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**Metabolic responses of dairy cows with fatty liver to treatment  
with glucagon**

by

**Arnold Ray Hippen**

A dissertation submitted to the graduate faculty  
in partial fulfillment for the requirements for the degree of  
**DOCTOR OF PHILOSOPHY**

**Major: Nutritional Physiology**

**Major Professors: Jerry W. Young and Donald C. Beitz**

**Iowa State University**

**Ames, Iowa**

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## ABSTRACT

To study the effects of glucagon on early-lactation dairy cows that have fatty livers and are susceptible to ketosis, glucagon was continuously infused via intravenous jugular catheters into dairy cattle in various physiological states. In preliminary dosage response studies, glucagon was infused into eight spayed Holstein heifers, four midlactation Holstein cows, and four early-lactation Brown Swiss cows in cross-over design trials at dosages of 20 mg/d or less for 48 h. Plasma glucose concentrations were increased by glucagon in a dose-dependent manner. Plasma nonesterified fatty acid and ketone body concentrations were not increased by glucagon at 5 mg/d or less, were increased in heifers at 10 mg/d, and increased in cows at 20 mg/d compared with preinfusion concentrations. Glucagon at 10 mg/d was the largest dosage tested in lactating cows that did not elicit a lipolytic or ketogenic response. Glucagon at 10 mg/d or vehicle was then infused for 14 d during a protocol to induce ketosis in 20 early-lactation Holstein cows already having fatty livers. Plasma glucose concentrations were increased by 42% compared with glucose concentrations in control cows. Plasma insulin concentrations increased during the first 4 h of glucagon infusion, thereafter they were not different than concentrations in plasma of control cows. Concentrations of nonesterified fatty acids and ketone bodies in plasma were decreased over time by glucagon compared with concentrations in plasma of control cows. In livers of cows treated with glucagon, concentrations of triacylglycerols rapidly and dramatically declined. At the end of glucagon treatment, triacylglycerol concentrations in livers of glucagon treated cows had been decreased by 71% compared with livers of control cows. Glucagon caused liver glycogen concentrations to transiently decrease and ultimately increase to 231% of the glycogen concentrations in livers of control cows. Production of milk and milk protein was decreased during glucagon treatment, but rapidly increased to levels of production by control cows after

treatment. No adverse effects of glucagon were observed on the health of the cows. Glucagon can be used as an effective treatment for fatty liver and increasing the resistance of early-lactation cows to ketosis.

## **GENERAL INTRODUCTION**

### **Dissertation Organization**

This dissertation is presented as two papers, each of which is in a form suitable for submission to the Journal of Dairy Science. These papers have been prepared from research performed to partly fulfill the requirements for a Ph.D. degree. Each paper is complete in itself, including an abstract, introduction, materials and methods, results, discussion, conclusions, and references. The first paper represents preliminary dosage response work upon which the dissertation research was established. The second describes the main body of research conducted for completion of the dissertation. The papers are preceded by a literature review and followed by a general discussion.

### **Literature Review**

Fatty liver and ketosis are related metabolic disorders that occur with discouraging frequency in early-lactation dairy cows. Cows that have the greatest potential for milk production are also the most susceptible to these disorders. Often associated with this disease complex are:

- decreased milk production, both acutely and chronically.
- depressed immune function, leading to increases in infectious disorders.
- calving related disorders: dystocia, retained placenta, and metritis.
- decreased reproductive efficiency: delayed estrus and failure to conceive.

- post-calving metabolic aberrations: milk fever, "downer cow syndrome", and displaced abomasums.
- a general state of malaise: "She just won't eat, Doc."
- and in extreme fatty liver: liver necrosis and loss of the cow along with her genetic potential.

Admittedly, this list is very general and seemingly any postparturient disorder could be attributed to fatty liver and ketosis, but the list serves to illustrate the complexity of the syndrome.

Because diagnosis of fatty liver is difficult, if not impossible without collection of a liver sample for quantification of triacylglycerol (**TAG**) content, fatty liver is sometimes referred to as a "hidden" disorder. Attempts to treat cows for the above postparturient problems, by necessity, focus on treatment of the immediate symptoms rather than on fatty liver itself, because no efficacious treatment has been found for fatty liver. Liver function may be badly impaired by fatty infiltration. Associated with fatty liver is a loss of endoplasmic reticulum and decreased numbers of mitochondria within the hepatocytes resulting in decreased synthetic and energetic functions by the liver (Baird, 1982). In the event of advanced fatty infiltration of the liver, the prognosis is poor, and the herdsman is usually advised to "put her on the truck" while the cow is still ambulatory.

Thus, economic losses attributable to fatty liver and ketosis can be substantial within individual herds. Various estimates have been made of the cost of fatty liver and ketosis to dairy farmers, but most estimates consider only the direct

costs of treatment for ketosis and milk production lost during clinical ketosis. The costs for treatment of related disorders such as surgical correction of a left displacement of the abomasum can be substantial. The true costs of the syndrome also should include treatments for these related disorders and decreased milk production related to a chronic condition of fatty liver and subclinical ketosis, a shortened productive life, costs of replacement animals, and a decrease in the rate of genetic improvement.

Efforts to elucidate the causes and etiology of fatty liver and ketosis have been numerous over the past 30 years, and many journal and popular press articles address this topic. The general consensus of published material is that ketosis is a result of an aberration in carbohydrate metabolism at a time when energy demands of the lactating dairy cow are at a zenith, and the resulting low concentrations of glucose are a primal factor in the etiology of ketosis and fatty liver. Therefore, the goal of this research was to remedy the hypoglycemic condition of ketotic cows having fatty livers through administration of the hormone that is directly responsible for glucose sufficiency, i.e., glucagon. What follows is an overview of significant recent gains in the understanding of the "fatty liver syndrome" and ketosis and a description of work that, hopefully, contributes meaningfully to this body of knowledge.

## Hormones of Carbohydrate and Lipid Metabolism

To discuss the derangements that occur during the development of fatty liver and ketosis, a brief review is warranted of the two major regulators of carbohydrate and lipid metabolism, insulin and glucagon. Insulin and glucagon have opposing and counter-balancing actions. Generally speaking, insulin is responsible for nutrient uptake by tissues and opposes gluconeogenesis and lipolysis. Glucagon promotes mobilization of nutrients by stimulating lipolysis, ketogenesis, and net glucose export from the liver via glycogenolysis and gluconeogenesis (Holtenius, 1993; Shade and Eaton, 1975). The effects of the two hormones may vary widely when comparisons are made across species, and the discussion that follows is an overview of commonly accepted activities and relationships of these hormones. Whenever species specific effects are pertinent, an effort has been made to elaborate in the discussion that follows. Throughout the discussions, it is important to keep in mind that insulin and glucagon are considered to be counterregulatory, and changes in the molar ratio of insulin to glucagon are considered to be important predictors of metabolic status.

***Insulin.*** Insulin is synthesized in the endoplasmic reticulum of the  $\beta$ -cells in the pancreatic islets of Langerhans. The secretion of insulin is increased in response to increases in concentrations of blood glucose entering the  $\beta$ -cells via GLUT-2 transporters, which are not insulin sensitive (Ganong, 1991). The  $\beta$ -cells also contain glucagon receptors, and glucagon is able to stimulate an increase in blood insulin concentrations (Ahrén et al., 1987). Other compounds that stimulate



insulin secretion include mannose, amino acids,  $\beta$ -keto acids, acetylcholine, intestinal hormones, and phosphodiesterase inhibitors such as theophylline. Insulin secretion is inhibited by somatostatin, epinephrine, norepinephrine, and insulin itself (Ganong, 1991).

The stimulatory effects of the above agents can be attributed to their effects on cellular  $\text{Ca}^{++}$  concentrations. An increase in cytosolic  $\text{Ca}^{++}$  stimulates insulin secretion. Cytosolic  $\text{Ca}^{++}$  is increased by glucose activation of ATP-sensitive  $\text{K}^+$  channels, glucagon,  $\beta$ -adrenergic agents, and phosphodiesterase inhibitors. Acetylcholine will increase cytosolic  $\text{Ca}^{++}$  by activating phospholipase C and generating inositol triphosphate (Voet and Voet, 1990).

Upon binding to receptors, insulin is internalized rapidly and degraded within cellular endosomes. Thus, the half-life of insulin in humans is only about 5 min. Insulin increases the uptake of glucose in most tissues, in particular muscle and adipose tissue, by increasing the number of glucose receptors on cell surfaces. Insulin also increases transport of amino acids and  $\text{K}^+$  into insulin-sensitive tissues. Protein synthesis is increased and degradation is decreased by insulin. Insulin increases nutrient uptake by virtually all tissues, but, in ruminant animals, the insulin-induced uptake of nutrients is slower than in nonruminant animals (Brockman and Manns, 1978). Additionally, insulin is not able to stimulate glucose transport into the mammary gland of dairy cows, and glucose transport is a passive process controlled by the relative concentrations of glucose in blood and utilization of glucose in mammary tissue.

Within the liver, insulin increases the activity of glycogen synthase and glycolytic enzymes and decreases the activity of phosphorylase and gluconeogenic enzymes. In ruminant animals, insulin is able to inhibit hepatic glucose production by only about 15%, and the inhibition seems to be limited to glycogenolysis rather than to gluconeogenesis (Brockman, 1978).

In adipose tissue as well as liver, insulin increases messenger RNA for lipogenic enzymes, stimulating lipogenesis. In vivo studies in sheep showed that insulin administration was associated with decreases in net output of glycerol and nonesterified fatty acids (NEFA) from adipose tissue, and the uptake of acetate by peripheral tissue was restored in alloxan-diabetic sheep (Brockman, 1978). Insulin is also responsible for increasing the uptake of ketone bodies in peripheral tissues (Robinson and Williamson, 1980).

A general summation of the effects of insulin on specific tissues follow (Ganong, 1991):

- Adipose tissue

- Increased glucose and  $K^+$  uptake.

- Increased fatty acid synthesis, glycerol phosphate synthesis, and TAG deposition.

- Activation of lipoprotein lipase.

- Inhibition of hormone-sensitive lipase.

- Muscle

- Increased glucose, ketone body, and amino acid uptake.

Increased glycogen and protein synthesis.

Decreased protein catabolism and release of gluconeogenic amino acids.

- Liver

Decreased ketogenesis.

Decreased glucose export by decreasing gluconeogenesis and increasing glycogen synthesis.

Increased protein and lipid synthesis.

The activity of insulin is directed primarily to extrahepatic tissues where it promotes nutrient uptake. In the absence of insulin, synthetic activity of tissues is decreased, and a net movement of metabolites toward the liver occurs (Brockman et al., 1978).

**Glucagon.** Glucagon, similarly to insulin, also is produced in the pancreas. The  $\alpha$ -cells of the islet of Langerhans are responsible for synthesis and secretion of glucagon. Partly because of the proximity of the  $\beta$ -cells, insulin is a major factor regulating glucagon secretion and inhibits glucagon gene expression in vivo and in vitro (Philippe, 1996). Declining production of insulin by the  $\beta$ -cells of the pancreas removes chronic inhibition of the  $\alpha$ -cells (Cryer, 1996). Norepinephrine, epinephrine, growth hormone, and cortisol also will stimulate glucagon production (Cryer, 1996). Similar to insulin, glucagon production also is stimulated by increases in intracellular  $\text{Ca}^{++}$ . The glucagon gene contains a cAMP response element that activates transcription when stimulated by  $\text{Ca}^{++}$  and protein kinase A

(Philippe, 1996). Thus, the  $\beta$ -adrenergic agents will stimulate secretion and  $\alpha$ -adrenergic agents inhibit secretion of glucagon.

Nutritional stimulation of glucagon secretion is caused by declining concentrations of blood glucose (Cryer, 1996). Glucagon secretion is stimulated after ingestion of protein or infusion of gluconeogenic amino acids and during starvation. The decreasing glucose concentrations also stimulate the autonomic nervous system, which act further on the  $\alpha$ -cells both neuronally and hormonally (Cryer, 1996). Accordingly, glucagon is secreted via the sympathetic nervous system in response to stress, exercise, and infection. Glucagon, similar to insulin, is secreted also in response to acetylcholine and phosphodiesterase inhibitors. In a counterregulatory fashion, glucagon stimulates the secretion of insulin, somatotropin, and somatostatin. Inhibition of glucagon secretion is caused by increasing concentrations of glucose, ketone bodies, and NEFA. Hormonal inhibitors of glucagon secretion include insulin and somatostatin.

The half-life of glucagon in blood of dairy cows is about 5 min (deBoer et al., 1986). Because glucagon is secreted into the portal vein, the liver is responsible for most of the removal of glucagon from circulation and concentrations in peripheral blood are low except in cases of cirrhosis (Ganong, 1993). Glucagon receptors have been identified also in white and brown adipose tissue, kidney, pancreatic islets, brain, heart, and intestinal tract tissues (Iwanij, 1996). The glucagon receptor is involved in the activation of adenylate cyclase, which is regulated by the guanosine triphosphate (GTP)-binding heterotrimeric G-protein complex (Iwanij,

1996). Therefore, much of the activity of glucagon can be attributed to the action of cAMP (Brockman, 1978).

Glucagon generally is considered to be gluconeogenic, glycogenolytic, lipolytic, and ketogenic (Brockman, 1978; Ganong, 1993); although, the ketogenic capacity of glucagon in ruminant livers has not been consistently observed (Cad6miga-Vali6o et al., 1997). Glucagon does, however, consistently increase rates of gluconeogenesis. In addition to increasing the rate of gluconeogenesis, glucagon also will decrease the rate of glycolysis. This stimulation is accomplished by a glucagon-induced activation of protein kinase A via the action of adenylate cyclase and cAMP. Protein kinase A inhibits glycolysis at two sites: the conversion of phosphoenolpyruvate to pyruvate by phosphorylation of pyruvate kinase and the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate by decreasing concentrations of fructose-2,6-bisphosphate. Glucagon also activates a cyclic-GMP-inhibited phosphodiesterase, increasing the dynamics of the cAMP-dependent systems by inducing rapid changes in protein kinase A activation and deactivation (Pecker and Pavoine, 1996).

Glucagon increases hepatic glucose production, primarily by stimulation of hepatic glucogenic enzymes (Cryer, 1996). The enhanced gluconeogenic activity of glucagon in sheep was associated with increased activity of pyruvate carboxylase (Brockman, 1978). Glucagon also stimulates extraction of gluconeogenic precursors from plasma, including pyruvate, lactate, alanine, and glutamate but not glycerol (Brockman, 1978; Cryer, 1996; Flakoll et al., 1994). Furthermore, glucagon

does not seem to increase mobilization of these precursors from peripheral tissues (Cryer, 1996; Flakoll, 1994).

Glycogenolytic effects of glucagon are caused by stimulation of glycogen phosphorylase and inhibition of glycogen synthase (Cryer, 1996). Glucagon stimulation of hepatic glycogenolysis is by  $\text{Ca}^{++}$  elevation as well as stimulation of cAMP (Pecker and Pavoine, 1996). Glucagon causes breakdown of phosphatidylinositol-4,5-bisphosphate to inositol triphosphate that, in turn, stimulates cytosolic calcium uptake (Iwanij, 1996). The true effector of the action of glucagon on  $\text{Ca}^{++}$  mobilization may be mini-glucagon (Pecker and Pavoine, 1996). Mini-glucagon is derived from processing of glucagon by an endopeptidase, and glycogenolytic activity is enhanced in the presence and diminished in the absence of mini-glucagon (Pecker and Pavoine, 1996). Glucagon and mini-glucagon also affect  $\text{Ca}^{++}$  translocation by potentiating the effects of calcium-mobilizing agonists such as vasopressin, phenylphrenine, and angiotensin (Pecker and Pavoine, 1996).

Adipose tissue responds to glucagon stimulation by an increase in lipolysis with a concomitant release of glycerol and NEFA (Iwanij, 1996). The lipolytic effects of glucagon in adipose tissue of ruminants is questionable at physiological concentrations, however. Etherton et al. (1977) failed to observe in vivo lipolytic responses to glucagon in adipose tissue from sheep and dairy steers. It is also possible that lipolytic effects can be attributed to a decrease in lipogenic activity induced by glucagon. The activity of the lipogenic enzyme, acetyl-CoA carboxylase,

is decreased with cAMP-stimulated phosphorylation that is promoted by glucagon (Foster and McGarry, 1992).

The effects of glucagon are directed mainly to the liver, where it promotes uptake of gluconeogenic substrate and increases hepatic output of glucose by glycogenolysis and gluconeogenesis (Brockman, 1978). A summation of the generally acknowledged effects of glucagon follows:

- Adipose tissue

Increased lipolysis.

Activation of hormone-sensitive lipase.

- Muscle

No direct effects.

Decreased protein synthesis via decreased plasma amino acid concentrations.

- Liver

Increase glucose export.

Increased gluconeogenesis.

Increasing glycogen degradation.

Increased amino acid uptake.

Increased ketogenesis.

The effects of glucagon in other tissues do not necessarily directly involve nutrient metabolism, but they are supportive of the role of glucagon as a regulator of energy

substrate. In the heart, glucagon has positive inotropic and chronotropic effects and activates L-type  $Ca^{++}$  channels, increasing the strength and duration of contractions (Iwanij, 1996). In the gastrointestinal tract, glucagon quiets contraction and relaxation of smooth muscle cells and inhibits gastric acid secretion; consequently, glucagon also has quieting effects on gastric motility (Iwanij, 1996).

### **Development of Fatty Liver**

***Prepartal nutrition.*** Nutrition of the dairy cow during the prepartal period seems to be a determinant of the postpartal development of fatty liver. Cows that are obese at parturition have a propensity for fatty liver. For this reason, fatty liver is often considered a component of "fat cow syndrome" (Morrow, 1976). Indeed, fatty liver may be the "gateway" disorder that leads to development of the peripartal diseases mentioned earlier.

A contributory factor for the postpartal development of fatty liver is the plane of energy intake during the early portion of the dry period. Van den Top et al. (1996) have shown that cows allowed ad libitum access to feed during the dry period developed severely fatty livers postpartum compared with cows that had limited access to feed during the dry period.

The nature of the feed, as well as the quantity, offered during the dry period also seems to have an effect on development of fatty liver. It has been hypothesized that increasing carbohydrates in the prepartal diet would increase insulin concentrations and inhibit mobilization of fatty acids from adipose tissue,



thereby preventing the development of fatty liver. This hypothesis is based partly upon research in which prepartal cows received an oral drench of propylene glycol daily (Studer et al., 1992). Cows in that experiment had decreased concentrations of liver TAG compared with that in control cows. Other researchers have observed that an increase in the ratio of concentrates to forage in the prepartal diet leads to increased insulin resistance typical of that observed in cows with fatty liver (Holtenius and Tråvén, 1990). They have concluded that overfeeding concentrate during late lactation and the dry period results in fat cows that are predisposed to postparturient diseases because of fatty infiltration of livers (Holtenius, 1993). In fact, cows fed 65% concentrate diets during the dry period did indeed have increased concentrations of insulin in plasma prepartum, but by 3 wk postpartum, insulin concentrations were decreased and concentrations of ketone bodies were increased in plasma compared with cows fed 50% concentrate during the prepartum period (Holtenius, 1993). On the basis of his research and that of others, Holtenius concluded that high levels of insulin-stimulating feedstuffs prepartum increases risk for postpartal insulin resistance, fatty liver, and ketosis.

Excessive energy concentration in the diet as early as midway through the previous lactation may be a factor also in the development of postpartal fatty liver and ketosis. Holstein cows fed diets containing 1.7 versus 1.6 Mcal/kg during the last 200 d of lactation had increased concentrations of plasma ketones and liver TAG during the first 28 d of the following lactation (Vazquez-Anon et al., 1997). Two high-energy diets were fed during that study, one with high concentrate levels

and one with supplemental tallow. The high-grain diet was responsible for an increase in plasma ketone body concentrations and both diets contributed to increases in liver TAG.

The addition of fat to the prepartal diet seems to hold promise for prevention of fatty liver by increasing energy intake of the cow immediately prepartum (Grum et al., 1996). Theoretically, the addition of fat in the form of TAG would provide glycerol for gluconeogenesis, but because dietary fat will increase concentration of NEFA in plasma, the benefit of the small amount of gluconeogenic substrate could be offset by increasing quantities of lipid that would have to be processed by the liver (Schultz, 1971). As early as 1956, it was observed that cows fed high-fat, low-protein diets prepartum were hypoglycemic after parturition but did not develop ketosis (Schultz, 1971). More recently, cows fed high-fat diets during the dry period had decreased liver TAG and plasma NEFA concentrations and increased hepatic capacity to oxidize fatty acids postpartum (Grum et al., 1996). But once again, results of prepartal fat feeding have been variable. Skaar et al. (1989) fed tallow during the dry period and observed slight increases in postpartal concentrations of TAG in livers of cows. Perhaps both timing of fat supplementation and the nature of the dietary fat affects the outcome of prepartal fat feeding. Feeding diets high in unsaturated fatty acids to preruminating calves resulted in hepatic secretion of very-low-density lipoproteins (VLDL) enriched in cholesteryl esters at the expense of TAG, which accumulated in the liver (Hermier et al., 1991; Leplaiz-Charlat et al., 1996). Even though bacterial biohydrogenation in the rumen limits such effects in

mature cows, the degree of unsaturation of dietary fats may have implications when protected fat supplements and diets high in corn grain are fed.

***Lipid metabolism in adipose tissue.*** The high-producing dairy cow is usually in a state of negative energy balance during the periparturient period (Coppock et al., 1974). Feed intake during this period is not adequate to keep pace with the energy demands of calving and lactation. Prevention of the dry matter intake (DMI) depression commonly observed during the peripartal period can alleviate the severity of fatty liver. When dietary refusals were collected and placed into rumens of fistulated cows, accumulation of TAG in liver was decreased during the postpartal period (Bertics et al., 1992).

An estimated 20 to 25% of the energy required for lactation must be met by mobilization of energy stores from adipose tissue (Holtenius, 1993). Control of the release of fatty acids from adipose tissue is under hormonal influence. Insulin is considered to be the predominant hormone controlling flux of fatty acids into and out of adipose tissue (Brockman, 1976). The role of insulin in adipose metabolism is to inhibit lipolysis and stimulate lipogenesis (Robinson and Williamson, 1980). Forms of insulin resistance are common, however, in obese animals and people (Kolterman et al., 1980; McCann and Reimers, 1986). In adipose tissue from ewes in early lactation, insulin failed to stimulate uptake of acetate in support of lipogenesis (Vernon and Taylor, 1988). Other researchers have noticed heightened responses of insulin to glucose tolerance tests in obese ewes, but glucose removal was not enhanced indicating peripheral insulin resistance (McNeill et al., 1997).

Similarly, during the early postparturient period, adipose tissue from dairy cows is adapted to increased rates of lipolysis to support milk production and is less susceptible to the effects of insulin (McNamara and Hillers, 1986). Therefore, the importance of insulin in inhibiting lipolysis during early lactation in ruminant animals is questionable because concentration and activity of insulin receptors on adipocytes are low at that time and rates of lipogenesis are negligible (Vernon and Taylor, 1988). Accordingly, in early lactation and particularly in obese cows and cows with fatty liver, an insulin resistance exists that prevents the insulin-induced inhibition of lipolysis, and the release of NEFA from adipose tissue is unrestrained (Holtenius, 1993; Holtenius and Tráven, 1990). Concurrent with the insulin resistance of adipose tissue during the postpartum period, lipolysis in adipose tissue of ewes is more responsive to the stimulatory effects of noradrenaline (Vernon and Finley, 1985). This increased sensitivity to noradrenaline is enhanced as adipocyte size is increased. Though this is a desirable adaptive mechanism relative to the nutritional requirements of the ewe, the same characteristic in an obese dairy cow would contribute to increases in postpartal plasma NEFA concentrations.

Glucagon generally is considered to be a lipolytic hormone, and it will stimulate release of fatty acids from adipose tissue of rats and poultry. In ruminants and dogs, which are species dependent on gluconeogenesis for supplying blood glucose, glucagon does not seem to stimulate release of fatty acids from adipose tissue if the normal ratio of insulin to glucagon is maintained (Brockman, 1978 and

Flakoll, 1994). In vivo infusions of glucagon have been observed to increase plasma fatty acids and glycerol, however, if insulin concentrations are not allowed to increase (Bassett et al., 1971; Brockman, 1976). Other in vivo studies, however, have not necessarily been able to confirm the lipolytic role of glucagon (Bauman, 1976).

In dairy cows, glucagon was observed to be unchanged between 3 wk before to 3 d after parturition, a time when fatty acid mobilization is increasing (Holtenius, 1993). Glucagon concentrations did increase in the cows by 3 wk after parturition, but no clear relationship of glucagon to plasma NEFA concentrations was shown. Other hormones have varying degrees of effects on fatty acid mobilization from adipose tissue (Ganong, 1993). Glucocorticoids have minimal lipolytic effects. Epinephrine, which is increased by the stress of parturition on the other hand, will activate hormone-sensitive lipase via the adenylate cyclase system and stimulate lipolysis.

The efflux of NEFA from adipose tissue supplies energy to peripheral tissues such as the mammary gland where it also supports milk fat synthesis. Fatty acids not removed from circulation by peripheral tissues are assimilated by the liver. The liver will esterify NEFA with glycerol to form TAG that are stored temporarily in cytosolic vacuoles.

