combination as expected (Section II), it might be argued that effects of feed restriction or butanediol separately would be different in cows in a greater state of tissue mobilization, such as those used in earlier experiments. Upon close inspection of the data, however, we feel that this is not justified. The subclinically-ketotic cows of the second experiment responded to FRBD in a manner that was qualitatively similar to the ketotic cows in earlier experiments. The response was different, however, from that of cows in experiment 1 to either feed restriction or dietary butanediol separately.

Three observations support the difference in response between the two experiments. First, lipid content of liver increased moderately in the subclinically-ketotic cows of experiment 2, whereas it decreased steadily
in cows given either feed restriction or butanediol separately in experiment 1. Second, increases in NEFA and BHBA in blood of cows in experiment 2 were more prolonged than the transient changes seen in cows of experiment 1. Third, two of the six cows assigned to the feed-restricted group in experiment 1 were judged to be in a state of tissue mobilization similar to earlier ketotic cows, but liver lipid content did not increase further and ketosis did not develop. It seems, therefore, that the response to the combined treatments in experiment 2 involved a synergism that was not present when the treatments were given separately, or when the treatments were given together later in lactation in experiment 1.

Results of experiment 2 will help to "fine-tune" the FRBD model and develop criteria for selection of cows for experiments. The triglyceride to glycogen ratio in liver is a potential marker to determine if cows will be susceptible to induction of fatty liver and clinical ketosis or if they will become only subclinically ketotic as in the present study. The usefulness of the hepatic triglyceride to glycogen ratio as a predictor of susceptibility to induction of clinical versus subclinical ketosis should be tested rigorously. The critical value above which cows are susceptible to induction of clinical ketosis needs to be defined closely. In the long term, the triglyceride to glycogen ratio may have value as a diagnostic tool for on-farm susceptibility to fatty liver and ketosis if associations can be found between the ratio and other more-easily measured indices. In the short-term, such a tool to predict response to the fatty liver and ketosis induction protocol will be enormously helpful in design and
conduct of experiments. Less waste of resources on "non-responding" cows would occur, and experiments could compare responses to treatments or other perturbations between cows induced into subclinical versus clinical ketosis. Comparisons should also be made between cows of differing body condition. The continued success of liver biopsy from high-producing cows will allow continued progress to be made by using these methods. We now have done well over 400 biopsies with only one cow death from hemorrhage and shock.

Tendencies for some changes in lipid metabolism were observed in subclinically ketotic cows of experiment 2. Oxidation of palmitate in liver slices was maintained at a greater level than in control cows. Tendencies for triglyceride in serum to be elevated, along with the early and transient increase in DSP-cholesterol and triglyceride, suggest that lipid transport may have been altered. It would be of interest to determine if these changes in subclinically-ketotic cows are repeatable and to compare such results with changes in cows that are induced into fatty liver and clinical ketosis. Future studies on lipid transport in cows with induced fatty liver could help to define the interactions of triglyceride synthesis, secretion, and accumulation in development of fatty liver.

Results of the two experiments in this dissertation strengthen the validity of the FRBD model of fatty liver and ketosis for several reasons. First, the protocol will not indiscriminantly cause fatty liver and clinical ketosis in all cows. Rather, the protocol seems to induce fatty liver and clinical ketosis only in cows that are relying extensively on
body reserves for energy-yielding nutrients. This probably is similar to
the situation for naturally-occurring fatty liver and ketosis. Second,
the effects seem to require both feed restriction and an exogenous source
of ketone bodies. This rules out both simple feed deprivation or influx
of dietary ketones as the usual causative factor. Third, induction is
specific for the very early postpartum period, which is similar to
naturally-occurring cases. Fourth, the decrease of in vitro gluconeogenic
capacity that occurs at ketosis is a specific aberration also associated
with clinical ketosis. Neither feed restriction nor dietary butanediol
separately decreased in vitro hepatic gluconeogenesis. In addition,
limited measurements showed no decrease in gluconeogenic capacity in the
subclinically ketotic cows.

With increased knowledge of the physiology of the ketosis-induction
protocol, researchers now should be in an excellent position to address
specific problems or hypotheses concerning development of fatty liver and
ketosis. Questions generated from the present experiments that deserve
attention are: 1) What is the timing of the period of susceptibility to
ketosis and fatty liver? 2) Are there changes in blood associated with
high ratios of triglyceride to glycogen in liver that might lead to
diagnosis of susceptibility without the need for liver biopsy? 3) Does
diet composition, specifically type or amount of protein, affect
susceptibility to fatty liver and ketosis? 4) What degree of energy
balance during early postpartum is associated with susceptibility, and is
this correlated with triglyceride to glycogen ratios? 5) Are there agents
that could counteract or prevent susceptibility to fatty liver and/or
ketosis? 6) What is the role of lipoprotein synthesis and/or secretion in development of fatty liver?