***Lipid metabolism in liver.*** The metabolism of NEFA in the liver is controlled by the bi-hormonal insulin and glucagon system (Brockman, 1976; McGarry et al., 1975). Insulin will stimulate the synthesis of TAG in the liver. In primary cultures of

hepatocytes from nonruminating calves, insulin increased the proportion of fatty acids esterified to TAG, but it did not increase uptake of fatty acids (Cad6rniga-Vali6no et al., 1997). This synthesis, however, requires a supply of both NEFA and glycerol, which are decreased by insulin (Holtenius, 1993). Because of the insulin resistance that occurs in cows during early lactation, NEFA are released continually from adipose tissue. If there is a concomitant increase in catecholamines or glucagon, hepatic gluconeogenesis will supply glucose for glycerol, and TAG synthesis will proceed (Figure 1; Holtenius, 1993). Once fatty acids have accumulated within the hepatocytes as TAG, they can either be metabolized by oxidation or exported within lipoprotein particles (Holtenius, 1993). Because adipose tissue, and not liver, is the primary site of fatty acid synthesis in dairy cows, the liver of the cow has limited ability to export lipid. An inability of the liver to keep pace with the influx of NEFA by oxidation or exportation in lipoproteins is considered to be the primary cause of fatty liver (also known as hepatic lipidosis or hepatic steatosis).

Triacylglycerol synthesis in hepatocytes begins with esterification of an activated fatty acid to glycerol-3-phosphate (Figure 1; Gruffat et al., 1996). The resulting acyl-glycerol-3-phosphate is transported to the smooth endoplasmic reticulum by a fatty acid binding protein, where a second acylation occurs to produce phosphatidic acid. Phosphatidic acid phosphatase then hydrolyzes the phosphate ester bond and creates diacylglycerol. The three preceding reactions are common also to the synthesis of phospholipids, a vital component for lipoprotein

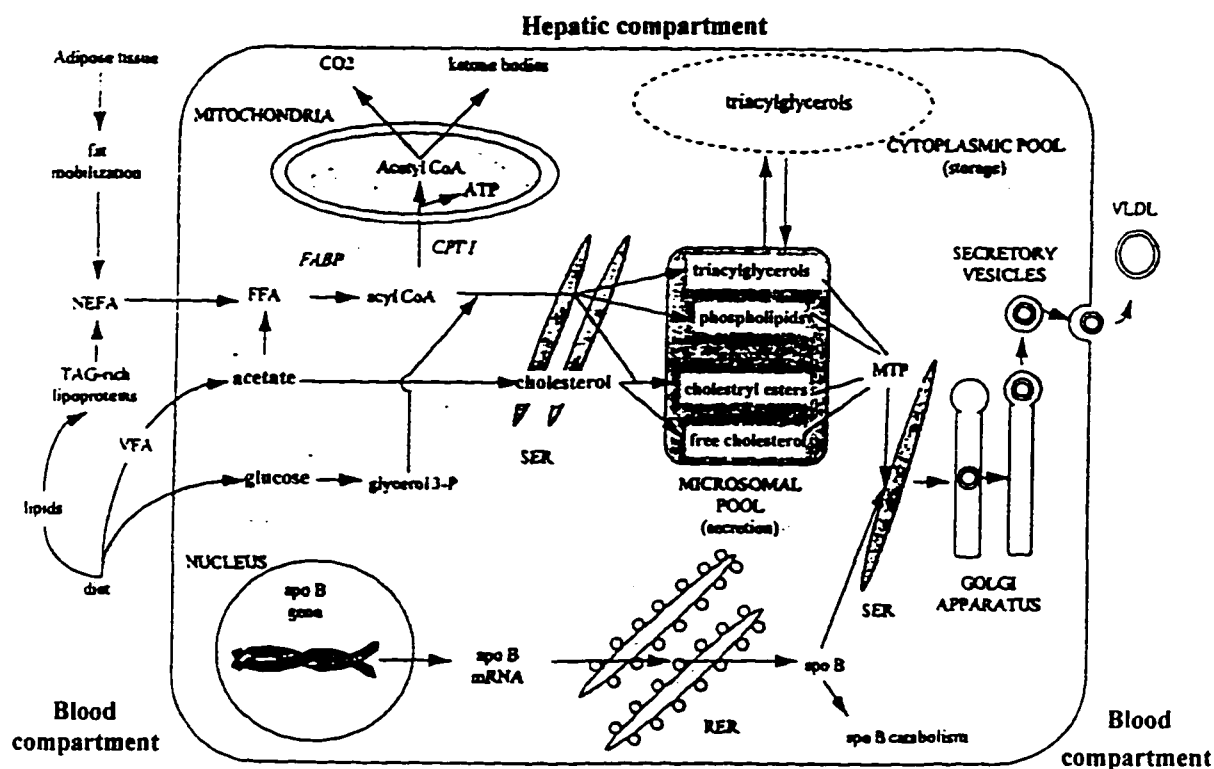


Figure 1. Schematic representation of the different processes involved in the synthesis and secretion of very-low-density lipoproteins (VLDL) in animal livers. apoB, apolipoprotein B; CPTI, carnitine palmitoyltransferase I; FABP, fatty acid binding protein; FFA, free fatty acids; MTP, microsomal transfer protein; NEFA, nonesterified fatty acids; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; VFA, volatile fatty acids. Adapted from Gruffat et al., 1996.

synthesis. The final reaction for the synthesis of TAG is the acylation of diacylglycerol by microsomal diacylglycerol acyltransferase. Activities of diacylglycerol acyltransferase have been shown to be increased in livers of cows during the first week postpartum in response to increasing concentrations of plasma NEFA, thereby increasing the rate of TAG formation (Van den Top et al., 1995). Time course studies conducted with sheep livers in vivo suggest that the newly synthesized TAG are not immediately incorporated into lipoprotein particles but are transferred to a temporary cytosolic pool instead (Pullen et al., 1988).

When TAG are exported from the liver, they are packaged as VLDL. Disturbances in lipoprotein metabolism and the subsequent production of VLDL are common causes of excessive TAG accumulation within hepatocytes and results in fatty liver (Herdt, 1988; Holtenius, 1989; Uchida et al., 1992). Concentrations of plasma lipids in the form of VLDL are decreased during the first week postpartum in dairy cows (Van den Top et al., 1995). Synthesis of VLDL is dependent upon an adequate supply of the appropriate amino acids for apoprotein synthesis, synthesis of phospholipids and cholesterol, mobilization of fatty acids from cytosolic TAG, reesterification of fatty acids to TAG in microsomes, and transport and assembly of all components into VLDL (Figure 1). These process also are regulated by the hormonal controls of insulin and possibly by glucagon.

The accumulated cytosolic TAG are not likely to be transported intact to microsomes for incorporation into lipoprotein particles, but instead, they must first be hydrolyzed to glycerol and fatty acids and then reesterified within the



microsomes (Figure 1; Mooney and Lane, 1981; Wiggins and Gibbons, 1992). The exact processes and agents involved in the hydrolysis and reesterification scheme have not been elucidated, but data do not support the involvement of lysosomal acid lipase, hormone-sensitive lipase, or lipoprotein lipase within the liver (Gruffat et al., 1996).

Phosphatidyl choline is the major phospholipid component of VLDL.

Synthesis of this phospholipid requires an adequate supply of choline. Choline deficiency limits VLDL secretion in hepatocytes from rats (Yao and Vance, 1988). Choline deficiency has been implicated also in the development of fatty liver in many species, and choline supplementation often is used as a remedy for fatty liver (Gruffat et al., 1996). Results of choline supplementation in lactating dairy cow diets, however, have been inconsistent. Likewise, methionine can restore impaired VLDL synthesis by promoting phosphatidyl choline synthesis (Robinson et al., 1989). Research involving methionine supplementation of the diet of peripartal dairy cows has been conducted, but no consistent results have been reported to date. On the other hand, intravenous infusion of L-methionine has been successful in stimulating VLDL secretion in cows (Durand et al., 1992; Juslin et al., 1965).

The principal protein necessary for VLDL synthesis is apolipoprotein B-100 (**apoB-100**). ApoB-100 messenger RNA has a half-life of 16 h and evidently is not subject to acute regulation (Pullinger et al., 1989). Hepatic apoB-100 secretion by rat hepatocytes in vitro is modulated by insulin and NEFA (Dashti et al., 1989; Kaptein et al., 1991; Pullinger et al., 1989). Newly synthesized apoB-100 is subject

to rapid degradation depending on pH, high temperatures, and ATP. This catabolism of apoB-100 is stimulated by insulin (Sparks and Sparks, 1991) and decreased by oleate (Dixon et al., 1991; Furukawa et al., 1992). In fact, the addition of oleate to HepG2 or McArdle-RH777 cell medium is highly stimulatory to apoB-100 secretion (Sakata et al., 1993; White et al., 1992).

Lipids are transported to newly synthesized apoB-100 by microsomal TAG transport protein (**MTP**; Atzel and Wetterau, 1994). Microsomal TAG transport protein catalyzes the transport of TAG, cholesteryl esters, and phospholipids from the endoplasmic reticulum to the site of VLDL synthesis (Figure 1; Gruffat et al., 1996). The role of MTP in lipoprotein assembly is supported by the observation that synthesis of MTP is stimulated by high fat diets in hamsters (Lin et al., 1994). Synthesis of MTP is decreased by up to 80%, however, by increasing insulin concentrations in HepG2 cells (Lin et al., 1995). The activity of MTP in HepG2 cells was not affected by insulin, probably because of the long half-life (4.5 d) of MTP protein (Lin et al., 1995).

Overproduction of VLDL is often a problem in humans with a form of noninsulin-dependent diabetes (Gruffat et al., 1996). Metabolic deviations associated with the overproduction of VLDL in this disorder are elevated plasma NEFA, hyperglycemia, and hyperglucagonemia (Howard, 1987). More closely related to disorders associated with fatty liver is the hyposecretion of VLDL. Gruffat et al. (1996) listed the possible mechanisms for lipoprotein abnormalities that lead to the development of fatty liver in humans (Table 1).

Table 1. Possible mechanisms involved in the development of liver steatosis<sup>1</sup>.

- 
- i. Increased supply of NEFA<sup>2</sup> to the liver resulting from:
    - higher NEFA mobilization from adipose tissue
    - higher NEFA synthesis in the liver
    - lower NEFA oxidation in the liver
  - ii. Impaired TAG<sup>3</sup> incorporation into VLDL<sup>4</sup> through:
    - inhibition of apoB<sup>5</sup> or MTP<sup>6</sup> synthesis
    - production of an inadequate supply of apoB or MTP molecules
    - higher intracellular degradation of apoB
    - competition with cholesteryl esters
  - iii. Defects in one or several steps in VLDL transport and secretion through:
    - alterations of VLDL transport from ER<sup>7</sup> to Golgi apparatus
    - impairment of final apoB glycosylation in Golgi apparatus
    - lower rate of secretory vesicle formation
    - impaired migration of secretory vesicles from Golgi apparatus to cell membranes
- 

<sup>1</sup>Taken from Gruffat et al., 1996.

<sup>2</sup>NEFA = long-chain fatty acids.

<sup>3</sup>TAG = triacylglycerol.

<sup>4</sup>VLDL = very-low-density lipoproteins.

<sup>5</sup>apoB = apolipoprotein B

<sup>6</sup>MTP = microsomal triacylglycerol transport protein.

<sup>7</sup>ER = endoplasmic reticulum.

Obviously, there are a multitude of derangements that can occur within lipoprotein metabolism that would lead to the development of fatty livers within dairy cows as well as in other species. In dairy cows, a decrease in apoB-100 concentrations in plasma were correlated with increasing TAG content of livers (Marcos et al., 1990). Similarly, when ethionine, an inhibitor of protein synthesis, was administered to dairy cows, fatty liver was induced, suggesting that apolipoprotein synthesis is a limiting step to VLDL secretion (Uchida et al., 1992). During the first week of lactation, correlations of hepatic concentrations of apoB, apoB messenger RNA, and TAG showed a negative relationship between apoB-100 gene expression and the development of fatty liver (Gruffat et al., 1994). More

recently, cows having liver TAG concentrations of 4 to 10% wet weight were found to have lower concentrations of apoB-100 in liver tissue than cows with normal livers (Gruffat et al., 1997). Because concentrations of messenger RNA were not different, the authors postulated that there is a posttranslational down-regulation of apoB-100 in fatty livers. This would indicate involvement of MTP in the etiology of a lipoprotein disturbance during fatty liver.

In early-lactation cows (2 wk postpartum) a general decrease in plasma lipoprotein concentrations was noted, but the decrease was predominately in low- and high-density lipoprotein fractions, not VLDL (Mazur et al., 1988). These effects were also relative to severity of fatty liver; cows with greater liver lipid concentrations had greater decreases in low- and high-density lipoproteins. The lack of significantly lower concentrations of VLDL may be attributable to the fact that VLDL concentrations in all cows were too small for detectable differences.

One form of fatty liver, with possible associations to fatty liver observed in dairy cows, is that which occurs in humans during parenteral nutrition (Fisher, 1989). The development of fatty liver is dependent upon the amount of intravenous glucose that the patient receives. Increasing amounts of infused glucose lead to hyperinsulinemia and inhibited VLDL secretion. Explanations for the development of fatty liver in these instances include increased peripheral lipolysis, decreased hepatic fatty acid oxidation, greater hepatic fatty acid synthesis, and greater apoB-100 degradation. The syndrome also has been related to inadequate intake of protein relative to carbohydrates. Infusion of lipids along with glucose decreased

insulin concentrations and also the molar ratio of insulin to glucagon and led to a decrease of lipid infiltration into the livers of rats (Nusbaum et al, 1992). Lastly, continual daily administration of glucagon at 33 µg/100 g of body weight to rats, along with parenteral nutrition, drastically decreased fatty infiltration of the livers as well as molar ratios of insulin to glucagon (Li et al., 1988).

### **Ketogenesis**

Fatty acids that are not exported from liver TAG as lipoproteins also can be removed by oxidation. Complete oxidation of fatty acids to CO<sub>2</sub> would supply energy to the liver for metabolic process and be an apparently efficient means of elimination of cytosolic TAG. The rate at which this occurs is insufficient, however, in the etiology of fatty liver and ketosis. One of the roles of the liver as an altruistic organ is to supply readily oxidizable metabolites to other tissues. Much of the fatty acids are, therefore, only partly oxidized to the ketone bodies: acetoacetate (**ACAC**), β-hydroxybutyrate (**BHBA**), and acetone, providing metabolic fuels for other tissues (Figure 2). This process is at its peak activity in dairy cows during the first 30 d of lactation (Aeillo et al., 1984). The resulting excess of ketone bodies is detectable in urine, milk, and plasma of ketotic cows. One of the obvious symptoms of ketosis is the readily detectable odor of acetone in the breath of affected cows because acetone is volatile and expired through respiration.

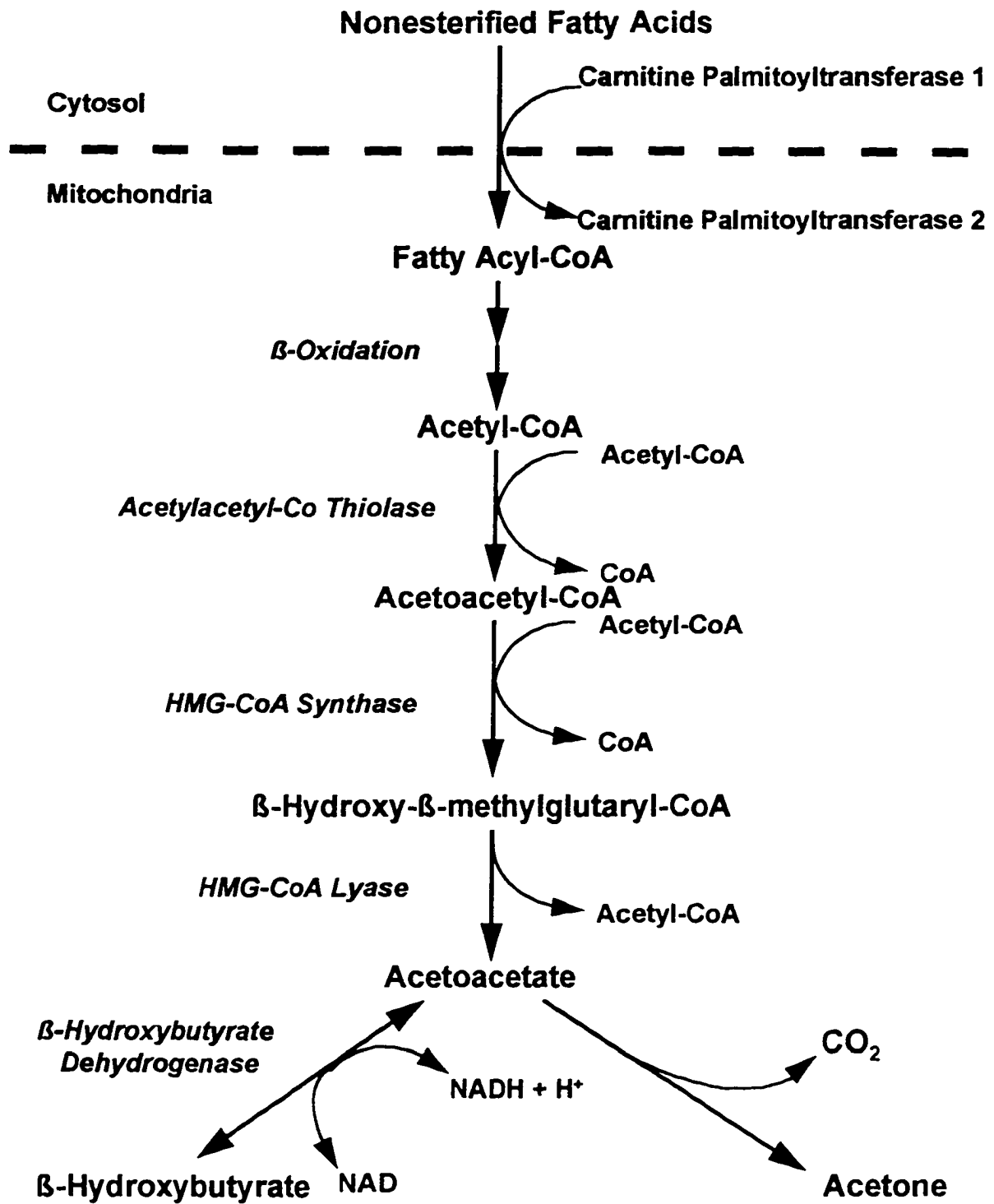


Figure 2. The synthesis of the ketone bodies; acetoacetate,  $\beta$ -hydroxybutyrate, and acetone from fatty acids in the bovine liver.

Ketone bodies also serve as metabolic regulators affecting transfer and utilization of other nutrients. Infusion of BHBA was found to decrease concentrations of alanine, the chief gluconeogenic amino acid, in plasma and urinary excretion of nitrogen in humans (Holtenius and Holtenius, 1996b). Negative correlations were observed also in concentrations of BHBA and alanine in phlorizin-treated lactating ewes and lactating cows (Holtenius and Holtenius, 1996b). Ketone bodies are capable also of sparing glucose from utilization by peripheral tissues during periods of negative energy balance in dogs (Umpleby et al., 1995). Most importantly, ketone bodies are capable of somewhat regulating their own production by decreasing rates of lipolysis and delivery of NEFA to the liver (Holtenius and Holtenius, 1996b).

For formation of ketone bodies to occur, fatty acids must be transported into mitochondria and undergo  $\beta$ -oxidation (Figure 2). During  $\beta$ -oxidation, fatty acids are degraded sequentially to a two carbon coenzyme A (CoA) derivative, acetyl-CoA. As acetyl-CoA accumulates, two acetyl-CoA molecules are condensed by acetyl-CoA thiolase to form acetoacetyl-CoA. Condensation of a third acetyl-CoA molecule to acetoacetyl-CoA by  $\beta$ -hydroxy- $\beta$ -methylglutaryl-CoA synthase produces  $\beta$ -hydroxy- $\beta$ -methylglutaryl-CoA. Finally, the action of  $\beta$ -hydroxy- $\beta$ -methylglutaryl-CoA lyase will remove the terminal acetyl-CoA molecule and form the four carbon molecule, ACAC. Acetoacetate can be readily and reversibly reduced to BHBA by the action of  $\beta$ -hydroxybutyrate dehydrogenase or it can decompose irreversibly to  $\text{CO}_2$  and acetone. The conversion of ACAC to BHBA by  $\beta$ -hydroxybutyrate

dehydrogenase is an NADH-dependent reaction. Because the reaction is reversible, the ratio of ACAC to BHBA is considered to be a measure of the mitochondrial redox potential and reflects relative concentrations of NADH and NAD<sup>+</sup> within mitochondria. Reflecting possible involvement of changes in mitochondrial redox capacity is the decrease in oxidative capacity of hepatocytes from ketotic cows. Rates of CO<sub>2</sub> production from propionate, lactate, alanine, aspartate, and glutamate have been observed to be decreased in liver slices from cows with an experimentally-induced ketosis (Mills et al., 1986b).

An aberration in fatty acid metabolism that has been proposed as a part of the etiology of ketosis is a carnitine insufficiency (Baird, 1982; McGarry et al., 1975, 1977, and 1980). Carnitine palmitoyl transferase I (CPT1) is an enzyme of the outer mitochondrial membrane that is responsible for transport of long-chain fatty acids across the mitochondrial membrane. A deficiency of CPT1 would prevent the  $\beta$ -oxidation of long-chain fatty acids to acetyl-CoA. The activity of CPT1 is inhibited by fatty acid synthetase and malonyl-CoA, another coenzyme A derivative formed by the action of acetyl-CoA carboxylase on acetyl-CoA (Brindle et al., 1985). Acetyl-CoA carboxylase is responsible for the first step in the synthesis of fatty acids from acetyl-CoA and, along with fatty acid synthetase, is stimulated by insulin. Logically, inhibition of  $\beta$ -oxidation prevents futile cycling of fatty acids during fatty acid synthesis. Fatty acid synthesis would not be expected to be great in hepatocytes of a fatty liver, but bovine malonyl-CoA has been shown to be a particularly potent inhibitor of CPT1. Propionate, a product of rumen fermentation



and the primary glucogenic precursor, is also thought to be an inhibitor of CPT1 through conversion to methylmalonyl-CoA (Grummer, 1993); however, Drackley et al. (1991) showed that propionate-induced decreases in fatty acid oxidation are greater than can be accounted for by inhibition of CPT1 alone. Even though a carnitine deficiency would not contribute directly to the ketotic state, it would decrease the ability of the liver to oxidize the fatty acids responsible for TAG accumulation.

Ketosis results from the excessive accumulation of acetyl-CoA and subsequent formation of ketone bodies. The excessive accumulation of acetyl-CoA has been hypothesized to be caused by an insufficiency of oxaloacetate (OAA), an intermediate in the citric acid cycle with which acetyl-CoA must condense before complete oxidation can occur (Baird et al., 1982; Krebs, 1966). The inability of all of the acetyl-CoA to condense with OAA will divert acetyl-CoA to ketone body formation as described already.

### **Gluconeogenesis**

Oxaloacetate also is an intermediate in the gluconeogenic process. It has been estimated that 47% of OAA is used for gluconeogenesis and the remainder enters the citric acid cycle (Young, 1977). Gluconeogenesis serves as the primary source of glucose for use by peripheral tissues in ruminants because little glucose is available for absorption after bacterial degradation of dietary carbohydrate in the rumen. A primary sign of lactation ketosis is hypoglycemia brought about by the

combination of poor DMI and increasing glucose demands to support milk lactose synthesis. Therefore, the drive to produce glucose may deplete the mitochondrial supply of OAA in support of gluconeogenesis.

Pyruvate carboxylase (PC) and phosphoenolpyruvate carboxykinase (PEPCK) are the first of four gluconeogenic enzymes required to bypass the irreversible reactions of glycolysis (Figure 3). These two enzymes reverse the action of pyruvate kinase by first converting pyruvate to OAA via PC and then converting OAA to phosphoenolpyruvate via PEPCK. Accordingly, increases in the activity of PC will increase the quantity of OAA, and increases in the activity of PEPCK will decrease concentrations of OAA. Hormonal regulations at this point of the gluconeogenic pathway include activation of the transcription of messenger RNA for PEPCK by glucagon and glucocorticoids and a strong inhibition of the PEPCK message by insulin (Granner and Pilkis, 1990). The increased rate of gluconeogenesis stimulated by glucagon has been attributed to increased activities of PC in sheep (Brockman, 1978).

Glucagon also stimulates hepatic extraction of pyruvate, lactate, alanine, and glutamate but not glycerol from blood (Brockman, 1978; Cryer, 1996; Flakoll et al., 1994). Compounds such as lactate and the amino acids that enter the gluconeogenic pathway via pyruvate require the activity of both PC and PEPCK and provide sources of OAA to the mitochondrial pool. Additionally, the presence of alanine or glucagon via cAMP phosphorylation will inhibit the opposing glycolytic enzyme, pyruvate kinase, thereby decreasing the rate of glycolysis, and insulin

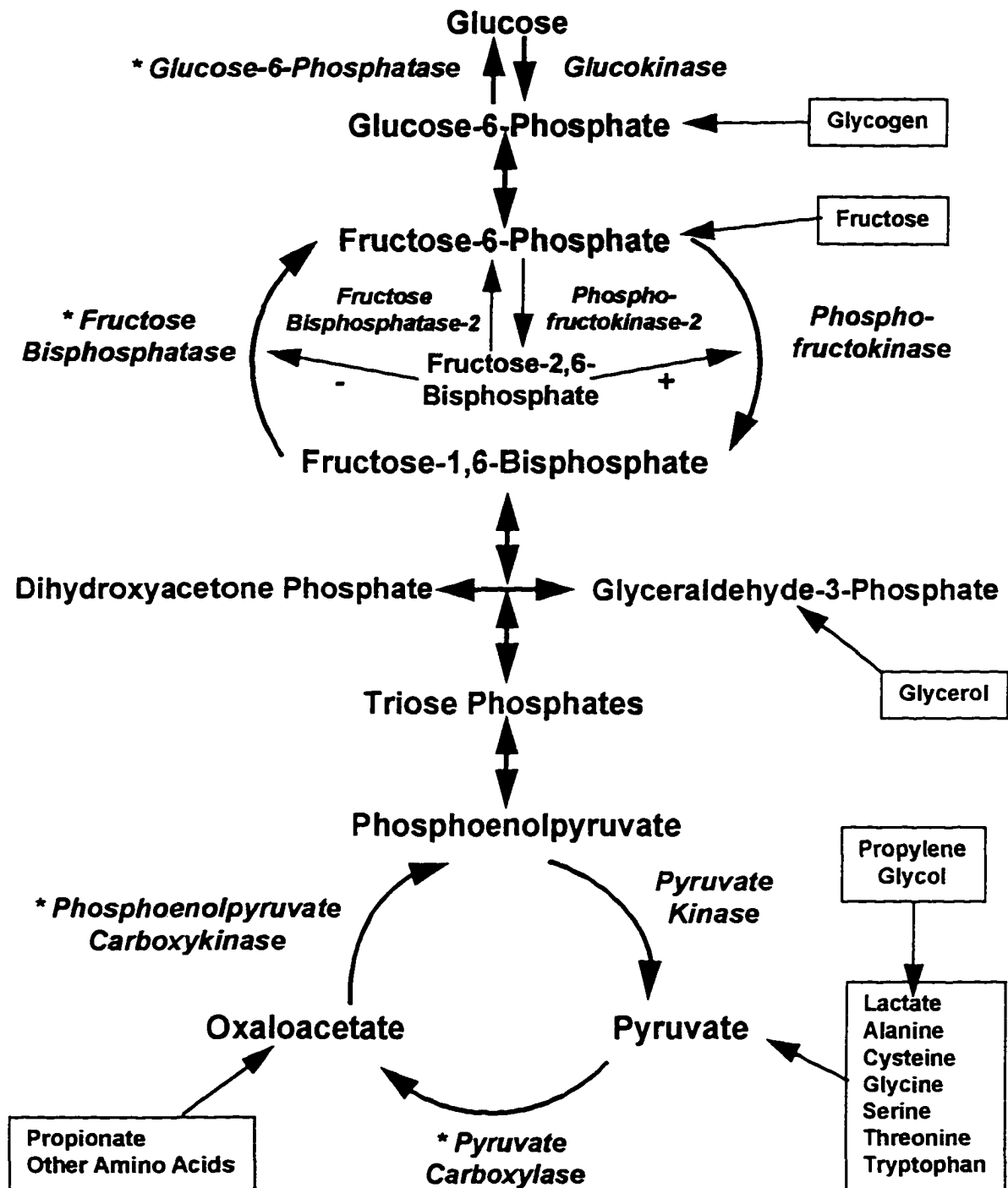


Figure 3. Pathways of glycolysis and gluconeogenesis in hepatocytes. Regulating enzymes are italicized. Gluconeogenic enzymes are marked with an asterisk. Gluconeogenic precursors are listed in boxes and their entry points into the gluconeogenic pathway are indicated.

will remove the glucagon induced inhibition (Pilkis and Granner, 1992). The conversion of other gluconeogenic amino acids and propionate, which is from rumen fermentation and is the primary gluconeogenic compound for ruminant animals, require only the activity of PEPCK to be converted to glucose and will contribute to depletion of the mitochondrial pool of OAA. The net uptake of propionate and conversion of propionate to glucose also are increased in sheep livers by treatment with glucagon (Ali and Jois, 1997). The role of glucagon in the metabolism of propionate to glucose is questionable, however. In vivo, glucagon failed to increase gluconeogenesis in sheep (Danfær et al., 1995).

In summary, during the hypoglycemic condition that accompanies ketosis, hepatic mitochondrial OAA is depleted for restoration of blood glucose concentrations. The lack of glucose further decreases the supply of pyruvate available for conversion to OAA, which initiates a vicious cycle that limits not only gluconeogenesis but also oxidation of fatty acids. Acetyl-CoA, the entry compound for oxidation of fatty acids in the citric acid cycle, is a powerful allosteric activator of PC; in fact, activity of PC is low without bound acetyl-CoA (Voet and Voet, 1990). The accumulation of acetyl-CoA, therefore, provides a signal that more OAA is needed for oxidation. Likewise, fatty acids converted to fatty acyl-CoA will activate PC. Alternatively, gluconeogenesis from dihydroxyacetone and fructose, "above" the level of PC in the gluconeogenic pathway, are unaffected by fatty acid concentrations in vitro, and the conversion of propionate to glucose also is inhibited by oleate (Brocks et al., 1980).

Fructose biphosphatase (**FBPase**) is responsible for the conversion of fructose-1,6-bisphosphate to fructose-6-phosphate (Figure 3; Voet and Voet, 1991). Glucagon stimulates and insulin inhibits transcription of messenger RNA for FBPase. The regulation of FBPase activity by glucagon occurs also by control of concentrations of fructose-2, 6-bisphosphate (Pilkis and Granner, 1992). When blood glucose concentrations are low, activation of FBPase occurs through glucagon-mediated phosphorylation of FBPase-2, which allosterically activates FBPase. Fructose biphosphatase-2 is inhibited by the presence of fructose-6-phosphate and activated by glyceraldehyde-3-phosphate, a downstream intermediate of the glycolytic pathway. Alternatively, the opposing glycolytic reaction by phosphofructokinase is activated allosterically by fructose-2,6-bisphosphate and inhibited by an excess of citrate from the citric acid cycle (Pilkis and Granner, 1992). Finally, glucose-6-phosphatase is responsible for the conversion of glucose-6-phosphate to glucose for export through the cytosolic membrane and is present only in gluconeogenic tissues. Glucose not exported from hepatocytes can be stored within the cytosol as glycogen.

All three of the described enzymes system could be deficient during the onset of clinical ketosis. Hepatic gluconeogenic capacity from propionate, lactate, alanine, aspartate, and glutamate has been shown to be decreased at the onset of an experimentally-induced clinical ketosis (Mills et al., 1986b).

## **Glycogen Metabolism**

Glycogen is synthesized from glucose by glycogen synthase after glucose is converted to uridine diphosphate glucose via glucose-6-phosphate (Voet and Voet, 1991). Glycogen synthesis is stimulated by ATP and the presence of glucose-6-phosphate. In hepatocytes from rats, the activity of glycogen synthase has been shown to be stimulated also by NEFA in the presence of glucose, and amino acids independently of glucose (Morand et al., 1992). The activity of glycogen synthase can be decreased by the actions of cAMP-dependent protein kinase, protein kinase C, and phosphorylase kinase activity when accompanied by increases in cytosolic concentrations of  $\text{Ca}^{++}$ .

The degradation of glycogen occurs through a bicyclic cascade controlling the activity of glycogen phosphorylase. Phosphorylase kinase activates glycogen phosphorylase in the presence of  $\text{Ca}^{++}$  after being activated by cAMP-dependent protein kinase (Pecker and Pavoine, 1996). Phosphoprotein phosphorylase will deactivate both glycogen phosphorylase and phosphorylase kinase. Glucose itself also is a strong inhibitor of glycogen phosphorylase (Salway, 1994). Additionally, the inhibitory effects of glucose on glycogen phosphorylase are enhanced in the presence of NEFA and amino acids in hepatocytes from rats (Morand et al., 1992).

Glucagon is considered to be the primary regulatory hormone of glycogen synthesis and degradation. The action of glucagon is directed through the adenylate cyclase system, which increases concentrations of cAMP, thereby

increasing the activity of glycogen phosphorylase and decreasing the activity of glycogen synthase (Pecker and Pavoine, 1996).

Low concentrations of glycogen in the liver seem to be a critical component in the development of ketosis. Cows having liver glycogen concentrations of more than one to each two parts TAG on a weight basis have not been susceptible to an experimental ketosis (Smith et al., 1997).

### **Relationships of Insulin and Glucagon During Fatty Liver and Ketosis**

Ketonemic cows have lower concentrations of plasma insulin than do normal cows, and insulin responses to glucose are diminished in ketotic cows compared with those in normal cows (Hove, 1978; Sakai et al., 1993). Because increases of plasma glucose in ketotic cows failed to elicit an insulin response, it is possible that the metabolism of glucose by pancreatic  $\beta$ -cells may be impaired (Newgard and McGarry, 1995). Accompanying the low insulin concentrations observed in ketonemic cows, glucagon concentrations are often elevated during ketosis (Holtenius et al., 1993; Sakai et al., 1993). The low insulin to glucagon ratio caused by decreased insulin concentrations is suspected of stimulating lipolysis in adipose tissue and ketogenesis in liver (Holtenius and Holtenius, 1996b). This relationship exists from a few weeks prepartum to about one month postpartum.

A similar hypoinsulinemia has been observed in lactating rats (Snell, 1991). Concurrent with the hypoinsulinemia was a diminished response of hepatic adenylate cyclase activation by glucagon, leading the author to speculate that

during lactation-associated hypoinsulinemia there exists either decreased numbers of glucagon receptors or attenuation of receptor coupling to adenylate cyclase. Therefore, glucagon resistance as well as diminished insulin responsiveness may be a significant part of the etiology of lactation ketosis and the development of fatty liver.

Poor insulin responses also were observed when glucagon was injected into ketonemic cows (Holtenius, 1993). Their cows also had a diminished response of glucose to glucagon. On the basis of those observations, Holtenius divided ketotic cows into two groups. One group consisted of hypoglycemic and hypoinsulinemic cows that had weak reactions to glucagon. Because this group seemed to have a limited gluconeogenic capacity, he referred to this group as Type I or a primary ketosis. The second group of ketotic cows responded to glucagon injection with increases of both glucose and insulin. He referred to these cows as Type II because of similarities to Type II diabetes in humans. The assumption used by Holtenius was that Type II ketosis is a form of insulin resistance that is associated with fatty liver and is often secondary to other disorders such as displaced abomasums.

Similar observations were made by other researchers (deBoer et al., 1985). At the onset of lactation, concentrations of ketone bodies and glucagon were increased compared with prepartal concentrations and concentrations of glucose and insulin were decreased. As cows became ketonemic from feed restriction, concentrations of glucagon and insulin decreased. Because the decrease in



insulin was more severe, the molar ratio of insulin to glucagon also decreased. In contrast to the ketonemic cows observed by Holtenius (1993), responses to glucose injections (500 ml of 50% dextrose) during the ketonemic periods caused by feed restriction were characterized by increased concentrations of insulin and decreased concentrations of BHBA (deBoer et al., 1985). Glucagon concentrations, however, decreased only slightly after injection of glucose.

### **Treatments and Preventatives for Lactation Ketosis**

Therapies for ketosis target the carbohydrate insufficiency by increasing blood glucose either by direct augmentation or stimulation of glucogenic pathways. Overall effects of these treatments are to increase concentrations of blood glucose and insulin and liver glycogen while simultaneously ameliorating ketogenesis (Baird, 1982).

**Glucose.** In thin, undernourished cows, glucose administered intravenously provides for replacement of inadequate carbohydrate. An injection of glucose will promptly increase blood glucose concentrations, but the effect is short-lived and repeated treatment is usually necessary. Hepatic production of glucose is actually decreased by treatment with glucose (Baird, 1982). Therefore, after the initial increases, blood glucose declines to concentrations that may be higher than before treatment but are still below normal. The administration of glucose is usually in conjunction with other therapeutic measures such as oral propylene glycol or the use of glucocorticoids (Merck, 1986).

Duodenal infusion of glucose during a ketosis induction protocol prevented increases of NEFA and BHBA that normally accompanied the ketotic state (Veenhuizen et al., 1991). Cows receiving glucose also were resistant to the development of fatty liver exhibited by other cows receiving the induction protocol. When administered intravenously for 6 d, glucose decreased concentrations of ketone bodies, NEFA, and glucagon in ketotic cows (Sakai et al., 1993). Simultaneous infusion of insulin with glucose resulted in sustained glucose concentrations after treatment, greater decreases in NEFA and ketone bodies, and more rapid restoration of appetite than glucose alone.

***Propylene glycol.*** Response of ketotic cows to oral propylene glycol is slower than response to glucose and provides the best therapy when administered as supportive therapy to glucose or glucocorticoids (Merck, 1986). Propylene glycol administered to heifers as an oral drench increased glucose and insulin and decreased BHBA and NEFA in blood in a linear fashion respective to dosage (Grummer et al., 1994). Propylene glycol seems to have beneficial effects whether administered as a drench or as a portion of concentrate fed once daily (Christiansen et al., 1997). During feed restriction in heifers, propylene glycol administered by either method increased plasma insulin and decreased NEFA concentrations. Administration as part of a total mixed ration, however, failed to deliver desired effects.

Following drenching with propylene glycol, there was a decrease in the ruminal acetate to propionate ratio, indicating conversion of the propylene glycol to

propionate within the rumen (Studer et al., 1993). Most propylene glycol, however, is absorbed intact through the rumen epithelium and subsequently converted to glucose by sequential conversion to lactate, pyruvate, and OAA (Schultz, 1971). Providing propionate or pyruvate to the liver is stimulatory towards gluconeogenesis; thus effects of propylene glycol are sustained over a greater periods of time than the effects of glucose.

Propylene glycol also may provide a preventative for the development of fatty liver if administered to dairy cows during the prepartal period (Studer et al., 1993). Drenching cows with 1 L of propylene glycol for 10 d prepartum, decreased concentrations of TAG in livers of cows at both 1 and 21 d postpartum. Associated with the decreased liver TAG concentrations were decreases in concentrations of NEFA and BHBA and increases in concentrations of glucose and insulin in blood during the prepartal period.

**Glucocorticoids.** An adrenal insufficiency has been postulated as a causative factor for ketosis (Baird, 1982). It has been observed that administration of adrenocorticotropin to ketotic cows elicits a normal response and also that steroid excretion is similar in normal and ketotic cows (Shultz, 1975). Glucocorticoids increase the release of glucose from the liver by stimulating gluconeogenesis and glycogenolysis. Glucocorticoids can directly stimulate gluconeogenesis by activating transcription of PEPCK and stabilizing PEPCK messenger RNA from degradation (Granner and Pilkis, 1990). Additionally, there is evidence that glucocorticoids have a stimulatory effect on PC (Jones et al., 1993), thereby

increasing the flux of pyruvate toward phosphoenolpyruvate. Glucocorticoids also inhibit the insulin-stimulated uptake of glucose by peripheral tissues. Similarly, inhibition of insulin-stimulated protein synthesis and increased protein catabolism in muscles by glucocorticoids serves to increase the supply of amino acids available to liver for gluconeogenesis (Holtenius and Holtenius, 1996a). Glucocorticoids also are ketogenic in the absence of insulin in monogastric species. Studies in sheep that used a somatostatin analogue to inhibit insulin release, however, have indicated that this ketogenic behavior may not be present in ruminants (Holtenius and Holtenius, 1996a).

Intramuscular injection of glucocorticoids will return blood glucose concentrations to normal within 8 to 10 h and may increase glucose to above normal concentrations within 24 h. Ketone body concentrations in plasma will decrease to normal by 3 to 5 d after glucocorticoid administration (Merck, 1986).

### **Induction of Ketosis**

A protocol to induce early-lactation cows into a ketotic state that resembles on-farm lactation ketosis has been described by others (Smith et al., 1997). Briefly, the protocol consists of the following: feed intakes are monitored during the first 2 wk of lactation; starting at 14 DIM, feed intake is restricted to 80% (DM basis) of previous daily consumption and 1,3-butanediol (BD) is introduced into the diet concurrent with feed restriction; increasing amounts of BD are added to the diet until a maximal amount of approximately 1 L is fed daily; and feed restriction and BD

supplementation continue until cows develop clinical ketosis, which usually occurs at 42 DIM on average (Smith et al., 1997).

Characteristics of cows during the feed restriction and BD supplementation protocol are listed in Table 2. Feed restriction alone is unable to induce cows into a ketosis that metabolically resembles the spontaneous ketosis observed in early-lactation cows. During progressive feed restriction starting 21 d postpartum, cows became transiently ketonemic but not clinically ketotic (deBoer et al., 1985). The addition of BD to the diet provides substrate for formation of ketone bodies. The metabolism of BD to ketone bodies has been described in detail by Smith (1993). 1,3-Butanediol is metabolized to BHBA by the sequential actions of alcohol and aldehyde dehydrogenases in the liver (Drackley et al., 1991). Physiological effects of dietary supplementation with BD resemble those of ketosis (Smith, 1993). These effects include increases in ketone bodies in plasma and urine, increased concentrations of NEFA and acetate in plasma, and decreased concentrations of plasma insulin. In cows, BD increased concentrations of lipid and decreased concentrations of glycogen in liver (Smith et al., 1997; Veenhuizen et al., 1991). Calves receiving supplementation with BD exhibited behavior similar to ketosis, displaying hyperexcitability, nervousness, and incoordination. Most importantly, the feed restriction, BD protocol increases concentrations of acetate in blood along with ketonemia. During starvation ketosis, acetate concentrations in blood are decreased by lesser rates of rumen fermentation (Smith, 1993). Increases in plasma acetate concentrations are a reflection of insulin insufficiency because

Table 2. History of ketosis induction at Iowa State University.

Publication	Parameter	Response
de Boer et al., 1985	Preconditioning	None
	Method	FR <sup>1</sup>
	# of cows ketotic	0 of 6
	Plasma NEFA	NC <sup>2</sup>
	BHBA	NC
	Glucose	NC
	Insulin	Decreased
Mills et al., 1986	Glucagon	Decreased
	Preconditioning	Overfed dry period
	Method	FR-BD <sup>3</sup>
	# of cows ketotic	4 of 5
	Plasma NEFA	Increased
	BHBA	Increased
	Glucose	Decreased
Veenhuizen et al., 1991	Insulin	NC
	Liver TAG	Increased
	Glycogen	Decreased
	Preconditioning	None
	Method	FR-BD
	# of cows ketotic	5 of 6
	Plasma NEFA	Increased
Drackley et al., 1992	BHBA	Increased
	Acetate	Increased
	Glucose	Decreased
	Insulin	Decreased
	Liver TAG	Increased
	Glycogen	Decreased
	Preconditioning	None
Smith et al., 1997	Method	FR-BD
	# of cows ketotic	6 of 10
	Plasma NEFA	Increased
	BHBA	Increased
	Glucose	Decreased
	Insulin	Decreased
	Glucagon	NC
	Liver TAG	Increased
	Glycogen	Increased
	Preconditioning	Overfed late lactation
	Method	FR-BD
	# of cows ketotic	6 of 10
	Plasma NEFA	Increased
	BHBA	Increased
	Glucose	Decreased
	Insulin	Decreased
	Glucagon	NC
	Liver TAG	NC
	Glycogen	Decreased
	Preconditioning	Overfed late lactation

<sup>1</sup>FR = feed restriction.<sup>2</sup>NC = not changed.<sup>3</sup>BD = 1,3-butanediol.

insulin is responsible for metabolism of acetate (Baird, 1982). Therefore, BD supplementation of the diet, along with feed restriction, produces a physiological condition that resembles on-farm lactation ketosis rather than the starvational ketonemia of feed restriction alone.

Development of a model of the etiology of ketosis through the use of the feed restriction and BD protocol has shown that the development of fatty liver precedes the occurrence of ketosis (Drackley et al., 1992; Smith et al., 1997; Veenhuizen et al., 1991). In fact, fatty liver seems to be a prerequisite for ketosis to occur. Cows made ketotic by use of the protocol had liver TAG to glycogen ratios greater than 1.8 to 1 on a weight basis (Drackley et al., 1992; Smith et al., 1997). Cows with livers having TAG to glycogen ratios less than 1.5 to were resistant to the onset of ketosis during this protocol.

Susceptibility to the development of fatty liver and ketosis during the ketosis induction protocol seems to be related to prepartal nutrition also. Cows that were not fed to a condition of obesity prior to parturition were generally resistant to the effects of the protocol (Drackley et al., 1992). Cows overfed to become obese during the latter part of the previous lactation were less susceptible to ketosis than cows that were overfed during the dry period (Mills et al., 1986a; Smith et al., 1997).

### **Summation of Literature**

Fatty liver is a "gateway" disorder that occurs in periparturient dairy cows and increases the susceptibility of cows to ketosis and other metabolic aberrations

during the postpartal period. Cows with fatty liver are characterized by obesity, inappetance, and insulin resistance. These conditions contribute to mobilization of fatty acids from adipose tissue to meet the increasing energy demands of early lactation. The efflux of fatty acids from adipose tissue must be metabolized or transported by the liver. The inability of the liver to do so increases fatty infiltration of the liver and contributes to ketogenesis. The metabolic fuel of the cow changes from carbohydrates to lipids during these events, and the cow suffers from an insufficiency of blood glucose at the same time that concentrations of ketone bodies increase, creating a state of ketoacidosis.

Glucagon is the primary hormone responsible for maintaining concentrations of glucose in blood of dairy cows through the processes of gluconeogenesis and glycogenolysis in the liver. If treatment with glucagon were to be able to elevate blood glucose concentrations in cows with fatty liver, insulin sensitivity may be restored and the cascade of fatty acid mobilization and ketogenesis could be reversed. Thus, carbohydrate metabolism in postparturient cows suffering from fatty liver could be normalized and lipid infiltration of the liver could be alleviated, restoring productivity.

A protocol to induce an experimental ketosis that resembles lactation ketosis observed in dairy cows has been described. This protocol provides a method to test the hypothesis that glucagon may be able to reverse the metabolic aberrations associated with fatty liver and ketosis under controlled conditions. Because the severity of the ketotic state can be manipulated by altering the quantity of BD fed,



use of the feed restriction and BD protocol can provide the most severe test for evaluation of the efficacy of glucagon as a remedy for fatty liver and lactation ketosis.

## **METABOLIC RESPONSES OF DAIRY CATTLE TO VARIOUS INTRAVENOUS DOSAGES OF GLUCAGON**

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### **Abstract**

To measure the capacity of glucagon to improve carbohydrate status in dairy cattle, glucagon was infused intravenously for 48 h into lactating dairy cows and nonpregnant heifers in three crossover experiments. During experiment one, 5 and 20 mg/d were infused into four midlactation Holstein cows. Experiment two consisted of infusion of 0, 2.5, 5.0, and 10 mg/d into eight nonpregnant, nonlactating, Holstein heifers with each receiving two of the dosages. Experiment three was comprised of four early-lactation Brown Swiss cows treated with 5 and 10 mg/d. Treatment with glucagon increased plasma glucose concentrations in a linear, dose-dependent fashion in each experiment for the duration of the 48-h treatment periods. Concentrations of insulin were increased in a nondose-dependent manner by glucagon. Plasma urea nitrogen concentrations were increased with glucagon at 5 mg/d during experiment two and tended to be decreased by glucagon during experiment three. Concentrations of nonesterified fatty acids in plasma were not affected on average; during experiments one and two, however, they were increased by 20 and 10 mg/d dosages, respectively. Concentrations of  $\beta$ -hydroxybutyrate were increased only by the 20 mg/d dosage.

During experiment one, liver glycogen concentrations decreased by 2.1% wet wt for both dosages, and concentrations of total lipid in livers were increased by 0.6% wet wt by 20 mg/d. Milk fat percentage was increased by glucagon, but milk and milk protein production was decreased. Glucagon improved carbohydrate status over an extended period, and lipolytic effects were evident only at the higher dosages.

### **Introduction**

Lactation ketosis is a metabolic disorder that occurs in early-lactation dairy cows and is characterized by hypoglycemia and hyperketonemia (Baird, 1982). The primary cause of ketosis in a lactating dairy cow is an insufficiency of blood glucose to support milk production at a time when feed intake is depressed. This decrease in carbohydrate status precipitates a decline in concentrations of plasma insulin, an increase in mobilization of fat from adipose tissue, and increased hepatic ketogenesis (Baird, 1982). The increase in mobilization of fatty acids leads to fatty infiltration of the liver, and this accumulation of fat in the liver is thought to occur prior to visible symptoms of ketosis (Veenhuizen et al., 1991). Clinical ketosis therefore is characterized by decreases of blood glucose and insulin and increases of blood NEFA, BHBA, and ACAC along with the development of fatty liver (Baird, 1982; Mills et al., 1986; Smith et al., 1997).

Treatments for ketosis generally address the primary cause of ketosis by attempting to restore blood glucose concentrations (Baird, 1982). Intravenous administration of glucose, oral administration of propylene glycol, and intramuscular

injection of glucocorticoids are commonly used methods for directly increasing blood glucose, supplying precursor for glucose synthesis, and stimulating gluconeogenesis, respectively. These effects also could be accomplished by administration of glucagon to ketotic cows. Glucagon may be able to restore blood glucose concentrations both immediately and long-term because it has both glycogenolytic and gluconeogenic activities (Cryer, 1996; Williamson et al., 1971).

Because of the decline in carbohydrate status, it has been postulated that an insufficiency of circulating glucagon may be partly responsible for onset of ketosis (Baird, 1982; de Boer et al., 1986). This postulate is supported by observations that glucagon concentrations are decreased in blood of cows that are obese at calving (Smith et al., 1997) and they decline further during periods of ketonemia (de Boer et al., 1986). Treatment of early lactation cows with a single injection of either 520 µg or 2.0 mg of glucagon was able to increase concentrations of glucose and insulin in both normal and ketonemic cows (de Boer et al., 1986; Holtenius and Tråvén, 1990). Glucagon, however, has a physiological half-life of only 5 min in dairy cattle (de Boer et al., 1986); therefore, glucagon therapy must be administered continuously for sustained effects.