Use of the FRBD protocol in cows of "defined" susceptibility, as indicated by increased ratios of triglyceride to glycogen in liver, should be coupled with further perturbations of metabolism as suggested by Veenhuizen (1988). Such manipulations might include giving phlorizin injections to cows induced into subclinical ketosis, to see if decreased availability of glucose would induce clinical ketosis. Changes in enzyme activities and concentrations of metabolic intermediates in liver in response to such perturbations should be measured. If it is discovered that activities of enzymes are altered during induction of fatty liver and/or ketosis, techniques of molecular biology could be used to study regulation of transcription or translation of the corresponding mRNA. Treatments or preventative measures for fatty liver and ketosis should be tested by using the FRBD model. Intestinal administration of glucose should be reevaluated because the cows treated with glucose by Veenhuizen (1988) had very low hepatic triglyceride to glycogen ratios. This suggests that the glucose-treated cows would have responded to FRBD similarly to cows in the present study even in the absence of exogenous glucose.

Although the results of experiments reported in this dissertation were "negative" in many instances, they have in fact strengthened the value of the FRBD protocol for inducing fatty liver and ketosis. Truly innovative research on biochemical development of fatty liver and ketosis has been slow to appear. It seems that by use of the FRBD protocol, the
capability now exists to make real progress in determining biochemical mechanisms responsible for development of fatty liver and ketosis.
REFERENCES


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Thanks to all of you.
TABLE A1. Analysis of variance for conversion of propionate to glucose by liver slices from cows in Section One.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>Type III SS</th>
<th>MS</th>
<th>F</th>
<th>Pr&gt;F</th>
<th>conserv.² Pr&gt;F</th>
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</thead>
<tbody>
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<td>Treatment</td>
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<td>Week</td>
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<td>7.26</td>
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<td>Treatment<em>Week</em>Addition</td>
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<td>67</td>
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<td>.94</td>
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<td></td>
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</tbody>
</table>

¹A similar model was used to analyze conversion of propionate to CO₂. Units are micromoles of propionate converted to product per h per g of tissue wet weight.

²Probability of a greater F calculated with conservative degrees of freedom (df) for week effects. Conservative df are calculated by dividing both terms of F test by df for week; thus, for Treatment*Week effect, probability of a greater F is determined by using 2 and 12 df, not 6 and 38 df.

³Error term for nonrepeated effect is Cow (Treatment).

⁴Error term for week effects is Week*Cow (Treatment).

⁵Additions to incubation media (none, carnitine, 1,3-butanediol, or nicotinic acid).

⁶Error for Addition effects is residual error.
TABLE A2. Analysis of variance for milk production (kg/d) by cows from experiment described in Section Two.

<table>
<thead>
<tr>
<th>Source of Conserv. variation</th>
<th>df</th>
<th>III SS</th>
<th>MS</th>
<th>F</th>
<th>Pr&gt;F</th>
<th>Pr&gt;f</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>1</td>
<td>434</td>
<td>434.8</td>
<td>6.24</td>
<td>.32</td>
<td></td>
</tr>
<tr>
<td>Error A</td>
<td>10</td>
<td>697</td>
<td>69.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week</td>
<td>6</td>
<td>89</td>
<td>14.8</td>
<td>2.20</td>
<td>.056</td>
<td>.17</td>
</tr>
<tr>
<td>Treatment*Week</td>
<td>6</td>
<td>261</td>
<td>43.5</td>
<td>6.47</td>
<td>.0001</td>
<td>.029</td>
</tr>
<tr>
<td>Error B</td>
<td>57</td>
<td>384</td>
<td>6.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Conditional comparisons:

SED 2.35  df\(^7\) = 22

1Presented as an example of the statistical model used to analyze repeated measures in both Sections One and Two.

2Probability of a greater F calculated with conservative degrees of freedom (df) for week effects. Conservative df are calculated by dividing both terms of F test by df for week; thus, for Treatment*Week effect, probability of a greater F is determined by using 1 and 10 df, not 6 and 56 df (Geisser and Greenhouse, 1958).

3Error term for nonrepeated effect is Cow (Treatment).

4Error term for repeated effect is residual error.

5Conditional comparisons between treatments of any week were made by t-tests as described by Gill (1986).

6Standard error of difference between any two treatment means at any week, calculated as described by Gill (1986) using harmonic mean of number of observations per treatment.

7Approximate df for conditional comparisons, calculated by procedure of Saitherwaite as described by Gill (1986).
REFERENCES