Glucagon also is known to increase mobilization of fatty acids from adipose tissue in nonruminant animals, and thus it can be ketogenic (Aeillo et al., 1984; Iwanji, 1996). Past research has shown that the lipolytic and ketogenic effects of glucagon observed in nonruminant animals (Williamson et al., 1971) may not be present or, at the least, are blunted in ruminant animals (Baird, 1982; Basset, 1971).

Furthermore, lipolytic effects were not present in dogs (Flakoll et al., 1994) or sheep (Brockman, 1978) when insulin to glucagon ratios were normalized while the animal was in a hyperglucagonemic state. If lipolysis is not stimulated in dairy cows by glucagon, this hormone may provide an effective means of increasing glucose concentrations, thereby alleviating lactation ketosis.

Our experiments were designed and conducted as preliminary investigations to determine the efficacy of glucagon as a treatment to improve the carbohydrate status of dairy cows. For glucagon to be an effective preventative or treatment for ketosis, the glucogenic capacity of glucagon must offset potentially harmful ketogenic effects. Therefore, the action of glucagon was observed first in midlactation cows and mature heifers before an experiment was conducted with early lactation cows.

## **Materials and Methods**

### **Experiment One**

***Experimental design.*** Two blocks of two midlactation (177 DIM average) Holstein cows from the Iowa State University dairy herd were infused with glucagon at 5 and 20 mg/d via jugular vein catheters for 48 h in a crossover design. Lyophilized bovine glucagon (donated by Eli Lilly and Co., Indianapolis, IN) was dissolved in 0.15 M NaCl (preadjusted to pH 10.25 with NaOH) at concentrations that would deliver 5 or 20 mg/d when infused at 30 ml/h. To prevent adherence of glucagon, all glassware and tubing to come in contact with the glucagon infusate

were rinsed with 2% (wt/vol) BSA in 0.15 M NaCl. Solutions of glucagon were prepared and stored for no more than 24 h at 4°C until infusion. All cows were handled and treated in accordance with guidelines established by the Iowa State University Committee on Animal Care.

***Samples and analysis.*** Milk production was recorded, and milk samples were collected for quantification of fat, protein, and total solids by midinfrared reflectance spectrophotometry (Fossomatic, Swiss Valley Farms, Hopkinton, IA) 2 d before, during, and 2 d after infusion of glucagon. Liver samples were collected via puncture biopsies about 48 h before infusions began (Smith et al., 1997). Samples of livers were blotted free of blood, placed in liquid nitrogen for transport, and stored at -80°C until analysis for concentrations of total lipid and triacylglycerol (Smith et al., 1997) and glycogen (Mills et al. 1986).

Bilateral jugular vein catheters for infusion and blood collection were placed in cows after the preinfusion liver biopsy. Catheters were kept patent with sodium heparin (20 IU/ml in 0.15 M NaCl) until infusions began and between collection of blood samples. To establish baseline concentrations of metabolites, blood samples were collected at 1-h intervals for 4 h just before glucagon infusions. After infusions began, blood samples were collected at 5, 10, 20, 30, 45, 60, 90, 120, 180, 240, 300, 360 min and every 6 h thereafter until infusions ended at 48 h. After each infusion, blood was collected for an additional 12 h at the same intervals as at the start; then, a second liver biopsy was taken. Five days after the first replication, liver biopsies were collected again, catheters were reinserted, and the previous

procedures were repeated for the second replication with the alternate dosage of glucagon.

Blood samples were transferred into three 10-ml culture tubes each containing 50 USP of heparin, one of which also contained 75  $\mu$ l of 4% NaF to prevent glycolysis, and placed on ice immediately. Plasma was prepared from samples within 1 h and stored at -20°C until analysis for concentrations of glucose (kit number 315, Sigma Chemical Co., St. Louis, MO), NEFA (Smith et al., 1997), and BHBA (Williamson and Mellanby, 1974). Insulin and glucagon were analyzed by radioimmunoassay (Amaral, 1988; Herbein et al., 1985). Plasma samples designated to be assayed for glucagon contained 2500 IU/ml aprotinin (Trasylo®; Mobay Chemical Corp., FBA Pharmaceuticals, New York, NY).

**Statistical analysis.** Repeated measures were summarized as means for each cow and period (e.g., preinfusion, infusion, and postinfusion periods) before statistical analysis. Measures from samples collected during the preinfusion period were compared with measures from samples collected at similar times of the day during infusion periods for effects of glucagon and compared with postinfusion measures for residual effects. Data were subjected to analysis of variance as a design for a split plot in time design by using the general linear models procedure of SAS (SAS, 1988). Block, glucagon dosage, order of dosage, and replication within block were considered as whole-plot effects and tested against the interaction of block, dosage, order of dosage, and replication within block as the error term. Period and dosage  $\times$  period interaction were subplot effects and were tested

against the residual error (Cochran, 1957). Orthogonal contrasts for effects of glucagon both during and after treatment (i.e., the preinfusion vs. the infusion period and the preinfusion vs. the postinfusion periods, respectively) were constructed from subplot effects.

Comparisons of the effects of glucagon at 5 vs. 20 mg of glucagon daily, order of dosage, and replication within block were conducted only on measures from blood samples collected from 12 to 48 h during infusions. The term for period effects was dropped from the model, and residual error was used to test for significance. Data from blood samples collected during the first 4 h of infusions were used to calculate dose response measures of peak heights, time to peak heights, and area under the curves of glucose concentrations, which were all compared as already described for dosage effects. Because only four cows were used in experiment one, significance was declared at  $P \leq 0.10$  and trends at  $P \leq 0.20$ .

## Experiment Two

**Experimental design.** Eight nonpregnant Holstein heifers at Eli Lilly & Co., Greenfield, IN were assigned randomly to either a high concentrate (HC) or low concentrate (LC) diet 4 mo earlier with four receiving each diet. Diets were fed to supply equal NE for average gains of about 0.35 kg/d for the heifers that weighed  $458 \pm 27$  kg at the start of the glucagon experiment. Diets consisted of a corn-soybean meal concentrate, corn silage, and alfalfa haylage. Assuming 50% of the



corn silage as concentrate, the HC diet contained 80% concentrate and the LC diet contained 43%.

The heifers were prepared for infusion of glucagon as described for experiment one. Following a 24-h sampling period to establish baseline blood measures, treatments of glucagon at 0, 2.5, 5.0, or 10 mg/d were conducted for 48 h, and blood sampling continued. Blood was collected for another 24 h after infusions ended to determine residual effects. At 24 d after the first replication, glucagon dosages were reassigned within each diet as the second period of a  $4 \times 4$  Latin square, and a second replication was performed. Thus, two replications were completed for each diet.

***Samples and analysis.*** Between blood samples collected at less than 30-min intervals, catheters were kept patent with sodium citrate (5% wt/vol in 0.15 M NaCl). For longer durations between samples, sodium heparin was used. After collection, blood samples were transferred into 10 ml tubes containing sodium EDTA, centrifuged for collection of plasma, and analyzed for concentrations of glucose, urea nitrogen (**PUN**), NEFA, BHBA, and ACAC by using a Microcentrifugal autoanalyzer (Monarch Plus Instrumentation Laboratories, Lexington, MA) usually within 24 h of collection.

***Statistical analysis.*** Statistical comparisons were conducted as described for experiment one. Diet (HC vs. LC) was substituted for block as a main effect and additional contrasts were constructed to determine linear and quadratic effects of

dosage of glucagon over the 48-h infusion period. Significance was declared at  $P < 0.05$  and tendencies at  $P < 0.10$ .

### Experiment 3

**Experimental design.** Two blocks of two early-lactation (35 DIM average) Brown Swiss cows from the Iowa State University dairy herd were infused with glucagon at 5 and 10 mg/d in a crossover design. Bilateral jugular vein catheters for infusion and blood collection were placed in each of the cows 24 h before the start of infusions. Catheters were kept patent with sodium heparin until the start of the infusions and between blood collections of greater than 30-min intervals. Between intervals of less than 30 min, catheters were kept patent with sodium citrate. After each cow received the dosage of glucagon assigned for the first 48-h, dosages were reversed abruptly, and infusion continued for another 48 h. Glucagon infusate was prepared as in experiment one at concentrations that would provide 5 and 10 mg/d when infused at 20 ml/h.

**Samples and analysis.** To establish baseline concentrations of metabolites, blood was collected at 30-min intervals for 2 h just before glucagon infusions. Thereafter, samples were collected as in experiment one. Plasma was prepared from blood, stored at  $-20^{\circ}\text{C}$  until completion of the experiment, packed in dry ice, and shipped to Greenfield, IN for analysis of concentrations of glucose, PUN, NEFA, BHBA, and ACAC.

**Statistical analysis.** Data were analyzed as described for experiment one with the following changes: Repeated measures were summarized as means for each cow and period (e.g. preinfusion, first 48-h infusion, second 48-h infusion, and postinfusion periods) before statistical analysis. Replication was dropped from the model. Block and order of dosage were considered whole-plot effects and tested against the interaction of block  $\times$  order of dosage as the error term. Period and order  $\times$  period interaction were subplot effects and were tested against the residual error. For comparison of effects of 5 vs. 10 mg of glucagon daily, only the two 48-h infusion periods were used and dose and dose  $\times$  order interaction were tested against residual error.

## Results

### Experiment One

Plasma immunoreactive glucagon concentrations were increased about 50% by glucagon infusions, but, because of great variability, they were not significantly different between cows receiving glucagon at 5 vs. 20 mg/d. The primary effect of both dosages of glucagon on metabolic responses was an increase ( $P = 0.01$ ) of plasma glucose during the infusion period (Figure 1A). With the initiation of glucagon, glucose concentrations increased dramatically, peaked 25 to 30 min after infusions began, and decreased during the next 4 to 6 h until elevated concentrations stabilized above the preinfusion period. Peak glucose

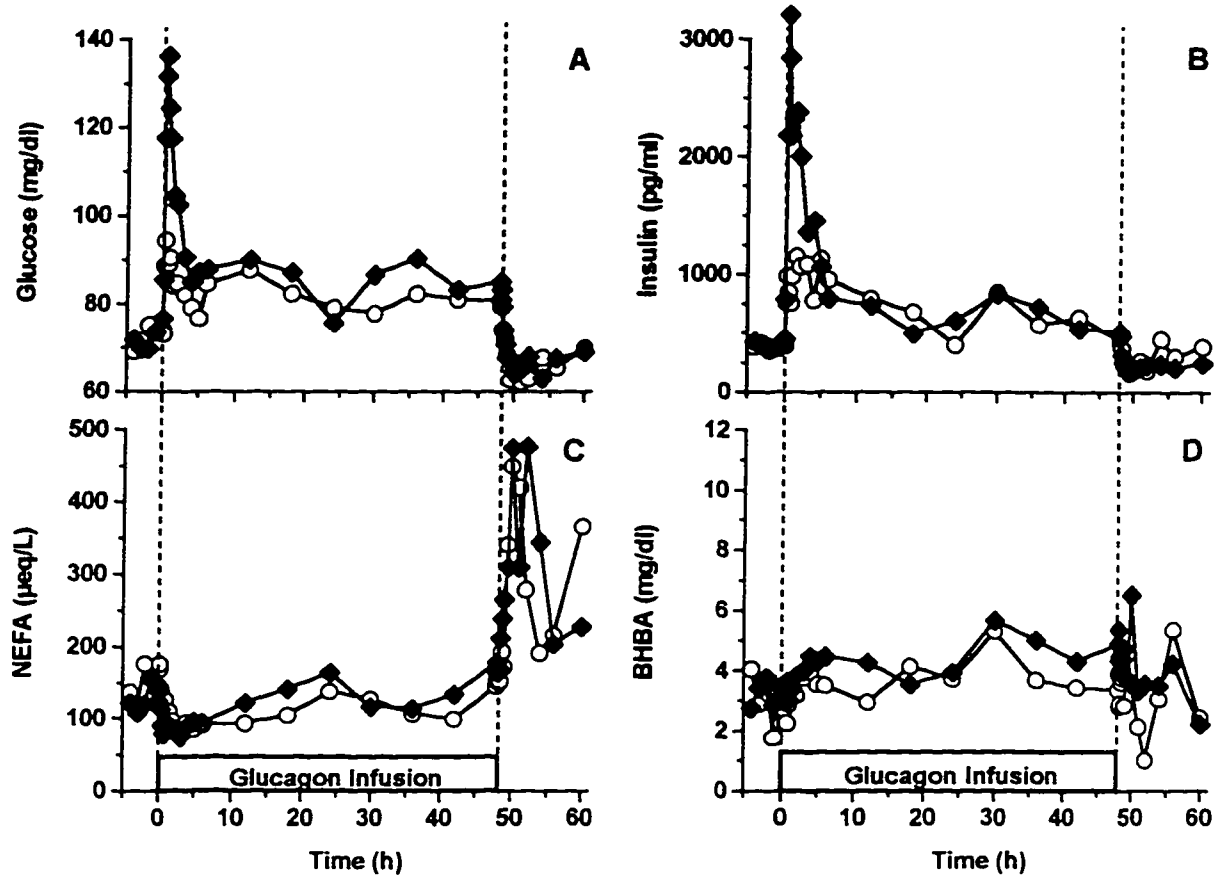


Figure 1. Plasma metabolite and insulin concentrations in cows in experiment one before, during, and after treatment with glucagon at 5 (O) and 20 mg/d (◆) for 48 h. **A. Glucose.** Effects in model: before vs. during glucagon ( $P = 0.002$ , SEM = 1.23), before vs. after glucagon ( $P = 0.61$ , SEM = 1.28), and glucagon at 5 vs. 20 mg/d ( $P = 0.10$ , SEM = 1.03). **B. Insulin.** Effects in model: before vs. during glucagon ( $P = 0.15$ , SEM = 56.4), before vs. after glucagon ( $P = 0.13$ , SEM = 54.3), and glucagon at 5 vs. 20 mg/d ( $P = 0.82$ , SEM = 111). **C. NEFA.** Effects in model: before vs. during glucagon ( $P = 0.67$ , SEM = 18.7), before vs. after glucagon ( $P = 0.03$ , SEM = 25.4), and glucagon at 5 vs. 20 mg/d ( $P = 0.44$ , SEM = 15.5). **D. BHBA.** Effects in model: before vs. during glucagon ( $P = 0.04$ , SEM = 0.22), before vs. after glucagon ( $P = 0.01$ , SEM = 0.20), and glucagon at 5 vs. 20 mg/d ( $P = 0.11$ , SEM = 0.19).

concentrations and total response area during the first 4 h of infusion were increased 2.5 times by glucagon at 20 compared with 5 mg/d (Table 1). The time to peak response did not vary between the two dosages of glucagon but occurred sooner (35 vs. 20 min,  $P = 0.09$ ) during the second replication of glucagon treatment, regardless of dosage. Increases of glucose concentrations from baseline to peak height in response to glucagon were also greater (53.8 vs 44.8 mg/dl,  $P = 0.10$ ) during the first replication of glucagon treatment compared with the second.

Throughout the remainder of the 48-h infusion period, glucose concentrations were higher ( $P = 0.10$ ) in cows receiving glucagon at 20 compared with 5 mg/d. Comparing the preinfusion period to the same time of day during the infusion period, glucose concentrations were increased by 7.5 and 10.4 mg/dl for 5 and 20 mg/d dosages. The order in which the two dosages were administered had no effect ( $P = 0.44$ ) on plasma glucose response; however, glucose concentrations tended to be greater ( $P = 0.16$ ) during the first replication regardless of dosage than during the second (84.9 vs. 81.7 mg/dl). After infusions, glucose concentrations rapidly declined to preinfusion levels ( $P = 0.61$  for residual effects).

Plasma insulin concentrations also were increased ( $P = 0.001$ ) by glucagon compared with preinfusion values (Figure 1B); the increases, however, were not different ( $P = 0.82$ ) between dosages. Insulin concentrations were not affected by the order of dosage administration. In contrast to glucose concentrations, plasma insulin concentrations tended ( $P = 0.13$ ) to be lower during the postinfusion vs. the preinfusion period.

Table 1. Response of plasma glucose in dairy cattle to glucagon at 0, 2.5, 5, 10, and 20 mg/d during the first 4 h of treatment.

Item	Dosage of glucagon, mg/d					SEM <sup>1</sup>	P > F <sup>2</sup>
	0	2.5	5.0	10	20		
Time to peak <sup>3</sup> , min							
Experiment 1	-	-	30	-	25	3.5	0.41
Experiment 2	-	90	146	20	-	31.8	0.20
Experiment 3	-	-	53	17	-	3.6	0.09
Peak height <sup>4</sup> , mg/dl							
Experiment 1	-	-	30	-	68	2.2	0.006
Experiment 2	11	27	38	54	-	7.2	0.02
Experiment 3	-	-	25	54	-	0.2	0.007
AUC <sup>5</sup> , mg/dl×h							
Experiment 1	-	-	51	-	138	14.5	0.05
Experiment 2	14	71	115	113	-	17.0	0.03
Experiment 3	-	-	63	133	-	2.3	0.03

<sup>1</sup> Experiments 1 and 2, number of cows at each dosage = 4; Experiment 3, number of cows at each dosage = 2.

<sup>2</sup> For linear effect of dosage of glucagon on glucose response.

<sup>3</sup> Time to peak concentration from the start of glucagon infusion.

<sup>4</sup> Peak height above baseline concentrations.

<sup>5</sup> AUC = area under the curve minus baseline concentrations.

Plasma NEFA concentrations were not significantly affected ( $P = 0.67$ ) by glucagon (Figure 1C); however, compared with preinfusion concentrations, they were decreased 9  $\mu\text{eq/L}$  during infusion of glucagon at 5 mg/d but were increased by 33  $\mu\text{eq/L}$  during the 20 mg/d infusion. After cessation of glucagon, NEFA concentrations increased rapidly for both dosages and remained elevated ( $P = 0.03$  for preinfusion vs. postinfusion) throughout the remainder of the sampling period.

Concentrations of BHBA in plasma were greater during both infusion ( $P = 0.03$ ) and postinfusion ( $P = 0.01$ ) periods than during the preinfusion period (Figure 1D). Glucagon administered at 20 mg/d elevated plasma BHBA concentration to a greater ( $P = 0.10$ ) extent than did 5 mg/d. Contrary to that observed with plasma

glucose concentrations, the increase in BHBA concentrations tended ( $P = 0.11$ ) to become greater during the second replication of each infusion.

Because of an inability to obtain a liver biopsy from one cow, only three cows were used to determine the effects of glucagon on liver composition (data not shown). Glycogen concentrations in livers were decreased ( $P = 0.05$ ) from 3.5 to 1.4% wet wt, on average, by both dosages of glucagon, and the magnitude of changes was not affected by dosage. Concentrations of total lipid in livers were increased (3.6 vs. 4.2% wet wt) by 20 mg/d of glucagon, but they were slightly decreased (3.9 vs. 3.5% wet wt) when 5 mg/d was given. Because of the small number of cows, dosage effects were nonsignificant; however, the increase in liver lipid concentrations after 20 mg/d of glucagon created a significant period effect ( $P = 0.04$ ). Liver triacylglycerol concentrations in these midlactation cows were very low (mean =  $0.40 \pm 0.03$ ), and no changes were detectable. The effects of glucagon on liver lipid content suggest that the optimal dosage of glucagon that will increase blood glucose concentrations and yet not adversely affect liver lipid metabolism is between 5 and 20 mg/d.

Data from first milkings immediately after the start and end of infusions were dropped from analysis because they represented transitional adjustments to treatment. Milk production was decreased ( $P = 0.09$ ) by both dosages of glucagon but returned to preinfusion levels during the postinfusion period (Table 2). Percentages of fat in milk were increased both during ( $P = 0.07$ ) and after ( $P = 0.002$ ) infusion. Percentages of protein in milk were decreased also ( $P = 0.0001$ ) by

Table 2. Production and composition of milk from dairy cows before, during, and after treatment with glucagon at 5 and 10 mg/d.

Item	Dosage of Glucagon, mg/d						SEM <sup>2</sup>	Contrast <sup>1</sup>		
	5			20				GLN	Dose	RES
	Before	During	After	Before	During	After				
Milk, kg/d	31.7	26.2	32.6	29.6	24.4	25.5	3.4	0.09	0.73	0.61
Fat										
%	3.22	3.32	4.09	3.24	3.31	5.22	0.58	0.07	0.99	0.002
kg/d	0.84	0.80	1.28	0.98	0.93	1.69	0.06	0.03	0.32	0.03
Protein										
%	3.27	2.76	3.32	3.00	2.36	3.00	0.13	0.0001	0.10	0.29
kg/d	1.01	0.68	1.18	0.90	0.64	1.09	0.08	0.08	0.67	0.55
Solids										
%	12.06	11.92	13.55	11.85	11.64	13.57	0.65	0.01	0.77	0.002
kg/d	3.51	2.89	4.43	3.52	3.20	4.53	0.22	0.08	0.57	0.23

<sup>1</sup> GLN = effect of glucagon, Dose = effect dosage of glucagon, RES = residual effect.

<sup>2</sup> Number of cows at each dosage = 4.

glucagon, and they were lower ( $P = 0.10$ ) in milk from cows receiving glucagon at 20 mg/d than at 5 mg/d. Postinfusion milk protein concentrations were similar to preinfusion concentrations indicating short-lived effects of glucagon on milk protein secretion. Percentage of total solids in milk was decreased ( $P = 0.02$ ) during infusion and increased ( $P = 0.002$ ) after infusion (data not shown).

## Experiment Two

Similarly to the effects of glucagon observed in experiment one, glucose concentrations increased dramatically at the start of glucagon treatments in experiment two; times to peak glucose concentrations, however, ranged from 20 min in heifers receiving glucagon at 10 mg/d to 146 min in heifers receiving 5 mg/d (Table 1). Dosage of glucagon did not affect time to peak concentrations of glucose



( $P = 0.20$ ). Peak concentrations and total response area during the first 4 h of infusion were increased linearly ( $P < 0.03$ ) by dosage of glucagon. Diet had no effect on peak times, peak heights, or area under the curves of glucose responses to glucagon. Replication did not affect any of the initial responses of blood glucose to glucagon.

During preinfusion and postinfusion periods, glucose concentrations were similar between heifers receiving the HC diet compared with the LC diet. During the infusion period, however, plasma glucose concentrations were 3.5 mg/dl higher in heifers receiving the LC diet than in heifers receiving the HC diet (Figure 2A). Glucagon increased plasma glucose concentrations in a linear fashion ( $P = 0.006$ ) with respect to dosage compared with preinfusion and 0 mg/d of glucagon (Table 1). Quadratic effects of glucagon dosages on plasma glucose concentrations were not significant ( $P = 0.20$ ). Plasma glucose concentrations were decreased slightly (5.7 mg/dl,  $P = 0.02$ ) during the second replication compared with the first replication. After treatments were completed, plasma glucose concentrations were 5 mg/dl lower ( $P = 0.002$ ) than preinfusion concentrations.

Plasma urea nitrogen concentrations tended to be greater ( $P = 0.10$ ) in heifers receiving the LC diet vs. the HC diet. This effect was amplified by glucagon ( $P = 0.04$ , Figure 2B). Glucagon increased ( $P = 0.0001$ ) PUN primarily because of the large increase observed in heifers fed the LC diet receiving glucagon at 5 mg/d. The effect of glucagon on PUN was neither linear nor quadratic. During the

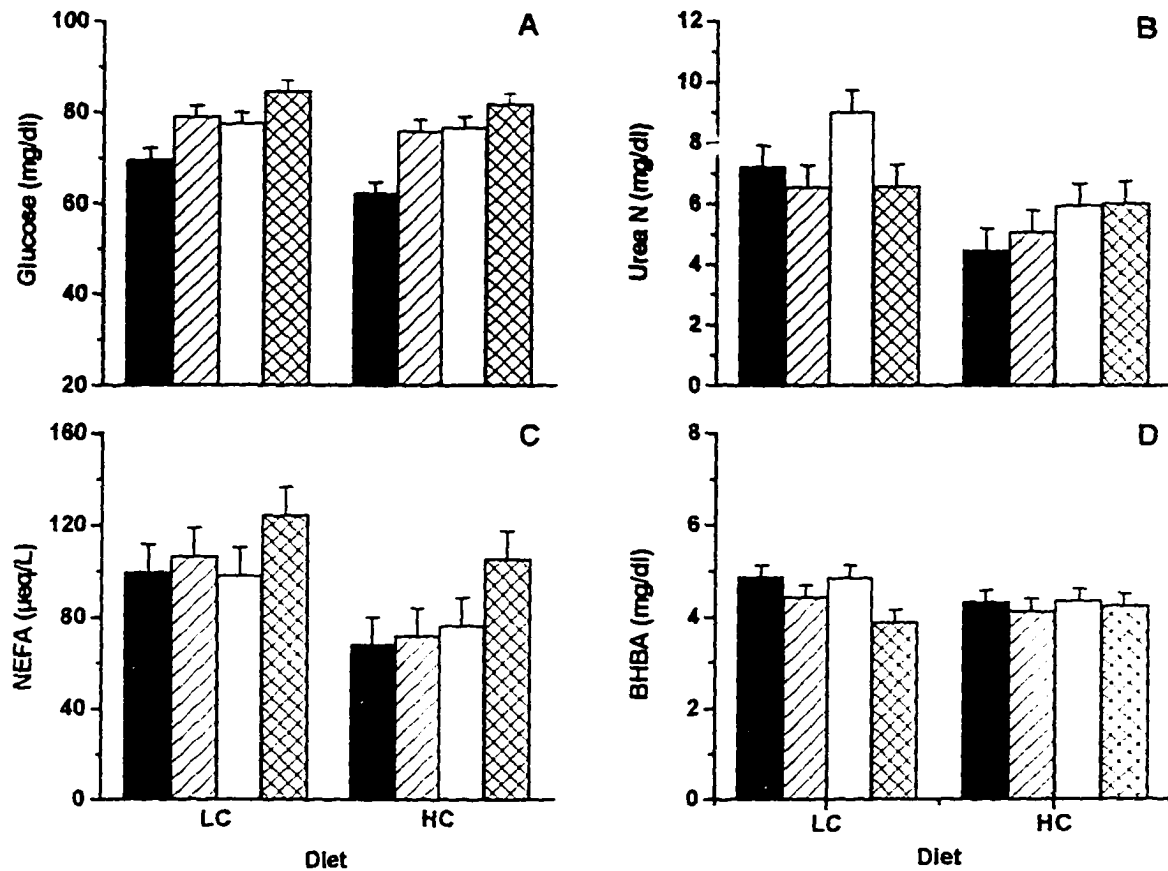


Figure 2. Plasma metabolite concentrations in experiment two with heifers fed low concentrate (LC) and high concentrate (HC) diets during 48 h infusions of 0 (■), 2.5 (▨), 5.0 (□), and 10 (▩) mg/d. **A. Glucose.** Effects in model: LC vs. HC diets ( $P = 0.04$ , SEM = 1.20), glucagon at 2.5, 5.0 and 10 vs. 0 mg/d ( $P = 0.006$ , SEM = 2.39), linear effect of glucagon dosage ( $P = 0.007$ , SEM = 2.39), and quadratic effect of glucagon dosage ( $P = 0.20$ , SEM = 2.39). **B. Urea nitrogen (N).** Effects in model: LC vs. HC diets ( $P = 0.04$ , SEM = 0.35), glucagon at 2.5, 5.0 and 10 vs. 0 mg/d ( $P = 0.32$ , SEM = 0.70), linear effect of glucagon dosage ( $P = 0.40$ , SEM = 0.70), and quadratic effect of glucagon dosage ( $P = 0.32$ , SEM = 0.70). **C. NEFA.** Effects in model: LC vs. HC diets ( $P = 0.07$ , SEM = 6.02), glucagon at 2.5, 5.0 and 10 vs. 0 mg/d ( $P = 0.27$ , SEM = 12.0), linear effect of glucagon dosage ( $P = 0.07$ , SEM = 12.0), and quadratic effect of glucagon dosage ( $P = 0.28$ , SEM = 12.0). **D. BHBA.** Effects in model: LC vs. HC diets ( $P = 0.38$ , SEM = 0.13), glucagon at 2.5, 5.0 and 10 vs. 0 mg/d ( $P = 0.29$ , SEM = 0.26), linear effect of glucagon dosage ( $P = 0.18$ , SEM = 0.26), and quadratic effect of glucagon dosage ( $P = 0.60$ , SEM = 0.26).

postinfusion period, PUN concentrations were not different ( $P = 0.78$ ) than during the preinfusion period.

Plasma NEFA concentrations tended to be greater ( $P = 0.07$ ) during infusions in heifers receiving the LC vs. the HC diet (Figure 2C). On average, glucagon did not increase NEFA concentrations ( $P = 0.27$ ), but there was tendency ( $P = 0.07$ ) for a positive linear effect of glucagon dosage on NEFA that could be attributed to increases of NEFA in heifers receiving 10 mg/d of glucagon. After infusion of glucagon, NEFA concentrations were increased an average of  $86 \pm 14$   $\mu\text{eq/L}$  for all three doses ( $P = 0.0001$ ).

Plasma BHBA concentrations were not affected by diet ( $P = 0.77$ ) or by glucagon ( $P = 0.29$ , Figure 2D). During the postinfusion period, concentrations of BHBA were increased ( $P = 0.0001$ ) when compared with preinfusion concentrations; however, this seems to be an infusion effect rather than a glucagon effect because the increase of  $0.64 \pm 0.1$   $\mu\text{eq/L}$  was consistent across all treatments, including the 0 mg/d dosage. Concentrations of ACAC (data not shown) tended to be decreased ( $P = 0.10$ ) by all dosages of glucagon when compared with the 0 mg/d control.

### Experiment Three

The initial response of plasma glucose concentrations to glucagon was identical to that in the first two experiments (Table 1). Values for peak increases of glucose concentrations and area under the curves during the first 4 h of treatment

were similar to those in experiment one and were increased ( $P < 0.03$ ) by dosage (Table 1). Time to peak concentrations occurred more quickly when cows received glucagon at 10 rather than at 5 mg/d. During glucagon treatments, plasma glucose concentrations increased by 9.2 mg/dl compared with preinfusion baseline measures ( $P = 0.01$ , Figure 3A).

Glucagon at 10 mg/d increased glucose concentrations more than did 5 mg/d throughout the infusion period (76.4 vs.  $70.8 \pm 1.3$  mg/dl,  $P = 0.09$ ). Cows receiving the 10 mg/d dosage of glucagon during the first 48-h of infusion tended to have a greater glucose response to 10 mg/d than did cows receiving 5 mg/d first ( $P = 0.17$  for dose  $\times$  order). During the postinfusion period, glucose concentrations were decreased ( $P = 0.001$ ) when compared with preinfusion concentrations. The postinfusion decrease in plasma glucose concentration were probably short-lived effect of the abrupt cessation of glucagon treatment. A more recent experiment showed that when the rate of glucagon infusion was decreased gradually over a 10-h period the decrease in plasma glucose concentrations was not observed (She, P., 1997).

Plasma urea nitrogen concentrations tended to be decreased by glucagon ( $P = 0.13$ , Figure 3B). Dosage of glucagon did not affect PUN ( $P = 0.26$ ), but concentrations were higher in cows receiving 10 mg/d during the first infusion period ( $P = 0.07$  for dose  $\times$  order). The PUN were decreased postinfusion compared with preinfusion concentrations (17.6 vs. 20.0 mg/dl,  $P = 0.04$ ).

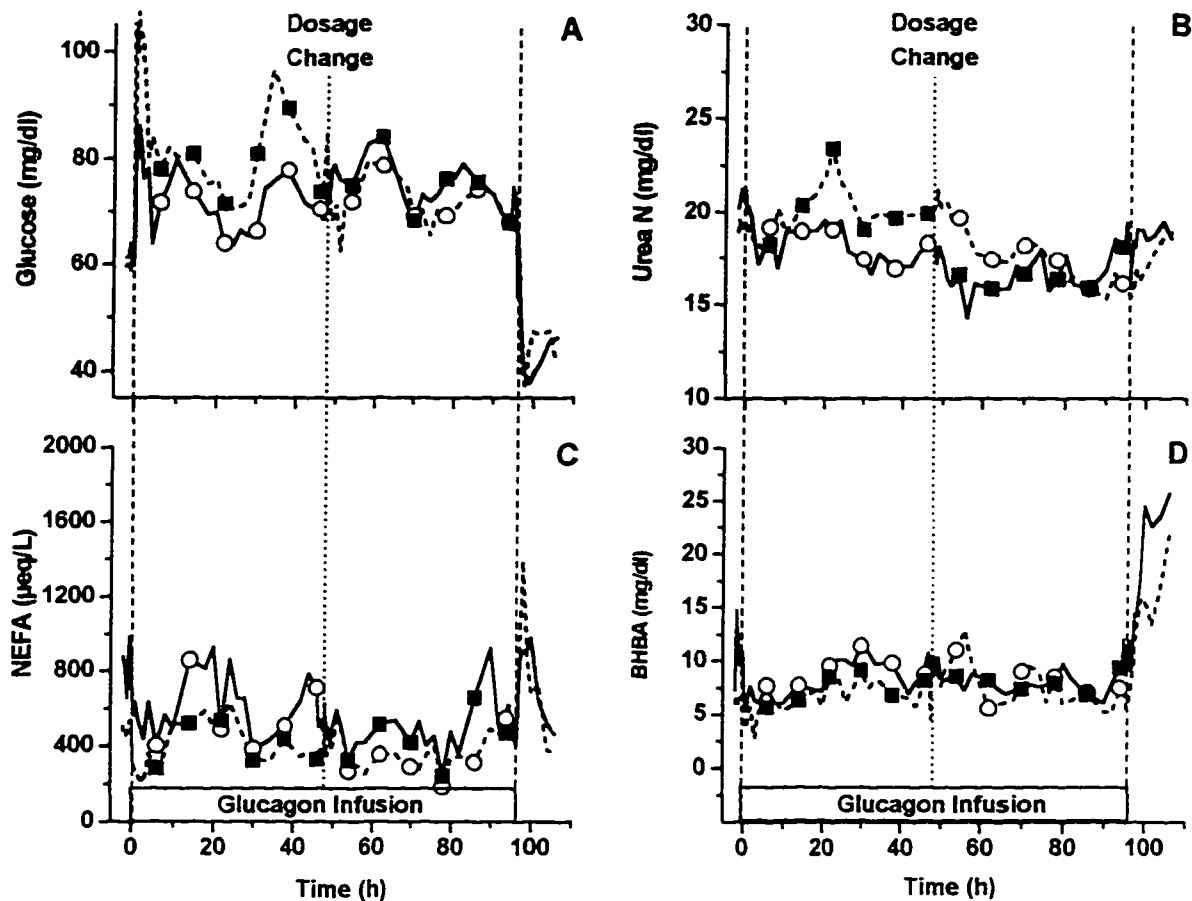


Figure 3. Plasma metabolite concentrations in cows in experiment three before, during, and after treatment with glucagon at 5 (O) and 10 (■) mg/d. Dosages were changed from 5 to 10 mg/d (—) and from 10 to 5 mg/d (--) at h 48. **A. Glucose.** Effects in model: before vs. during glucagon ( $P = 0.01$ , SEM = 1.34), before vs. after glucagon ( $P = 0.0001$ , SEM = 1.17), and glucagon at 5 vs. 10 mg/d ( $P = 0.09$ , SEM = 1.27). **B. Urea nitrogen (N).** Effects in model: before vs. during glucagon ( $P = 0.13$ , SEM = 0.85), before vs. after glucagon ( $P = 0.04$ , SEM = 0.67), and glucagon at 5 vs. 10 mg/d ( $P = 0.26$ , SEM = 0.48). **C. NEFA.** Effects in model: before vs. during glucagon ( $P = 0.007$ , SEM = 22.7), before vs. after glucagon ( $P = 0.99$ , SEM = 20.48), and glucagon at 5 vs. 10 mg/d ( $P = 0.22$ , SEM = 13.3). **D. BHBA.** Effects in model: before vs. during glucagon ( $P = 0.77$ , SEM = 0.51), before vs. after glucagon ( $P = 0.0002$ , SEM = 0.55), and glucagon at 5 vs. 10 mg/d ( $P = 0.66$ , SEM = 0.53).

Concentrations of plasma NEFA were decreased compared with preinfusion by both dosages of glucagon (496 vs. 660  $\mu\text{eq/L}$ ,  $P = 0.007$ ), but dosage of glucagon and the order that dosages were administered did not influence NEFA ( $P = 0.22$ , Figure 3C). Cows receiving glucagon at 5 mg/d the first 48 h, however, had increased NEFA compared with cows receiving 10 mg/d first (613 vs. 439  $\mu\text{eq/L}$ ,  $P = 0.04$  for dose  $\times$  order). After the infusion, plasma NEFA decreased to concentrations similar to preinfusion.

Neither glucagon or dosage of glucagon had any effect ( $P > 0.66$ ) on concentrations of plasma BHBA (Figure 3D) or ACAC (not shown). During the postinfusion period, concentrations of BHBA and ACAC increased dramatically through the end of the sampling period ( $P > 0.01$ ).

## Discussion

Our experiments were the first to measure effects of chronic, low level administration of glucagon into dairy cattle. Previous studies with dairy cows consisted of administration of bolus doses of glucagon followed by response measures similar to those observed during the first 4 h and after cessation of treatment in our experiments (de Boer et al., 1986, Holtenius and Tråvén, 1990).

During the first hour of glucagon treatment, glucose concentrations increased rapidly, creating a temporary hyperglycemic state (Table 1). The severity and duration of the hyperglycemic state was dose dependent in all three experiments. All dosages of glucagon tested increased plasma glucose concentrations in a linear,

dose-dependent fashion in each experiment throughout the 48-h treatment periods (Figures 1A, 2A, and 3A). After glucagon treatment, plasma glucose concentrations declined to pretreatment concentrations in midlactation cows but descended below pretreatment concentrations in heifers and early lactation cows.

The initial hyperglycemic conditions occurring during the first hour of glucagon treatment were alleviated by corresponding increases in plasma insulin concentrations. Insulin response to glucagon was immediate and preceded increases in glucose concentrations (Figure 1B). This effect also was observed in other experiments (de Boer et al., 1986; Holtenius and Tråvén, 1990; Williamson et al., 1971), indicating a direct action of glucagon on pancreatic  $\beta$ -cells that stimulates insulin secretion independently of blood glucose (Cryer, 1996). Throughout the remainder of the treatment periods, concentrations of insulin were increased relative to basal concentrations in a nondose-dependent manner by glucagon.

Plasma urea nitrogen concentrations were increased variably during experiment two and tended to be decreased by glucagon during experiment three (Figures 2B and 3B). Increases in PUN may be attributable to increased uptake and deamination of amino acids as precursors for hepatic glucose production (Brockman, 1978; Flakoll et al., 1994). The lack of effect of glucagon on PUN in lactating cows in our study does not support the concept of increased use of amino acids as gluconeogenic substrates. Similar results were observed when ovine livers were perfused with glucagon (Gill et al., 1985). Concentrations of urea in the

perfusate actually decreased under the influence of glucagon when livers were perfused with propionic acid and threonine. By use of carbon-14 labeling, they determined that the use of propionate for glucose synthesis was increased by glucagon treatment and there was a sparing effect on threonine. In the presence of adequate propionate, as was probably the case for the heifers fed the HC diet and the cows receiving lactation diets in our experiments, use of amino acids as gluconeogenic substrates was probably minimal. Alternatively, the lack of observable changes in PUN concentrations in our cows may result from the capacity of lactating cows to remove and recycle urea and transfer PUN into the mammary gland at rates greater than hepatic ureagenesis stimulated by the increased gluconeogenesis. The possibility of this effect was strengthened by the observation that milk protein production was decreased during treatment with glucagon (Table 2) possibly because gluconeogenic amino acids normally utilized for milk production were diverted to the liver to support increased rates of gluconeogenesis. Effects of glucagon on milk urea nitrogen and protein fractions warrant further studies.

Glucagon has been considered to be a powerful lipolytic agent, which is supported by results of experiments in which glucagon was administered as a single bolus dose (de Boer et al., 1986) or into rats (Williamson et al., 1971). During our experiments, concentrations of plasma NEFA were not increased by glucagon except by the 20 and 10 mg/d dosages in experiments one and two, respectively (Figures 1C and 2C). On the other hand, NEFA increased dramatically after all



dosages of glucagon, probably as a result of the removal of lipolytic inhibition by falling insulin concentrations. The coordination of insulin along with glucagon concentrations is important in controlling peripheral lipolysis because insulin seems to have stronger regulatory effects on peripheral metabolism than does glucagon (Brockman, 1978). The increase in NEFA concentrations after stopping glucagon infusion was reflected by increased milk fat percentages probably as a result of increased precursor availability for milk fat synthesis.

When 520  $\mu\text{g}$  of glucagon was administered as a single bolus injection to early lactation and ketonemic cows, small increases of NEFA were observed within 15 min, but the greatest increases in NEFA concentrations occurred 1.5 to 2 h after injection of glucagon, corresponding with times that insulin concentrations were lowest after removal of glucagon-stimulated insulin secretion (de Boer et al., 1986). In early lactation cows, demands for nutrients for milk synthesis are satisfied by a low insulin-to-glucagon ratio (Herbein et al., 1985) and abnormally low concentrations of insulin cause this ratio to be decreased further in cows susceptible to ketosis (de Boer et al., 1986; Smith et al., 1997).

Taken together, our results and those of de Boer et al. (1986) and Basset (1971) indicate that the insulinotropic action of glucagon may provide a mechanism to normalize insulin concentrations in ketonemic cows and to simultaneously suppress the lipolytic action of glucagon. Similar results were observed when insulin-to-glucagon ratios were held constant during glucagon infusions into dogs (Flakoll et al., 1994). In those experiments, there were no increases in

concentrations of either NEFA or BHBA in plasma. Additionally, in early lactation, ketotic dairy cows, simultaneous administration of glucose and insulin, mimicking the effects of glucagon observed in our experiments, decreased concentrations of NEFA and BHBA in plasma to a greater extent than treatment with glucose alone (Sakai et al., 1993). During our experiments, concentrations of BHBA were increased only by the 20 mg/d dosage used in experiment one (Figure 1D). Similar to, and probably as a result of, increased NEFA concentrations post-treatment, concentrations of ketones were increased by all dosages of glucagon during the post-treatment period (Figures 1D and 3D).

Measures of effects of glucagon on liver composition were only collected during experiment one. As anticipated, glucagon caused mobilization of glycogen from livers to increase plasma glucose concentrations (Cryer, 1996; Iwani, 1996). The quantity of glycogen contained in livers would be inadequate to supply the plasma glucose concentrations observed throughout the 48-h infusion periods. Hepatic glycogenolysis most likely was responsible for the initial glucose response, and the elevated glucose concentrations during the remainder of the treatment periods were likely the result of increased and sustained gluconeogenesis (Cryer, 1996; Williamson et al., 1971).

Total lipids in livers were increased only by the 20 mg/d treatment. The increase in plasma NEFA observed during administration of the 20 mg/d dosage of glucagon was probably responsible for this effect. Increases in NEFA concentrations caused by lipid mobilization from adipose tissue is considered to be

responsible for fatty infiltration of livers of ketotic cows (Baird, 1982; Mills et al., 1986; Smith et al., 1997).

Our results suggest that 10 mg/d may be the appropriate maximal dosage of glucagon for increasing circulating concentrations of glucose in ketosis susceptible lactating dairy cows without having detrimental lipolytic and ketogenic effects. No detrimental effects from glucagon were observed in any of the animals involved in our experiments neither during nor after infusions. Additionally, one early lactation Brown Swiss cow showing signs of ketonemia was infused along with the four experimental cows during experiment three. Observations on this cow revealed that glucagon increased plasma concentrations of glucose from 31.3 to 53.3 mg/dl and decreased plasma concentrations of NEFA and BHBA from 777  $\mu$ eq/L and 43.2 mg/dl to 666  $\mu$ eq/L and 34.8 mg/dl, respectively.

### **Conclusion**

Continuous treatment of dairy cows and heifers with glucagon is able to sustain increased plasma glucose concentrations by both glycogenolytic and gluconeogenic activity. Lipolytic effects of glucagon evidently occur only at the higher dosages tested and after abrupt cessation of glucagon. Our experiments indicate that treatment of early lactation dairy cows with low, chronically administered dosages of glucagon may provide a way to improve carbohydrate status and decrease lipid flux during periods of fatty liver and susceptibility to ketosis.

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## **METABOLIC RESPONSES OF DAIRY COWS WITH FATTY LIVERS TO 14-DAY INTRAVENOUS INFUSIONS OF GLUCAGON**

A paper to be submitted to the Journal of Dairy Science

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L. F. Richardson, and R. W. Tucker

### **Abstract**

Twenty multiparous Holstein cows were offered additional dietary concentrate during the final 30 d prepartum to create a postpartum susceptibility to fatty liver and ketosis. From 14 to 42 days in milk, all cows were subjected to a protocol to induce ketosis. To test the use of glucagon as a treatment for fatty liver, either glucagon at 10 mg/d or vehicle was infused continuously via jugular vein catheters from 21 to 35 days in milk. All cows became ketonemic and hypoglycemic during ketosis induction. Glucagon increased plasma glucose to 142% of concentrations in controls throughout the 14-d treatment period. The hypoinsulinemia present in these susceptible cows, however, was not affected by glucagon. Plasma  $\beta$ -hydroxybutyrate and nonesterified fatty acids were decreased over time by glucagon. At 6 days in milk, liver triacylglycerol concentrations averaged 12.9% of the wet weight of livers. Compared with controls, glucagon had decreased triacylglycerol content of livers by 71% at d 35. Glycogen content of livers was 1.0% of the wet weight of livers at 6 days in milk and was decreased by glucagon to 0.5%, 2 d after glucagon treatment started. Glycogen then increased linearly in livers of cows treated with glucagon until at 38 days in milk glycogen

content was 3.7% compared with 1.6% in livers of controls. Our results indicate that glucagon successfully decreases the degree of fatty liver and improves carbohydrate status in early-lactation dairy cows.

### **Introduction**

Fatty liver occurs in varying degrees in most dairy cows during the peripartal period. Development of fatty liver occurs when fatty acid uptake and TAG synthesis by the liver exceeds the liver's capacity to either oxidize fatty acids or to hydrolyze and export TAG as VLDL (Grummer, 1993). During the final days prepartum and immediately postpartum, feed intakes of dairy cows are depressed and energy requirements for parturition and initiation of lactation are greatly increased. The resulting negative energy balance overloads the liver with NEFA mobilized from adipose tissue, and TAG accumulates in the liver because ruminant liver has a relatively limited capacity to synthesize and export VLDL (Grummer, 1993). The severity of fatty liver can be decreased by avoiding the peripartal DMI depression (Grummer, 1993), but it also can be accentuated by overfeeding during the early prepartal period (Mills et al. 1986a; Van den Top et al., 1996).

Cows having moderate to severe fatty liver are more susceptible to metabolic disorders and immunosuppression (Baird, 1980; Franklin et al., 1991). The metabolic disorder most commonly associated with fatty liver is lactation ketosis. In fact, fatty liver seems to be a prerequisite for development of ketosis. Under an experimentally induced ketosis, the development of fatty liver preceded the

occurrence of ketosis, and cows without fatty liver were resistant to ketosis (Mills et al., 1986a; Smith et al., 1997; Veenhuizen et al., 1991).

Lactation ketosis is characterized by hypoglycemia and hyperketonemia (Baird, 1982). The primary cause of ketosis in a lactating dairy cow is an insufficiency of blood glucose to support milk production and fatty acid oxidation. This decrease in carbohydrate status causes 1) a decrease in concentrations of plasma insulin, 2) an increase in mobilization of fat from adipose tissue, and 3) increased hepatic ketogenesis (Baird, 1982; Grummer, 1993). Clinical ketosis, therefore, is characterized by decreases of blood glucose and insulin, and increases of NEFA, BHBA, ACAC, and severity of fatty liver (Baird, 1982; Grummer, 1993; Mills et al., 1986a; Smith et al., 1997).

The predominant hormones responsible for maintaining glucose homeostasis are insulin, and glucagon. Glucagon generally is considered to be counter-regulatory to insulin and is responsible for increasing blood glucose concentrations during hypoglycemia. Glucagon concentrations are decreased in blood of cows that are obese at calving, and they decline further during periods of ketonemia (Baird, 1982; de Boer et al., 1986; Smith et al., 1997). Even though glucagon is lipolytic and ketogenic in many nonruminant animals (Aiello et al., 1984; Iwanji, 1996; Williamson et al., 1971), these effects may not be present or are blunted in ruminant animals (Baird, 1982; Basset, 1971; Brockman, 1978). When insulin to glucagon ratios were normalized during glucagon administration, lipolytic effects were not present in dogs (Flakoll et al., 1994) or sheep (Brockman, 1978). Additionally,



Etherton et al. (1977) failed to observe lipolytic effects of glucagon during in vitro studies with adipose tissue from sheep and dairy steers.

Previous studies involving treatment of early lactation cows with single, bolus injections of glucagon have shown that concentrations of glucose and insulin were increased by glucagon in both normal and ketonemic cows (de Boer et al., 1986; Holtenius and Tråvén, 1990). Furthermore, this glucogenic activity of glucagon in normal early and mid-lactation dairy cows can be sustained for at least 48 h during continual intravenous infusions of glucagon (Hippen et al., 1997). Not only were blood glucose concentrations increased for the duration of glucagon treatment; concentrations of NEFA and BHBA were not increased by dosages of glucagon less than 20 mg/d.

Glucagon has not been considered as a practical treatment for lactation ketosis because it has a physiological half-life of only 5 min (de Boer et al., 1986). If delivery of exogenous glucagon could be sustained over time, glucagon may be able to restore blood glucose concentrations in ketotic cows, both immediately and long-term, because it has glycogenolytic and gluconeogenic activities (Cryer, 1996; Hendrick et al. 1990; Williamson et al., 1971).

The purpose of our experiment was to examine the effects of a 14-d intravenous infusion of glucagon into early-lactation dairy cows with fatty liver that were subjected to an experimentally induced ketosis. The effects of glucagon on normal early-lactation cows are reported elsewhere (She et al. 1998), and they will

be discussed at the end of this paper relative to effects observed in cows with fatty liver.

## **Materials and Methods**

### **Experimental Design**

To create a postpartal fatty liver and susceptibility to lactation ketosis, 20 multiparous Holstein cows were offered 5 to 6 kg of cracked corn daily in addition to their regular prepartal diet (Table 1), which was fed to meet recommendations for energy (National Research Council, 1988) during the final 30 d prepartum (Van den Top, et al. 1996). Cows were selected for overfeeding on the basis of having BCS of 3.5 or above or symptoms of ketonemia in an earlier lactation (Edmondson et al., 1989). Liver samples were collected at 6 d postpartum by puncture biopsy (Smith et al., 1997) for determination of concentrations of total lipid, TAG (Mills et al., 1986a), and glycogen (Deriling et al., 1987). Cows having ratios of liver TAG to glycogen of greater than 2 to 1 were assumed to be susceptible to ketosis (Smith et al., 1997) and were assigned alternately as susceptible control cows (**SC**) or susceptible cows treated with glucagon (**STG**).

During the first 14 d postpartum, all cows were fed a typical lactation diet allowing ad libitum consumption formulated to meet recommendations (National Research Council, 1988; Table 1). Feed intakes were recorded from 6 to 49 DIM. From 14 to 42 DIM (Figure 1), all cows were subjected to a ketosis induction protocol (Mills et al., 1986a; Smith et al., 1997). This protocol consisted of

Table 1. Ingredient composition of diets fed to cows during the 30 d prepartal and 49 d postpartal periods.

Ingredient	Diet	
	Prepartum	Postpartum
	— kg/d —	- % of DM -
Corn silage	1.75	30.70
Soy hulls	0.83	14.59
Corn grain	6.20	12.18
Alfalfa haylage	0.41	7.15
Alfalfa hay	0.41	7.15
Soybean meal, 48%	0.38	6.57
SoyPLUS® <sup>1</sup>	0.35	6.08
Corn gluten feed	0.32	5.60
Whole cottonseed	0.29	5.00
Fish meal	0.10	1.69
Choice white grease	0.10	1.69
	— g/d —	
Dicalcium phosphate	39.07	0.68
Potassium chloride	20.00	0.35
Sodium bicarbonate	10.73	0.19
Limestone	6.95	0.12
Salt	5.31	0.09
Magnesium oxide	5.31	0.09
X-CEL Ruminant Trace Mineral® <sup>2</sup>	3.78	0.07
Grass hay	ad libitum	-

<sup>1</sup>Mechanically extracted soybean meal, West Central Coop, Ralston, IA

<sup>2</sup>Contains FeSO<sub>4</sub>, 2.5%; CuSO<sub>4</sub>, 0.5%; MnO<sub>2</sub>, 10.0%; FeCl, 2.5%; CuO<sub>2</sub>, 2.0%; I<sub>2</sub>EDTA, 0.4%, CoCl, 0.5%; ZnO, 10.0%; MgO, 15.0%. West Central Coop, Ralston, IA.

restricting feed intakes to 80% of NRC recommended intakes for energy and supplementation of the diet with 1,3-butanediol. The butanediol was introduced gradually into each cow's daily ration, starting at 0.25 L/d at 14 DIM and increasing to 1 L/d by 21 DIM. The quantity of butanediol was maintained at 1 L/d from 21 through 28 DIM. From 28 to 35 DIM, butanediol in the diet was increased again to 1.4 L/d and remained at that quantity until the end of the ketosis induction protocol

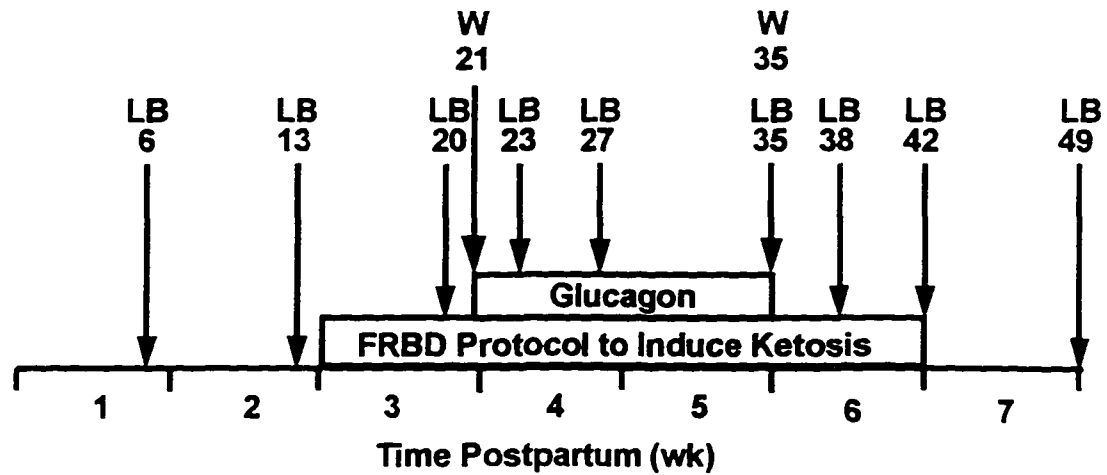


Figure 1. Experimental design. Parturition occurred at d 0. Liver biopsies (LB) were conducted on days indicated. Ketosis induction, consisting of feed restriction and diet supplementation with 1,3-butanediol (FRBD), was imposed on all cows during weeks 3 through 6 (14 to 42 DIM). Treatment with glucagon or vehicle was administered to STG and SC cows, respectively, during weeks 4 and 5 (21 to 35 DIM). Intensive window sampling of blood (W) occurred at 21 and 35 DIM, the start and end of glucagon infusions.

at 42 DIM. Butanediol was removed from the diet if a cow became clinically ketotic during ketosis induction, and the cow was treated as necessary to restore feed intakes to 80% of NRC recommendations. After appetite was restored, butanediol was reintroduced into the diet as before, increasing the quantity to either 1.4 L/d or the maximum amount the cow could consume without exhibiting clinical ketosis (whichever was lesser) until 42 DIM. Thus, all cows were under metabolic pressure to be ketonemic and borderline ketotic. From 42 to 49 DIM, diets were fed for ad libitum intakes without butanediol supplementation, and recovery from the ketosis induction protocol was monitored.

Starting at 21 DIM, glucagon or vehicle was infused intravenously (STG and SC groups, respectively) until 35 DIM (Figure 1), and all cows were confined to their stalls. Cows were prepared for infusion by bilateral insertion of catheters into jugular veins at 20 DIM. Catheters were kept patent with sodium heparin (200 USP in 0.15 M NaCl) until infusion. Glucagon was prepared for infusion by dissolving lyophilized glucagon (donated by Ely Lilly & Co., Indianapolis, IN) in 0.15 M NaCl (preadjusted to pH 10.25) at concentrations that would provide 10 mg/d of glucagon when infused at 20 ml/h. To prevent adherence and loss of glucagon, all glassware and utensils were rinsed with 1% BSA in 0.15 M NaCl before use. Preliminary studies indicated that glucagon was stable in solution for at least 24 h at ambient temperatures; therefore, fresh infusate was added to source vessels at 8 h intervals throughout the infusion periods. At 35 DIM, infusions were ended by decreasing the infusion rate 2 ml/h over a 10 h period.

Cows were monitored daily for severity of ketosis. Symptoms of ketosis included lethargy or nervousness, acetone on breath, and depression of feed intakes and milk production. Additionally, plasma collected each day was tested for concentrations of ketone bodies by use of strip ketone detectors (Ketostix®, Miles Inc., Diagnostics Division, Elkhart, IN). Severity of ketosis was scored as follows: 0 = no symptoms of ketosis and plasma ketones less than 5 mg/dl, 1 = plasma ketones between 5 and 40 mg/dl, 2 = plasma ketones greater than 40 mg/dl, and 3 = plasma ketones greater than 40 mg/dl and DMI decreased by greater than 30% of the assigned amount. Cows were diagnosed as clinically ketotic when their ketosis

score was three and symptoms of acetonemia, behavioral changes, and decreased milk production were present. Clinically ketotic cows then were examined by the herd veterinarian for confirmation of diagnosis and to ensure that ketosis was not secondary to a displaced abomasum or other disorder. Upon confirmation of clinical ketosis, 1,3-butanediol was removed from the diet to help restore feed intakes, and cows were given an oral drench of 454 g of propylene glycol, infused intravenously with 50% dextrose, or treated intramuscularly with dexamethasone, progressively, until recovery from ketosis as indicated by decreased ketone concentrations in plasma and urine and restoration of feed intake and milk production.

Cows were milked three times daily, and milk production was recorded during the trial and for 3 wk thereafter. Samples of milk were collected for analysis of fat, protein, and lactose (Milk-O-Scan 203, Foss Food Technology, Eden Prairie, MN) at 12, 13, 19 to 23, 33 to 37, 48, and 49. Body weights were recorded weekly, and BCS were evaluated by three individuals at -30, -15, 1, 14, 34, and 49 DIM. All cows were treated in accordance with guidelines established by the Iowa State University Committee on Animal Care.

### **Samples and Analysis**

During the first two of the 10 replications, blood was sampled from a jugular vein catheter at 30-min intervals from 0800 to 1500 on 13, 20, 27, 34, 41, and 49 DIM and daily during the infusion period. During all other replications, the window

sampling was discontinued and blood samples were collected daily from 7 to 49 DIM from the coccygeal vein because this allowed better monitoring of the development of ketosis. Samples from the first two replications collected within 1 h of the prescribed daily sampling time in later replications were considered representative of the daily samples and included in the overall analysis. Window sampling of blood from all cows was conducted from jugular catheters at 30-min intervals for 4 h before and 5, 10, 20, 30, 45, 60, 90, 120, 180, and 240 min after the start of infusions (Figure 1). Blood samples also were collected at 30-min intervals for 4 h prior to and throughout the period of decreasing infusion rates at the end of the treatment period for both SC and STG.

Blood was collected into 10-ml vacuum tubes containing Na<sub>2</sub>-EDTA and stored on ice until preparation of plasma within 2 h. Aprotinin (Boehringer-Mannheim, Indianapolis, IN) was added at 500 KIU to one ml of plasma to be analyzed for concentrations of glucagon (Diagnostics Product Corporation, Los Angeles, CA). Plasma was stored at -20°C until analysis for concentrations of glucose, BHBA, ACAC, acetate, NEFA, and urea nitrogen with a microcentrifugal autoanalyzer (Monarch Plus Instrumentation Laboratories, Lexington, MA, at Eli Lilly & Co., Greenfield, IN), glucagon, and insulin (Coat-a-Count®, Diagnostics Product Corporation, Los Angeles, CA). In addition to the initial biopsy at 6 DIM, liver samples also were collected at 13, 23, 27, 35, 38, 42, and 49 DIM (Figure 1) and stored at -80°C until quantification for total lipid, TAG, and glycogen.

## Statistical Analysis

Data from daily measures such as plasma metabolites, feed intakes, and milk production were summarized as weekly means before statistical analysis. The data then were analyzed as a complete randomized block with a split-plot in time by using the general linear models of SAS (SAS, 1988). Main effects of replication and treatment were tested by using replication  $\times$  treatment as the error term. Subplot effects of time and time  $\times$  treatment interactions were tested against residual error. Additionally, data from periodic samples (i.e., liver and milk composition, BW, and BCS) and weekly means of daily samples were compared separately across treatments after covariant adjustment by the pretreatment means of the respective data. One cow assigned to STG developed an infection around a catheter and was removed from the study at 28 DIM. Data collected from the cow until that time were used; thus, after 28 DIM,  $n = 9$  and  $10$  for STG and SC, respectively. Values presented in figures are least square means, and significance was declared at  $P \leq 0.05$ .

## Results

### BW, BCS, and DMI

Body condition scores of all cows averaged  $4.06 \pm 0.52$  during the prepartal period and were not different between SC and STG (Figure 2A). They declined to a low of  $2.24 \pm .53$  at 34 DIM ( $P = 0.0001$  for day), but they did not differ between



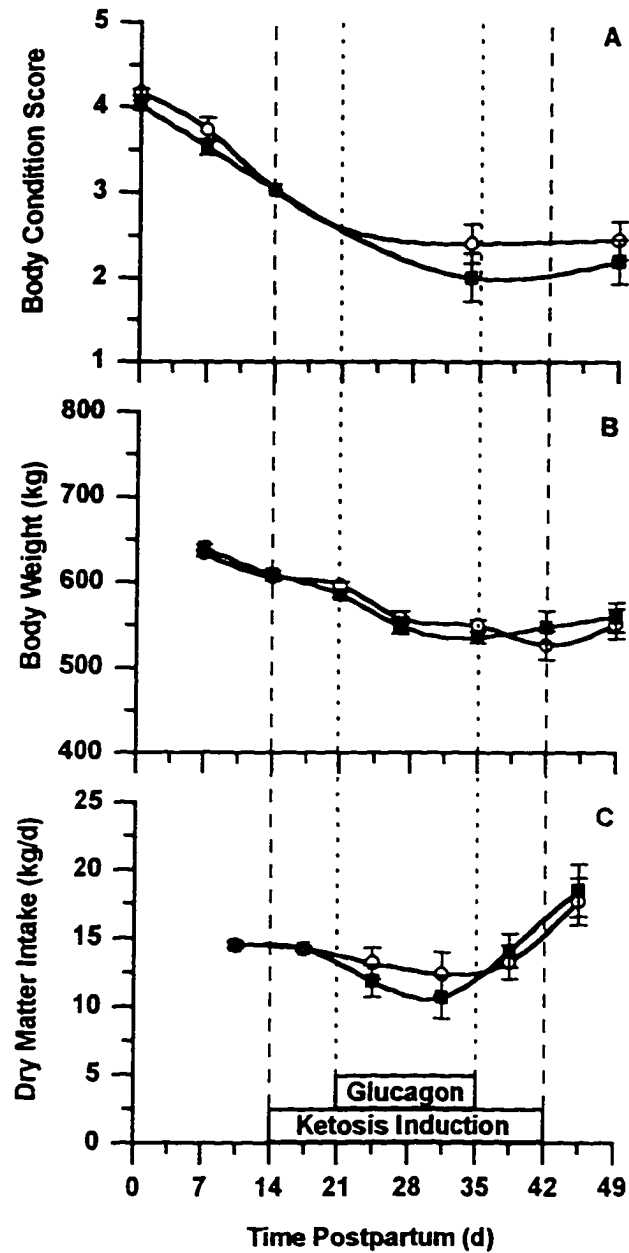


Figure 2. Body condition scores, body weights, and weekly means of feed intakes of ketosis susceptible control cows (O) and ketosis susceptible cows treated with glucagon (■). A. Body condition score. Effects in model: glucagon ( $P = 0.42$ ), day ( $P = 0.0001$ ) and glucagon  $\times$  day ( $P = 0.27$ ). B. Body weight. Effects in model: glucagon ( $P = 0.98$ ), day ( $P = 0.0001$ ) and glucagon  $\times$  day ( $P = 0.29$ ). C. Dry matter intake. Effects in model: glucagon ( $P = 0.87$ ), day ( $P = 0.001$ ) and glucagon  $\times$  day ( $P = 0.0003$ ).

treatments ( $P = 0.40$ ). Body weights of cows responded similarly to BCS (Figure 2B). Body weights averaged  $623 \pm 83$  kg during wk 1 and declined to  $533 \pm 81$  at 42 DIM ( $P = 0.0001$  for day). Glucagon did not affect BW ( $P = 0.98$ ).

Dry matter intakes averaged  $14.5 \pm 3.7$  kg/d during wk 2 of lactation (Figure 2C). Feed restriction to 80% of NRC recommendations for  $NE_L$  did not further limit the already depressed intakes. As cows developed clinical ketosis, DMI were depressed further between 21 and 35 DIM. After ketosis induction ended, DMI increased in both groups. Treatments did not have significant effects on DMI at any individual day. Treatment  $\times$  day interaction, however, was significant ( $P = 0.0003$ ) because STG had lesser intakes during glucagon treatment and a greater rate of increasing DMI after treatment.

Intakes of 1,3-butanediol were less than prescribed in the design because they were dependent on the ability of individual cows to resist ketosis (data not shown). The average amount of butanediol consumed by individual cows was  $0.6 \pm 0.1$  L the first week of ketosis induction and increased to  $0.8 \pm 0.6$  for the second week. There were no significant differences between groups in butanediol intakes at any time ( $P = 0.52$ ).

### **General Health and Ketosis**

Of the 20 cows started on this trial, two SC and two STG cows required treatment for milk fever during the first week of lactation (Table 2). Over the course of the experiment, eight SC and five STG cows, in addition to the STG cow

Table 2. Incidence of metabolic and physiological disorder in ketosis susceptible control cows and ketosis susceptible cows treated with glucagon during the first 49 DIM.

Treatment <sup>2</sup>	MF <sup>3</sup>	Ketosis by DIM <sup>1</sup>				DA <sup>4</sup>	Metritis	Mastitis	Infection <sup>5</sup>
		<21	21-27	28-35	>35				
SC	2	2	4	1	4	1	1	2	5
STG	2	4	3	1	0	0	1	0	4

<sup>1</sup>Incidence of ketosis classified by DIM. Repeated occurrences of ketosis are included.

<sup>2</sup>Treatments were control cows susceptible to ketosis (SC) and cows susceptible to ketosis that were treated with glucagon from 21 to 35 DIM (STG).

<sup>3</sup>MF = milk fever.

<sup>4</sup>DA = displaced abomasum.

<sup>5</sup>Infections include foot rot, winter dysentery and unexplained fevers.

removed at 28 DIM, exhibited disorders of an infectious nature. Milk fever and depressed immune function often are associated with fatty liver syndrome (Goff and Horst, 1997; Franklin et al., 1991); therefore, affected cows were retained in the study. All cows were checked for displaced abomasums whenever clinical signs of ketosis were exhibited. Only one cow, assigned to SC, developed a confirmed displaced abomasum that required surgical intervention. Because the displacement occurred after ketosis induction was completed at 45 DIM and was not associated with clinical ketosis, this cow was considered to have completed the study and all data collected from her until d 45 were included.

All cows in both groups became subclinically ketotic (i.e., appetites were poor and the presence of ketones was detectable in plasma and urine) both before and during the ketosis induction protocol. The incidence of clinical ketosis is reported in Table 2. In total, five STG and eight SC cows became clinically ketotic during the experiment and two cows in each group had multiple occurrences. Only one STG

cow was diagnosed as ketotic after the first week of glucagon treatment (28 DIM); however, four SC cows became ketotic after that time.

Concentrations of ketones in plasma as estimated daily by Ketostix® were slightly higher in SC than in STG during wk 2 of lactation (5.7 vs. 4.5 mg/dl,  $P = 0.04$ , Figure 3A). Ketosis induction increased ketone concentrations in plasma of all cows. Even though concentrations of ketones in plasma of SC increased further during the wk 4, they did not continue to increase in plasma of STG after glucagon treatment was initiated. Thereafter, ketone concentrations declined more rapidly in plasma of STG than in SC ( $P = 0.0001$  for treatment  $\times$  day).

Ketosis scores (Figure 3B), assigned as described, increased as concentrations of plasma ketones increased and reflect the incidence of clinical ketosis within groups. Ketosis scores of all cows were increased by ketosis induction. After treatment with glucagon began, ketosis scores of STG declined and scores of SC remained elevated until ketosis induction ceased ( $P = 0.0001$  for treatment  $\times$  day).

### **Milk Production and Composition**

The normal increase in milk production during early lactation was blunted by the ketosis induction protocol, which started at 14 DIM (Figure 4A). Milk production decreased in both the SC and STG groups as ketosis induction progressed. The decline in milk production was greater in STG than in the SC

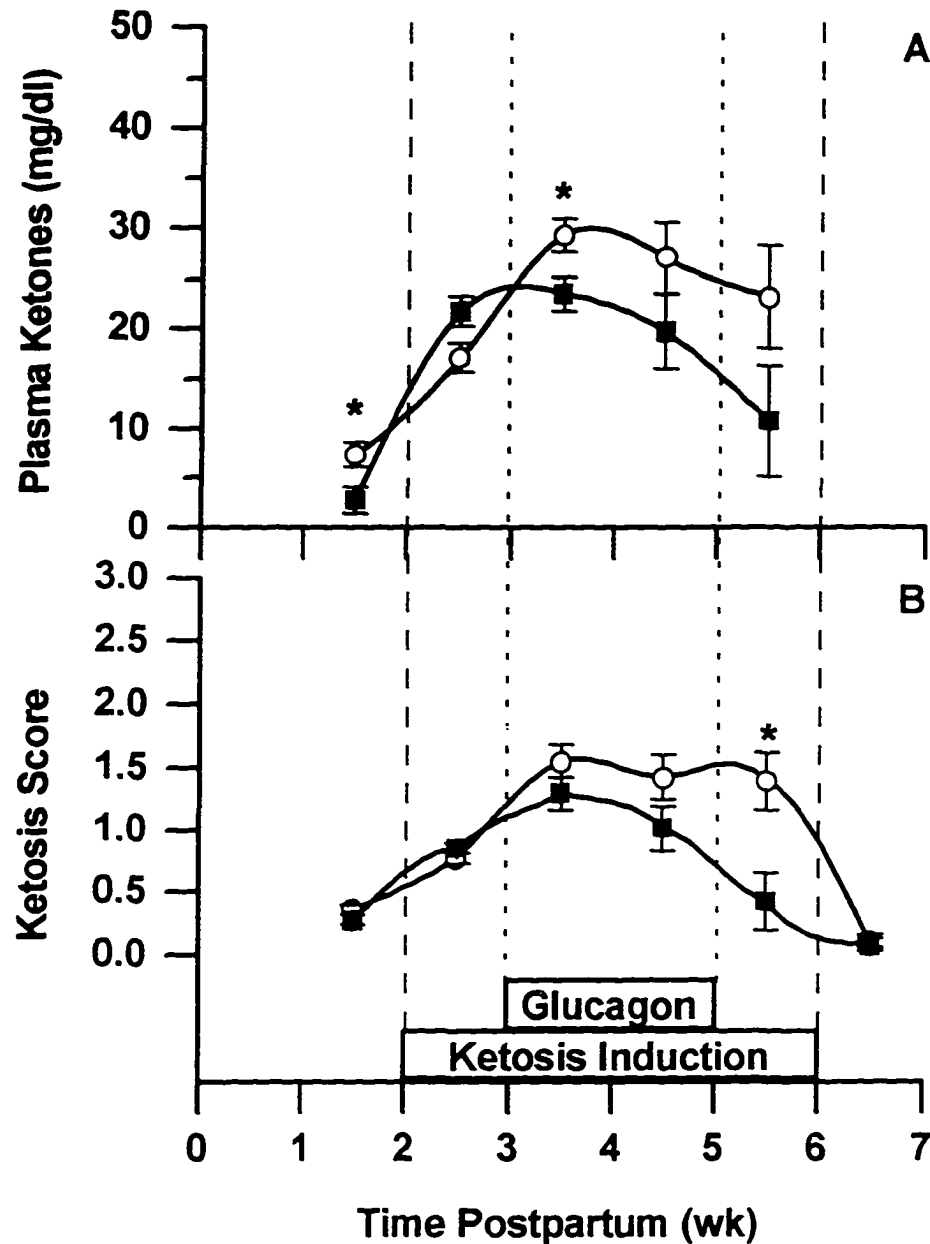


Figure 3. Weekly means of ketones in plasma and ketosis scores of ketosis susceptible control cows (O) and ketosis susceptible cows treated with glucagon (■). **A. Plasma ketones** as measured daily by Ketostix®. Effects in model: glucagon ( $P = 0.62$ ), day ( $P = 0.0001$ ) and glucagon  $\times$  day ( $P = 0.27$ ). **B. Ketosis scores.** Effects in model: glucagon ( $P = 0.24$ ), day ( $P = 0.0001$ ) and glucagon  $\times$  day ( $P = 0.0001$ ). Significant differences at individual times are indicated by \*  $P \leq 0.05$ .

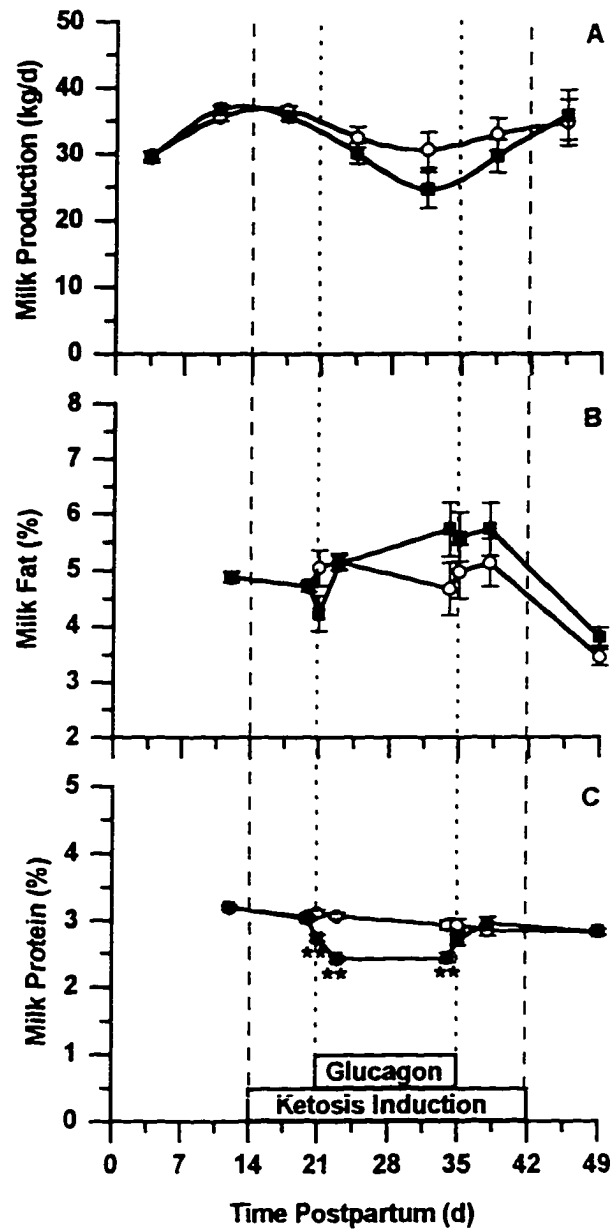


Figure 4. Weekly means of milk production and composition of milk from ketosis susceptible control cows (O) and ketosis susceptible cows treated with glucagon (■). **A. Milk production.** Effects in model: glucagon ( $P = 0.82$ ), day ( $P = 0.0001$ ) and glucagon  $\times$  day ( $P = 0.0001$ ). **B. Percentage of fat in milk at day indicated.** Effects in model: glucagon ( $P = 0.56$ ), day ( $P = 0.0004$ ) and glucagon  $\times$  day ( $P = 0.08$ ). **C. Percentage of protein.** Effects in model: glucagon ( $P = 0.007$ ), day ( $P = 0.0001$ ) and glucagon  $\times$  day ( $P = 0.0001$ ). Significant differences at individual times are indicated by \*\*  $P \leq 0.01$ .

group during glucagon treatment ( $P = 0.0001$  for treatment  $\times$  day), but milk production increased rapidly in the STG group after treatment ended. Overall, there were no treatment effects of glucagon on milk production ( $P = 0.56$ ).

Percentages of fat in milk produced by the STG group tended to increase during the last week of glucagon treatment compared with the SC group ( $P = 0.09$  for treatment  $\times$  day, Figure 4B) and decreased in milk from both the SC and STG groups after ketosis induction ended. Quantities of milk fat produced daily decreased along with milk production for both groups ( $P = 0.0001$  for day, data not shown) and were actually decreased at 34 DIM from the STG compared with the SC group (1.1 vs 1.6 kg/d,  $P = 0.05$ ). Average production of milk fat was not significantly affected by glucagon.

Protein percentages in milk were decreased immediately by glucagon and remained below controls until treatment ended at 35 DIM at which time their increase was as rapid as the initial decrease (Figure 4C). During treatment, percentages of protein in milk produced were  $2.4 \pm 0.3\%$  for the STG group compared with  $3.0 \pm 0.2\%$  for the SC group ( $P = 0.0001$  for treatment  $\times$  day). Milk protein production declined proportional to concentrations (data not shown). After treatment with glucagon ended, there were no differences in concentrations or quantities of protein in milk produced by the SC or STG groups.

Milk lactose production was not affected overall by either ketosis induction or glucagon ( $P = 0.23$  for day, data not shown). Concentrations of lactose, however, were increased sharply in milk produced by STG cows during the day glucagon

infusions began ( $4.7$  vs  $4.9 \pm 0.1\%$  for SC and STG,  $P = 0.01$ ) and were still slightly, but nonsignificantly, elevated 2 d later.

### Plasma Constituents

Infusion of glucagon at 10 mg/d increased concentrations of plasma immunoreactive glucagon (IRG) from 154 to  $705 \pm 201$  pg/ml (Figure 5A). Plasma IRG in the STG group declined to concentrations similar to SC cows upon stopping glucagon infusion. Concentrations of IRG were unchanged in the SC group until after ketosis induction at 42 DIM. During the wk 7 of the experiment, IRG increased in plasma of both SC and STG, but the increase was greater in STG than in SC ( $P < 0.008$ ).

Concentrations of insulin were  $215 \pm 80$  pg/ml during wk 2 postpartum (Figure 5B). Plasma insulin concentrations increased variably during the first 4 h of glucagon infusion, peaking 57 min after the start of glucagon at  $672 \pm 400$  pg/ml (Figure 5B insert), and they returned to preinfusion concentrations the following day. Glucagon infusion did not affect average weekly plasma insulin concentrations ( $P = 0.53$ ). Plasma insulin concentrations gradually increased in groups throughout the trial ( $P = 0.0001$  for day).

Glucose concentrations in plasma averaged only  $47.0 \pm 7.0$  mg/dl during wk 2 of lactation and were not different between groups ( $P = 0.51$ , Figure 6A). They decreased further in both groups during the first week of ketosis induction ( $39.5 \pm 7.1$  mg/dl). In SC, the decline in glucose continued through the second week of



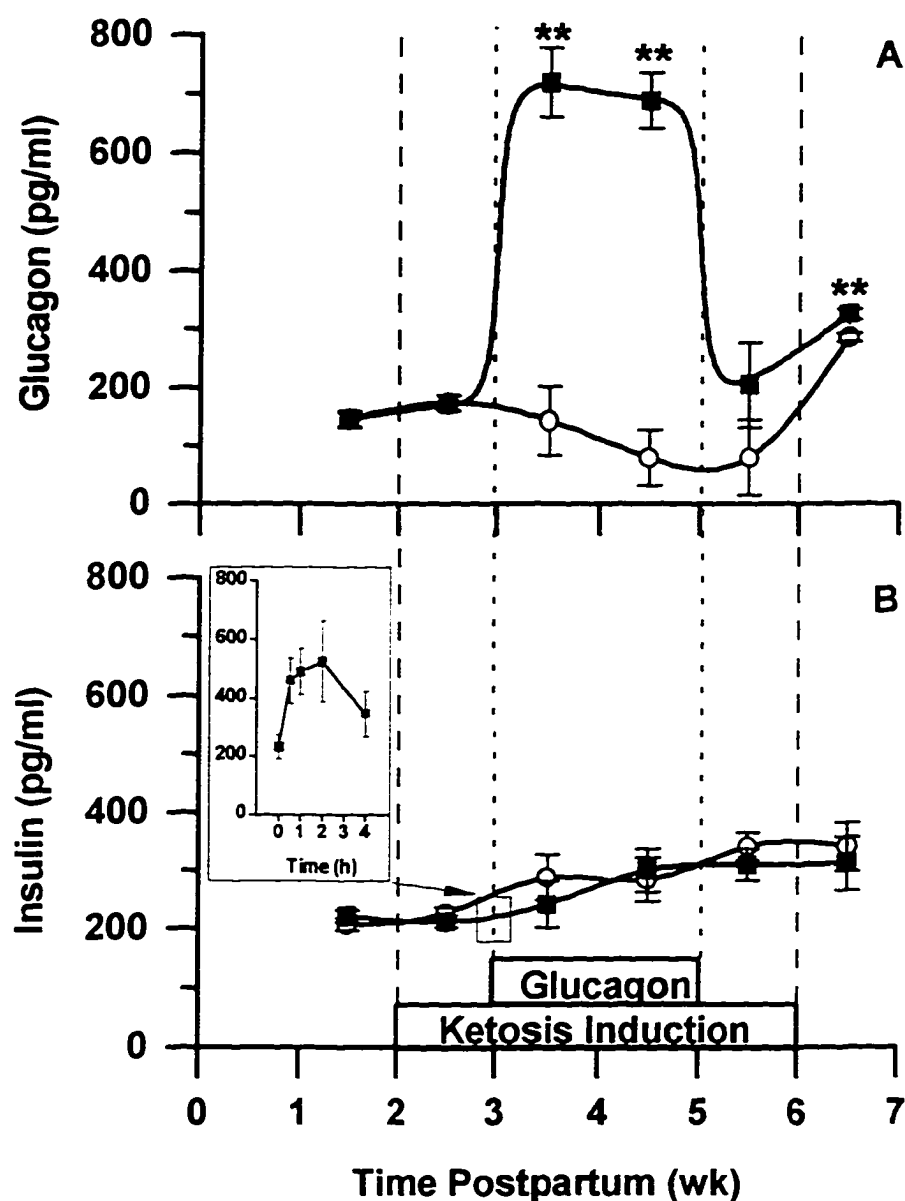


Figure 5. Weekly means of concentrations of immunoreactive glucagon and insulin in plasma of ketosis susceptible control cows (O) and ketosis susceptible cows treated with glucagon (■). **A. Immunoreactive glucagon.** Effects in model: glucagon ( $P = 0.0001$ ), day ( $P = 0.0001$ ) and glucagon  $\times$  day ( $P = 0.0001$ ). **B. Immunoreactive insulin.** Effects in model: glucagon ( $P = 0.83$ ), day ( $P = 0.0001$ ) and glucagon  $\times$  day ( $P = 0.53$ ). Significant differences at individual times are indicated by \*\*  $P \leq 0.01$ . **B insert.** Response of insulin to glucagon during the first 4 h of infusion.

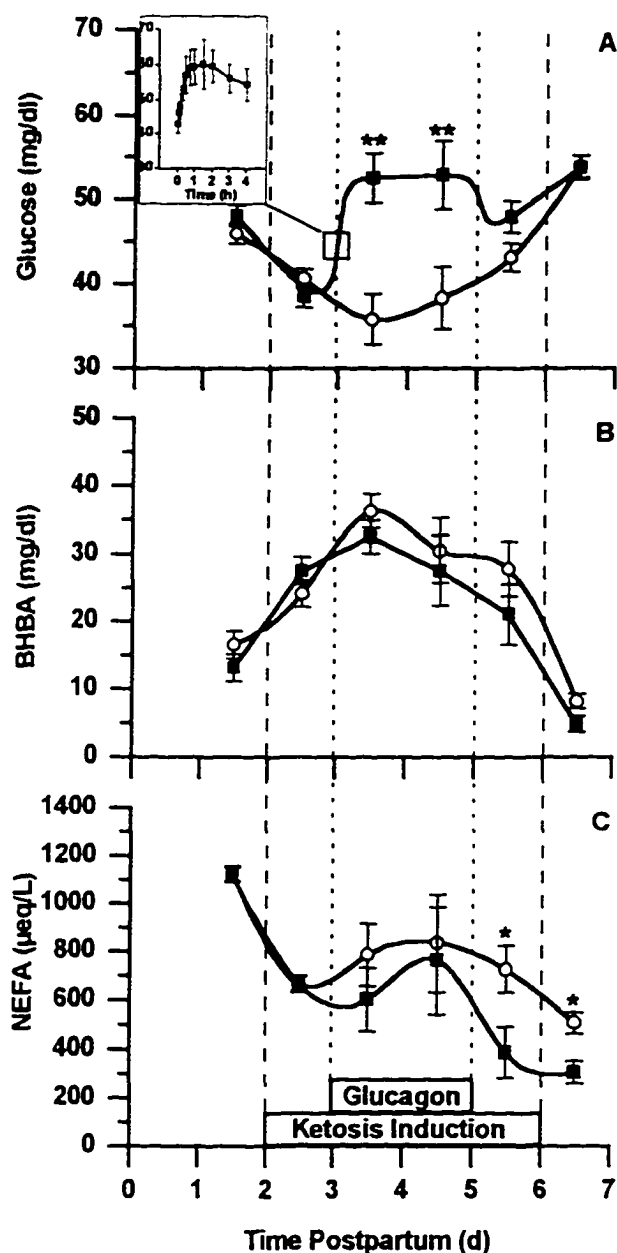


Figure 6. Weekly means of concentrations of glucose, BHBA, and NEFA in plasma of ketosis susceptible control cows (O) and ketosis susceptible cows treated with glucagon (■). **A. Glucose.** Effects in model: glucagon ( $P = 0.01$ ), day ( $P = 0.0001$ ) and glucagon  $\times$  day ( $P = 0.0001$ ). **A insert.** Effects of glucagon on plasma glucose during the first 4 h of infusion. **B. BHBA.** Effects in model: glucagon ( $P = 0.33$ ), day ( $P = 0.0001$ ) and glucagon  $\times$  day ( $P = 0.0002$ ). **C. NEFA.** Effects in model: glucagon ( $P = 0.88$ ), day ( $P = 0.0001$ ) and glucagon  $\times$  day ( $P = 0.008$ ). Significant differences at individual times are indicated by \*  $P \leq 0.05$  or \*\*  $P \leq 0.01$ .

ketosis induction and then gradually increased throughout the remainder of the trial. Glucagon abruptly increased glucose concentrations by  $22.7 \pm 12.5$  mg/dl in the STG group within 3 h of the start of infusions, and this average time to peak concentrations was  $92 \pm 57$  min. Glucose concentrations in plasma of STG averaged  $52.5 \pm 11.2$  mg/dl during the first week of infusion and were maintained until the end of glucagon treatment. During the week following infusions, plasma glucose in the STG group tended to be greater than in the SC group (47.9 vs 43.1 mg/dl,  $P = 0.10$ ).

Plasma urea nitrogen concentrations were not different at any time between the SC and STG groups (data not shown). Initial concentrations averaged  $13.9 \pm 3.7$  mg/dl during wk 2 and decreased during the ketosis induction protocol to a minimum of  $12.0 \pm 3.5$  mg/dl ( $P = 0.0001$  for wk). After the ketosis induction protocol ended, urea nitrogen concentrations increased in both groups.

Concentrations of BHBA in plasma averaged  $15.5 \pm 8.5$  mg/dl for all cows at the start of the experiment (Figure 6B). Ketosis induction increased BHBA concentrations until, during wk 4, concentrations averaged 36.3 and 32.4 for the SC and STG groups, respectively ( $P = 0.32$ ). Thereafter, BHBA concentrations declined in both groups through the end of the experiment. The rate of decline was greater in STG than in SC ( $P = 0.0002$  for treatment  $\times$  day), and during wk 7, BHBA concentrations were 3.4 mg/dl less in STG than in SC ( $P = 0.07$ ).

Nonesterified fatty acids in plasma of all cows averaged  $1120 \pm 505$   $\mu$ eq/L during wk 2 of lactation for both groups of cows combined (Figure 6C).

Concentrations of plasma NEFA declined during wk 3 and then increased under the pressure of ketosis induction. There were no significant differences in NEFA concentrations between groups during glucagon treatment ( $P > 0.35$ ). Plasma NEFA concentrations, however, declined more rapidly after glucagon treatment in STG than in SC ( $P = 0.008$  for treatment  $\times$  day), and they were lower in STG than in SC during the last 2 wk of the experiment ( $P < 0.05$ ).

### **Liver Composition**

Liver TAG content averaged 12.9% of the wet weight of the liver for both the SC and STG groups at 6 DIM and was not different between the two groups ( $P < 0.90$ , Figure 7A). Concentrations of TAG did not change in either group until the start of the glucagon treatment. Glucagon caused an immediate and dramatic decrease in concentrations of liver TAG in STG ( $P = 0.0001$  for glucagon  $\times$  day). Two days after starting treatment, TAG concentrations decreased to 8.6% in livers of STG and remained unchanged in livers of SC. By the end of the treatment period at 35 DIM, liver TAG concentrations in STG were 4.7% compared with 15.5% in SC. Triacylglycerol concentrations remained unchanged after glucagon treatment until the end of the ketosis induction protocol, at which time TAG in livers of all cows declined.

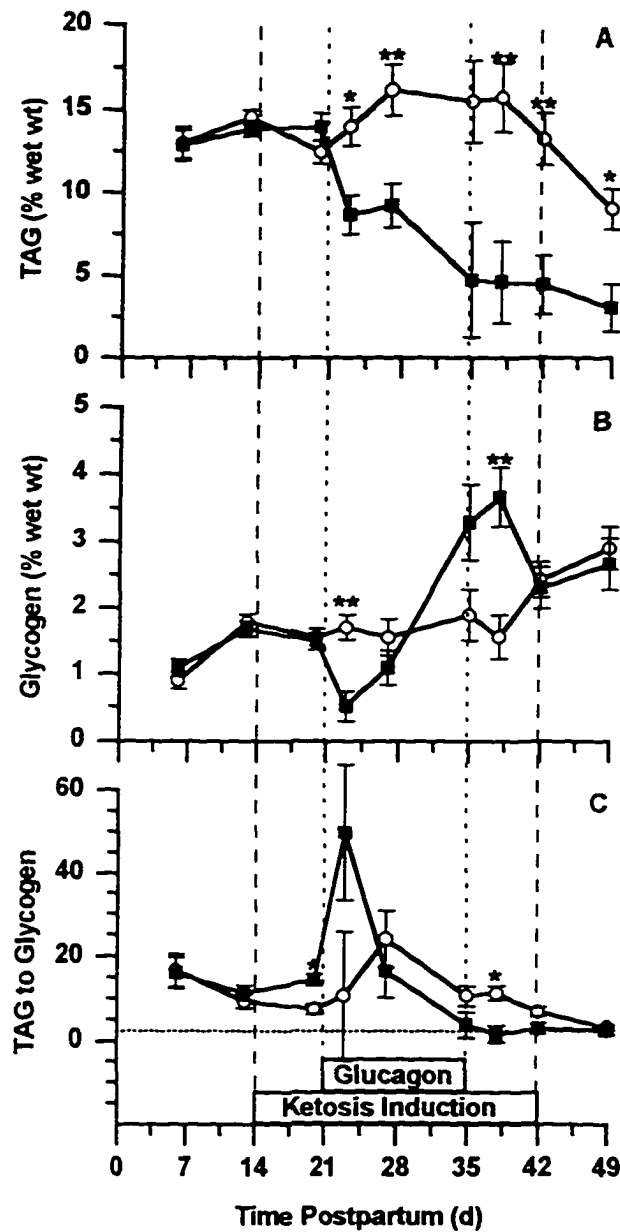


Figure 7. Concentrations of triacylglycerol (TAG) and glycogen in livers of ketosis susceptible control cows (O) and ketosis susceptible cows treated with glucagon (■). **A. Triacylglycerol (TAG).** Effects in model: glucagon ( $P = 0.51$ ), day ( $P = 0.0001$ ) and glucagon  $\times$  day ( $P = 0.0001$ ). **B. Glycogen.** Effects in model: glucagon ( $P = 0.46$ ), day ( $P = 0.0001$ ) and glucagon  $\times$  day ( $P = 0.0001$ ). **C. Ratio of TAG to glycogen.** Effects in model: glucagon ( $P = 0.66$ ), day ( $P = 0.009$ ) and glucagon  $\times$  day ( $P = 0.25$ ). Significant differences at individual times are indicated by \*  $P \leq 0.05$  or \*\*  $P \leq 0.01$ .

Liver glycogen content averaged 1.0% of the wet weight of the liver at 6 DIM for all cows and glycogen concentrations were not different until the start of treatments (Figure 7B). Concentrations of glycogen were unchanged in livers of SC until after 39 DIM, at which time glycogen increased gradually through 49 DIM. Treatment with glucagon initially caused glycogen concentrations in livers of STG to decrease to 0.5% of the wet weight of the liver. Thereafter, glycogen increased in livers of STG throughout, and until 3 d after, glucagon treatment. At 38 DIM, glycogen concentrations were 3.7% of livers' wet weight in STG compared to 1.6% of livers in SC ( $P = 0.009$ ). At 42 DIM, glycogen concentrations in livers of STG dropped to levels similar to those of SC and the groups were not different throughout the remainder of the experiment.

Ratios of liver TAG to glycogen averaged 16.4 to 1 at 6 DIM (Figure 7C). The decrease in liver glycogen content induced by glucagon at 23 DIM increased the ratio to 47.3 to 1 in livers of STG and the ratio remained unchanged in SC livers. As glycogen increased and TAG decreased in STG livers after 23 DIM, the ratio declined until at 38 DIM livers of STG had a ratio of 2.0 to 1 compared with 11.4 to 1 in SC livers ( $P < 0.02$ ). By 49 DIM ratios of both groups averaged 2.9 to 1 and were not different ( $P < 0.31$ ).

## Discussion

This experiment was conducted jointly with an experiment to determine the effects of 14-d infusions of glucagon on normal, early-lactation Holstein cows (She

et al., 1997). Because the two experiments were conducted concurrently as a  $2 \times 2$  factorial, the normal cows studied by She et al. (1998) can provide additional comparisons for evaluation of the current experiment. Comparisons to normal control cows (NC) and normal cows treated with glucagon (NTG) that follow were determined by analysis of the combined data as a  $2 \times 2$  factorial with condition (normal vs. susceptible cows) and treatment with or without glucagon as main effects and time of sampling as a subplot effect.

Compared with the normal cows (She, 1997), the overall effects of the ketosis induction protocol on SC and STG during the first week of induction were not dissimilar to those observed by others using this technique (Drackley et al., 1992; Mills et al., 1986a; Smith et al., 1997; Veenhuizen et al., 1991).

Concentrations of NEFA and BHBA increased in plasma of the susceptible cows as ketosis induction progressed, and, at the onset of clinical ketosis, milk production and DMI intakes decreased sharply as in spontaneous on-farm ketosis. In the current study however, ketotic cows were not allowed to recover fully, but they were pressured to remain in a ketonemic state by continuation of the induction protocol. Because cows used for this study had severe fatty liver before the induction protocol, further increases in liver TAG were not observed during ketosis induction. The severity of the ketosis induction protocol, as used in the current study, allowed this model to be an extreme test for studying efficacy of a possible treatment for fatty liver and on-farm ketosis.

### **BW, BCS, and DMI**

The BCS of susceptible cows (Figure 2A) were not increased by overfeeding during the last 30 d prepartum and were only 0.4 units greater at parturition than those of the normal cows used by She et al. (1997). Loss of body condition postpartum in susceptible cows was severe, and, by 14 DIM, BCS were similar to the normal cows, and they declined further to 2.3 at 34 DIM compared with 2.7 for the normal cows. Likewise, susceptible cows (Figure 2B) were 30 kg heavier than normal cows at 7 DIM, had similar BW at 14 DIM, but weighed 58 kg less at 42 DIM ( $P = 0.01$ ). The losses of BW and BCS in the susceptible cows are consistent with those observed in earlier work with this ketosis induction protocol in which average BW losses were 3.1 kg/d (Mills et al., 1986a).

Dry matter intakes of the susceptible cows (Figure 2C) were 5.2 kg/d less than those of the normal cows (She, 1997) during wk 2 postpartum. The experimental design originally stipulated that during ketosis induction DMI would be restricted to 80% of maximal DMI during wk 2, but, because DMI was already depressed in the susceptible cows compared with the normal cows, DMI was restricted to 80% of NRC recommendations for energy intake at quantities of milk produced during wk 2. Thus, during ketosis induction, DMI of the susceptible cows averaged only 56% of that of the normal cows. During the week after stopping feed restriction, DMI of the susceptible cows increased to 73% that of the normal cows. Glucagon decreased DMI transiently in both the NTG and STG groups, but DMI increased rapidly for both groups as soon as glucagon ended.



### Milk Production and Composition

Despite low DMI in susceptible cows during the first 14 DIM, milk production of SC and STG during that time (Figure 4A) was not significantly lower than that of the normal cows ( $P = 0.07$ ). The ketosis induction protocol, however, blunted the normal early-lactation increase in milk production of the susceptible cows to only 71% that of the normal cows ( $P = 0.0001$ ). After ketosis induction ended, milk production increased rapidly in the susceptible cows (Figure 4A) and was not different from the normal cows (She, 1997) by wk 10 of lactation. Similarly to DMI, milk production also decreased transiently for both the STG and NTG groups during wk 2 of glucagon treatment compared with controls, but they recovered rapidly after glucagon ended. During the month after the experiment, milk production in STG and NTG was numerically higher by 1.8 kg/d than in SC and NC ( $P = 0.51$ ).

Milk fat percentages (Figure 4B) were higher by 1.1% in milk from susceptible cows by compared with normal cows (She, 1997) throughout the experiment ( $P = 0.0001$ ), which would be consistent with decreased milk production and increased concentrations of NEFA in plasma of the susceptible cows. The amount of milk fat produced each day, however, was not different for normal and susceptible cows ( $P = 0.12$ ).

Milk protein concentrations (Figure 4C) were decreased similarly in both the susceptible and normal cows ( $P = 0.96$  for condition  $\times$  treatment). Daily production of milk protein was, on average, 0.3 kg less for susceptible cows than for normal cows ( $P = 0.0001$ ). Glucagon decreased milk protein production in both the NTG

and STG groups by 0.3 kg/d compared with controls ( $P = 0.0001$ ). Decreases in milk protein secretion may be attributable to a glucagon-mediated increase in uptake and deamination of amino acids as precursors for hepatic gluconeogenesis (Brockman, 1978; Flakoll et al, 1994). Assuming that two mols of amino acids are required to synthesize one mol of glucose, the diversion of milk protein to glucose via hepatic gluconeogenesis would have contributed no more than 245 g of glucose per day. It is doubtful that this glucose alone would make a significant contribution to the 20 mg/dl increase observed in blood glucose concentrations. When glucose was infused intravenously at 342 or 737 g/d into lactating dairy cows, blood glucose concentrations failed to increase (Amaral et al., 1990).

Milk lactose concentrations and yields (data not shown) averaged 0.15% and 0.64 kg/d less in the susceptible cows than in the normal cows ( $P < 0.009$ ). Lactose concentrations in milk increased transiently for STG and NTG groups during the first 2 d of glucagon treatment ( $P = 0.002$ ); yields of lactose, however, were not affected at any time by glucagon in either normal or susceptible cows.

### **Plasma Constituents**

Immunoreactive glucagon concentrations (Figure 5A) in plasma of susceptible cows were numerically lower than in plasma of normal cows (145 vs. 274 pg/ml,  $P = 0.19$ ). Increases in IRG during glucagon treatment were similar between STG and NTG groups (551 vs. 520 pg/ml,  $P = 0.79$ ); however, both were significantly increased above their respective controls ( $P = 0.0001$  for treatment).

Insulin concentrations in plasma (Figure 5B) were initially low in the susceptible cows compared with the normal cows (215 vs 288 pg/ml,  $P = 0.006$ ), and the susceptible cows remained hypoinsulinemic relative to the normal cows (She, 1997) throughout the experiment (220 vs 448 pg/ml,  $P = 0.0001$ ). Low concentrations of plasma insulin are typical of cows during ketosis induction (Veenhuizen et al., 1991). Unlike in the susceptible cows, glucagon increased concentrations of insulin in the normal cows by  $190 \pm 73$  pg/ml, but the degree of response was variable and not all normal cows responded (She, 1997). In previous studies (de Boer et al., 1986; Hippen et al., 1997), increases in insulin concentrations were consistent with increases in glucose concentrations. The lack of response observed in the susceptible cows in the current experiment may be attributable to glucose concentrations being below a minimum threshold for stimulation of insulin secretion. During glucagon treatment, the average glucose concentration in plasma from STG was equal to the basal concentrations of glucose in plasma of the cows used by de Boer (1986) and Hippen (1997). Furthermore, an immediate increase of insulin concentrations was observed in the STG group during the first 4 h of glucagon infusion when glucose concentrations increased by more than 22 mg/dl over baseline. Overall, the effects of glucagon on insulin secretion are similar to those of Holtenius et al. (1990) who observed heterogeneous responses of insulin to glucagon, particularly among cows overfed prepartum or with displaced abomasums.

Even though plasma glucose concentrations (Figure 6A) in our susceptible cows were 12.7 mg/dl less than in normal cows (She, 1997) the week before glucagon treatment began, glucose in plasma from this STG group increased to concentrations similar to those in plasma from NC throughout glucagon treatment (52.5 vs. 52.7 mg/dl) and the magnitude of the increases over the 2 wk treatment period were greater in STG than in NTG (14.6 vs 10.2 mg/dl). The time to peak heights, area under the curves, and peak concentrations of plasma glucose during the first 4 h of glucagon infusion were not different for STG versus NTG; however, the responses were delayed and decreased compared with earlier work with glucagon infusions in early-lactation cows [44.4 vs 133.3 mg/dl×h<sup>-1</sup> for area under the curves, (Hippen et al., 1997)]. These effects of glucagon on plasma glucose suggest that low concentrations of liver glycogen may limit immediate hepatic glucose production by glycogenolysis, but they also suggest that gluconeogenic capacity is not diminished in fatty livers of dairy cows. These results seem to differ from those observed in vitro; i.e., gluconeogenic capacity of bovine liver with propionate, lactate, and amino acids as substrates is diminished at the onset of ketosis (Mills et al., 1986b; Veenhuizen et al., 1991). The in vitro results; however, are a better measure of basal gluconeogenesis than gluconeogenic capacity because hormonal stimulation was not present. Also, increases in plasma glucose reported in the current study are weekly averages and do not reflect decreased glucose concentrations that occurred with the onset of clinical ketosis, even in cows treated with glucagon. Mills et al. (1986b) observed no correlation between in vitro

gluconeogenic capacity and hepatic TAG concentrations. Therefore, ketogenesis may have a greater inhibitory effect on hepatic gluconeogenesis than does hepatic lipidoses.

Plasma urea nitrogen concentrations were 15 mg/dl lower in the susceptible cows compared with those in the normal cows throughout the experiment (data not shown,  $P = 0.0001$ ). This difference should be expected because protein intakes were decreased by over 40% in susceptible cows compared with normal cows. Similar to observations in short-term glucagon trials (Hippen et al., 1997), glucagon did not affect PUN concentrations in either category of cows. If all the additional glucose in plasma was derived from amino acids and clearance rates of glucose and urea were similar, the maximal contribution to PUN from ureagenesis and subsequent gluconeogenesis would have been 4.7 mg/dl, which is slightly greater than the observed standard deviation of PUN. In support of increased utilization of amino acids for glucose synthesis, glucagon is a powerful stimulant of hepatic ureagenesis in bovine hepatocytes (Zhu et al., 1997).

Plasma BHBA concentrations (Figure 6A) were 3- to 4-fold greater in susceptible cows than in normal cows (She, 1997) from wk 1 through wk 6 of lactation. By wk 7, BHBA in STG had decreased to concentrations similar to that in the normal cows (4.8 mg/dl,  $P > 0.48$ ) whereas those in SC remained somewhat elevated (8.2 mg/dl  $P = 0.03$ ). Plasma NEFA concentrations in the susceptible cows (Figure 6B) were initially two-fold greater than those in the normal cows. Similarly to NEFA in susceptible cows, NEFA concentrations in normal cows

declined after wk 2 of lactation. In normal cows, however, NEFA continued to decline throughout the trial. During wk 7, even though NEFA had declined greatly in the susceptible cows, concentrations were still greater than those in the normal cows (412 vs. 184  $\mu\text{eq/L}$ ,  $P < 0.05$ ). Neither NEFA or BHBA concentrations in plasma of normal cows were affected by glucagon. Our experiment did not show any indications of an enhancement of lipolysis from adipose tissue or of a resultant ketogenesis in either the NTG or STG groups when glucagon was administered at 10 mg/d, even though insulin concentrations were unaffected by glucagon in STG.

### **Liver Composition**

At 6 DIM, TAG concentrations were 4-fold greater in livers of susceptible cows (Figure 7A) than in livers of normal cows (She, 1997). Similarly to results of earlier trials using this ketosis induction protocol (Drackley et al., 1992; Mills et al., 1986a; Smith et al., 1997; Veenhuizen et al., 1991), the TAG content of livers in the SC group remained elevated or even increased for individual cows. In normal cows and STG, TAG content of livers declined throughout the trial. Because of the decline of TAG in livers of NC, effects of glucagon were not obvious in livers of NTG. At the end of glucagon treatment, TAG in livers of STG, however, tended to be only slightly higher than in livers of the normal cows (5.7 vs 1.1% wet wt,  $P > 0.07$ ) where as TAG remained significantly elevated in livers of SC (14.4% wet wt). In our opinion, these TAG data are the most important findings in the experiment.

Both glucagon and glucose has been demonstrated to be capable of preventing the accumulation of TAG in livers in other studies. Administration of glucagon at 33  $\mu\text{g}/100\text{ g BW/d}$  (approx. 10 times that used in our study) to rats receiving total parenteral nutrition prevented the development of hepatic steatosis (5.7 vs 20.3% of liver wet wt for glucagon treated vs. controls) that is commonly observed under those conditions (Shujun et al., 1988). It should be noted that glucose concentrations were not allowed to increase in the parenteral nutrition experiment. Similarly, duodenal infusion of glucose at 484 g/d during ketosis induction prevented increases in liver TAG content in dairy cows (Veenhuizen et al., 1991). The glucagon-induced removal of TAG from liver reported here, however, is the first reported occurrence known to us for any species.

### **Mechanisms for TAG Removal**

Glucagon and cAMP generally are considered to be inhibitory towards lipoprotein synthesis and secretion which has been verified in rat hepatocytes (Björnsson et al. 1992), but discussions of the effects of glucagon on lipoprotein assembly and secretion in ruminant livers are scarce. It is known that insulin inhibits apoB assembly through inhibitory effects on transcription of microsomal triglyceride transfer protein and that fatty acids stimulate transcription of the protein, enabling apoB assembly and VLDL secretion (Gruffatt et al., 1996). It also is unlikely that TAG from NEFA are incorporated directly in VLDL, but they instead are stored temporarily in a cytosolic pool and must be hydrolyzed by a hepatic lipase for

transfer to microsomes and subsequent incorporation into VLDL (Gruffatt et al., 1996). Therefore, it is not unreasonable to assume that glucagon may stimulate transcription of microsomal triglyceride transfer protein either in a counter-regulatory fashion to insulin or via hydrolysis of hepatic TAG and increasing cellular concentrations of NEFA. Two other explanations for decreases of TAG could be either an increased availability of amino acids for lipoprotein synthesis caused by glucagon-induced partitioning of plasma amino acids to the liver or an increase in rate of oxidation of fatty acids hydrolyzed from cytosolic TAG.

At 6 DIM, glycogen concentrations in livers of the normal cows (She, 1997) were more than two-fold ( $2.6 \pm 1.1\%$  wet wt) those in livers of susceptible cows (Figure 7B). The effects of glucagon in livers of the NTG group paralleled those reported here for the STG group. At 38 DIM, glycogen content of livers in STG was greater than in NC (3.6 vs 2.5% wet wt,  $P = 0.02$ ), and glycogen in livers of NTG was greater still (4.0% wet wt). This increase in liver glycogen content could be attributable to increased concentrations of glucose which serves as substrate for glycogen synthesis in hepatocytes. At 49 DIM, 14 d after glucagon treatment, glycogen content in livers of all four groups of cows was not different. Previous research using this ketosis induction protocol has shown that cows having liver TAG to glycogen ratios of less than 2 to 1 are resistant to ketosis (Drackley et al., 1992; Smith et al., 1997; Veenhuizen et al., 1991). This proved to be the case in the current experiment also; no STG became ketotic when the ratio of TAG to glycogen



(Figure 4C) decreased to 2 to 1 after 35 DIM. And, the incidence of ketosis was greatest in STG during 21 to 27 DIM when the ratio was at its peak.

### **Antiketogenic Activities**

Two aberrations in fatty acid oxidation and ketogenesis have been postulated as possible contributory factors for lactation ketosis. The first is an insufficiency of carnitine palmitoyltransferase. Fatty acid oxidation and ketogenesis are regulated by entry of long-chain fatty acids into mitochondria (Aiello et al., 1984), and carnitine palmitoyltransferase is responsible for this transfer. The activity of bovine carnitine palmitoyltransferase is sensitive to inhibition by concentrations of malonyl-CoA which, in turn, is increased by the activity of acetyl-CoA carboxylase and fatty acid synthetase, which as lipogenic enzymes are both induced by insulin and inhibited by glucagon (Grummer, 1991).

Second, oxaloacetate is necessary for entry of acetyl-CoA into the Krebs cycle and its subsequent oxidation to  $\text{CO}_2$ . Sufficiency of oxaloacetate is one factor regulating the extent of oxidation of acetyl-CoA to  $\text{CO}_2$  or formation of ketone bodies (Baird, 1982). Mills (1986b) hypothesized that the defect in hepatic metabolism affecting gluconeogenic and oxidative functions during ketosis were at the level of oxaloacetate because oxidation of fatty acids to BHBA was decreased.

Oxaloacetate is also an intermediate in glucose synthesis from lactate, pyruvate, propionate, and glucogenic amino acids. Therefore, increases in urea cycle activity exhibited by glucagon (Zhu, 1997) should serve to increase

concentrations of oxaloacetate, and, more importantly, oxaloacetate is formed during the conversion of pyruvate to phosphoenolpyruvate by pyruvate carboxylase. The activity of pyruvate carboxylase also is stimulated by fatty acyl-CoA and acetyl-CoA, both of which are increased after hydrolysis of free fatty acids from hepatic TAG by the action of a glucagon-stimulated lipase (Williamson et al., 1971). This activity is evidenced by the fact that addition of oleate to perfused rat livers (Williamson et al., 1971) and to sheep hepatocytes (Chow and Jesse, 1992) increased gluconeogenesis from pyruvate, lactate, and alanine but not from dihydroxyacetone, which enters the gluconeogenic pathway beyond the level of phosphoenolpyruvate. Additionally, glucagon increases conversion of pyruvate to phosphoenolpyruvate via pyruvate carboxylase activity and thus increases concentrations of oxaloacetate both in rats (Williamson et al., 1971) and in sheep (Brockman and Manns, 1973).

In 1971, Williamson stated, "Gluconeogenesis by glucagon is mediated by enhanced oxidation of fatty acids liberated from endogenous triacylglycerol through the activation effect of cyclic AMP on a hepatic lipase." Perhaps his explanation for increases in hepatic gluconeogenesis also help to explain the ability of glucagon to promote clearance of TAG from fatty livers of dairy cows.

### **Conclusion**

Taken together, this work along with that of She (1997) represents the first known attempt to examine the use of glucagon as a treatment for metabolic

disorders in livestock, specifically fatty livers and ketosis in dairy cows. Glucagon administered continuously for 14 d promoted clearance of TAG from severely fatty livers in early-lactation cows and simultaneously increased blood glucose concentrations. Furthermore, rates of lipolysis from adipose tissue and ketogenesis within the liver seem to be decreased over time by glucagon. Therefore, glucagon has potential for not only alleviating the hypoglycemic condition of dairy cows that have lactation ketosis but also for treatment or prevention of fatty liver, which is the gateway disorder for lactation ketosis and several other postpartal metabolic aberrations.

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## GENERAL DISCUSSION

Cows used in this study displayed the highest degree of fatty infiltration of the liver observed by researchers using the feed restriction and 1,3-butanediol ketosis induction protocol. In preparation for this experiment, cows were offered excess energy in the form of carbohydrates during the 30 d immediately preceding parturition. Overfeeding during the period immediately preceding parturition increased susceptibility to fatty liver and ketosis more than did overfeeding cows in late lactation, as had been done in the previous study (Smith et al., 1997). Furthermore, this study represents the first time the ketosis induction protocol was continued after the onset of clinical ketosis and thus provided an extreme test of the efficacy of glucagon as an antiketogenic agent. All cows used in this study were ketonemic from the onset of lactation, and the ketosis induction protocol served to maintain elevated concentrations of liver TAG and ketonemia in the control cows. Cows treated with glucagon, similarly to control cows, were kept in a state of ketonemia by the induction protocol, but concentrations of TAG in livers of the glucagon-treated cows were decreased dramatically even during the induction protocol. The return to normalcy was accelerated in glucagon treated cows after stopping the induction protocol. The beneficial effects of glucagon during recovery may be attributed largely to the decrease in the severity of fatty liver. Postulated mechanisms of beneficial effects of glucagon are illustrated in Figure 1 and discussed under the following topic.

**Figure 1. Proposed mechanisms of glucagon effects on hepatic lipid and glucose metabolism. Pathways increased by glucagon are indicated with bold arrows. Allosteric or indirect activation of enzymes by metabolites are indicated with narrow arrows and + or -. Postulated effects not documented are indicated with ?.**



### **Triacylglycerol Clearance**

Glucagon, in conjunction with insulin therapy, has been postulated to speed liver regeneration and has been used in animal models to improve outcome of liver failure (Johnson and Zaloga, 1996). In human medicine, elevated concentrations of insulin relative to glucagon during parenteral nutrition lead to excessive carbohydrate storage, decreased rates of lipid removal, and eventually result in hepatic steatosis. Glucagon has been found to ameliorate the development of steatosis in rats during parenteral nutrition (Johnson and Zaloga, 1996); however, its use for the prevention of fatty liver has not been explored in clinical situations.

Glucagon has not been observed to increase lipoprotein synthesis and VLDL export in animal models tested thus far. Species differences in regulation of VLDL export from liver, however, are known to exist. In fat loaded bovine hepatocytes, insulin decreased TAG export by 49% (Cad6rniga-Vali6no et al., 1997). Therefore, it would not be unreasonable to suspect that glucagon would increase lipoprotein synthesis and VLDL export as part of its counterregulatory effects toward insulin. Hepatic lipolytic activity of glucagon also may have enhanced VLDL export in the cows in this experiment. Hydrolysis of TAG would increase cytosolic NEFA concentrations, which in turn are known to stimulate synthesis of MTP and apoB-100, thereby increasing VLDL secretion. Determination of the effects of glucagon on lipoprotein metabolism in ruminants remains to be elucidated. The antilipogenic effects of glucagon on liver in dairy cows may be easier to explain through a

coordinate shift in liver metabolism towards increased lipolysis, fatty acid oxidation, and gluconeogenesis.

### **Gluconeogenic and Antiketogenic Effects**

The cows in our study exhibited a decreased ability to secrete insulin in response to stimulation by both glucose and glucagon. Insulin secretion in humans is decreased when glucose concentrations in plasma are less than about 83 mg/dl (Cryer, 1996). The threshold concentrations for plasma glucose to induce stimulation of insulin secretion in dairy cows is unknown, but it is probably greater than the 52 mg/dl observed in our study. Diminished insulin responses to glucose and glucagon also were observed in other studies when glucagon was injected into ketonemic cows (Holtenius, 1993).

Cows used by Holtenius (1993) had a diminished glucose response to glucagon also, but he only measured glucose concentrations for 40 min after injection of 2 mg of glucagon. Peak glucose concentrations in what he referred to as Type I ketotic were about 55 mg/dl. Holtenius interpreted this diminished glucose response to glucagon as a lack of gluconeogenic capacity. On the basis of our observations, what he observed was more likely a reflection of low concentrations of glycogen in the livers of the ketotic cows. If glucagon treatment had been continued, the increase in glucose concentrations probably would have been sustained. A similar response was observed to glucagon injections in humans with Type II diabetes (Clare et al., 1992). Glucagon injections elicited a stronger

glucose response in the diabetic subjects than in nondiabetic subjects in spite of low hepatic glycogen stores.

In Holtenius' research, ketotic cows that responded to glucagon injection with increases of both glucose and insulin (160 mg/dl and 6,000 pg/ml, respectively) were referred to as Type II because of similarities to Type II diabetes in humans. Therefore, Holtenius concluded that Type II ketosis is a form of insulin resistance that is associated with fatty liver; however, the concentrations of liver TAG, if measured, were not reported. Our data would suggest that Type II ketosis is indeed secondary to other metabolic disturbances but is not associated with fatty liver. In fact, Type II ketonemic cows in the study reported by Holtenius had initial plasma glucose concentrations of 59 mg/dl which is similar to that observed in the normal cows studied by She (1997), and the responses could be considered typical of a healthy, high-producing cow in early lactation.

Based upon our research, dairy cows with fatty liver evidently have gluconeogenic capacity equal to that of normal dairy cows in spite of lower basal rates of gluconeogenesis. Cows with fatty liver, however, do not have the glycogen reserves necessary to offset the sudden and severe hypoglycemia associated with clinical ketosis. Glucagon was able to restore liver glycogen concentrations simultaneously with clearance of triacylglycerols from the liver, thereby, decreasing the ratio of triacylglycerols to glycogen. A ratio of liver triacylglycerol to glycogen greater than 2 to 1 is a primary factor in the development of spontaneous lactation ketosis (Smith et al., 1997). The shift in liver composition induced by glucagon

along with the increase blood glucose concentrations in dairy cows allowed resistance to ketosis and increased rates of recovery from ketosis.

The increases in hepatic glycogen concentrations not only continued throughout glucagon treatment but also continued for 2 d beyond the end of glucagon treatment. Increases in liver glycogen concentrations probably are attributable to increases in hepatic glucose concentrations. The presence of glucose in hepatocytes is a strong inhibitor of glycogen phosphorylase (Salway, 1994). Additionally, the inhibitory effects on glycogen phosphorylase by glucose are enhanced by the presence of NEFA and amino acids in hepatocytes from rats (Morand et al., 1992). The same study showed that glycogen synthase was stimulated by NEFA by the presence of glucose, and that amino acids had stimulatory effects on glycogen synthase independent of glucose. Increases in concentrations of glucose-6-phosphate as a result of increased hepatic glucose concentrations have a stimulatory effect on glycogen synthase also. Therefore, the gluconeogenic and lipolytic activity of glucagon along with enhancement of hepatic amino acid uptake would drive the synthesis of glycogen as glucose concentrations increased and  $\text{Ca}^{++}$  stimulation of phosphorylase waned. Upon stopping glucagon treatment, however, the inhibitory effects of glucose and insulin on phosphorylase should have declined also. Because alterations in enzyme activity by phosphorylation of proteins generally have comparatively rapid effects on enzyme activity, it seems as though rates of synthesis of the enzymes regulating glycogen metabolism were altered by glucagon treatment also.

The net effects of glucagon observed on metabolism in this study were most likely driven by both lipolytic and gluconeogenic stimulation (Figure 1). Hydrolysis of cytosolic TAG would increase formation of fatty acyl-CoA and acetyl-CoA, promoting the activity of PC. Associated with increased activity of PC would be increased concentrations of OAA permitting greater rates of oxidation of fatty acids, possibly decreasing partial oxidation of fatty acids to ketone bodies. Concentrations of OAA also would have been increased by increases in hepatic extraction of amino acids from blood and activity of the urea cycle as amino acids are converted to gluconeogenic precursors. This diversion of amino acids from milk protein to hepatic gluconeogenesis would, therefore, not only increase blood glucose by providing gluconeogenic substrate but also increase concentrations of OAA and provide greater entry of acetyl-CoA into the citric acid cycle. Simultaneously, the activity of glucagon would decrease concentrations of malonyl-CoA, thereby removing inhibition of carnitine palmitoyltransferase and increasing entry of fatty acids into the mitochondria. Thus, glucagon is able to address the two predominate theories of the etiology of lactation ketosis, OAA insufficiency and decreased activity of CPTI.

### **Glucagon and Insulin Interactions**

One of the concerns raised at the conception of this experiment was that the lipolytic and ketogenic effects ascribed to glucagon would worsen the effects of the ketosis induction protocol by increasing lipolysis in adipose tissue of the susceptible

cows, leading to further increases in plasma NEFA and ketone body concentrations. It was postulated that increases in plasma insulin concentrations may be sufficient to counteract these potentially harmful lipolytic and ketogenic effects of glucagon. The insensitivity of  $\beta$ -cells to glucose and glucagon observed in STG cows, however, prevented the anticipated glucagon-induced increase in plasma insulin concentrations. Therefore, the dosage of glucagon used in this experiment may have been a critical factor in preventing increases in lipolysis and ketogenesis. The dosage chosen was only 1.7  $\mu\text{g}/100\text{ g}$  of BW/d, which is far below concentrations that have had ketogenic and lipolytic activities ascribed to glucagon for other species. It is also possible that insulin resistance of adipose tissue in early-lactation cows was sufficient that lipolytic rates could not be further increased by any agent.

Related to the insensitivity of  $\beta$ -cells and hypoinsulinemia observed in cows in our experiment and those of other researchers is the occurrence of hyperglucagonemia. Cows in our study did not have elevated concentrations of glucagon prior to glucagon treatment, but because insulin concentrations were low, the molar ratio of insulin to glucagon was such that the cows could be considered hyperglucagonemic. Furthermore, glucagon concentrations normally are elevated in portal compared with peripheral blood because the liver is primarily responsible for extraction of glucagon from blood (Ali and Jois, 1997). Rates of glucagon extraction are diminished, however, by necrotic or severely fatty livers. Thus, decreased extraction of glucagon by liver in ketotic cows would allow greater

concentrations of circulating glucagon, which would create an extrahepatic hyperglucagonemia. Additionally, it was postulated by deBoer (1986) that an impairment of glucagon uptake by the liver may be partly responsible for the onset of lactation ketosis. If deBoer's hypothesis is correct, the supplementation of endogenous glucagon by infusions of exogenous glucagon in our study may simply have increased glucagon uptake by the liver to a more normal rate.

Finally, if secretion of insulin by pancreatic  $\beta$ -cells had increased in response to glucagon treatment, the outcome of glucagon treatment may not have been positive. Increases of insulin concentrations in plasma would have limited availability of amino acids for hepatic metabolism, thus decreasing ureagenesis, gluconeogenesis, regeneration of hepatic proteins, and possibly lipoprotein synthesis. Insulin itself has been shown to inhibit transcription of MTP, which is vital for apoB-100 synthesis. Insulin also would serve to limit CPTI activity via increasing acetyl-CoA conversion to malonyl-CoA. The increase in hepatic lipogenesis also would have increased cytosolic TAG formation. It seems that decreased responsiveness of  $\beta$ -cells for insulin secretion was not so much a metabolic aberration in the ketotic cow but instead an appropriate response to fatty liver and declining carbohydrate status.

### **Summary**

This study has shown that treatment of early-lactation dairy cows with glucagon for 14 d was not only able to restore blood glucose concentrations and

alleviate the symptoms of ketosis, but glucagon also was able to remove accumulated TAG from the livers of cows with severely fatty livers. The discovery of the ability of glucagon to treat fatty liver in dairy cows is a major breakthrough in the study of fatty-liver syndrome. Glucagon could not only provide therapy for a heretofore untreatable condition but also may provide a means with which to prevent the development of the disorder in periparturient dairy cows and other livestock predisposed to fatty liver. Limited evidence of an antilipogenic effect of glucagon in liver is present in the literature, but, until this time, the possibilities for its use in commercial livestock production has not been examined. The use of glucagon as a metabolic conditioner for high stress periods in dairy cows and other livestock species is a prospect that deserves further investigation.



## APPENDIX. LIVER TRIACYLGLYCEROL MEASURES

Questions have been raised whether a 5 g sample of liver obtained during a single biopsy is representative of the composition of the liver as a whole. In an effort to address this question, four livers with varying degrees of fatty infiltration were obtained from Holstein cows after slaughter at a meat processor and two livers were collected from Holstein cows from the Iowa State University (ISU) dairy farm that had died of periparturient complications indicating involvement of fatty livers. Four samples of  $\approx 5$  g each were collected via biopsy cannula from widely dispersed sites within the livers collected from the meat processor. Similar samples were collected from 10 different sites within the livers of the cows from the ISU dairy farm.

The livers had average TAG concentrations that ranged from 1.1 to 12.5% of the wet weight of the livers. Within the liver having the lowest average TAG concentration, TAG ranged from 0.6 to 1.7% wet weight and the coefficient of variation was 53%. At the other extreme, the coefficient of variation in the liver with the greatest TAG content, 12.5% wet weight, was 34.9% wet weight. This liver had large regions of necrotic tissue that, interestingly, did not have the characteristic yellow tint of fatty infiltration and had TAG concentrations of approximately 5% compared with approximately 20% in the nonnecrotic tissue. The liver having the second highest TAG content, 10.8% wet weight, had a coefficient of variation of only 4.8% across locations. The average coefficient of variation for the 6 livers was 28.9% and was not correlated to TAG concentrations ( $R^2 = 0.21$ ,  $P = 0.36$ ). By

observation, if livers are not necrotic, the relative accuracy of determination of TAG content improves as TAG concentration of the liver increases. Analysis of the data by ANOVA indicated that location of biopsy did not have a significant effect upon the TAG concentration measured ( $P = 0.12$ ).

It has also been suggested that quantification of total lipid (TL) provides a reasonable estimate of the TAG concentrations in liver. This assumption is based upon the fact that the difference between TL and TAG consists primarily of structural lipids that are fairly constant within a range of TL concentrations. During the course of the ketosis experiment, 464 liver samples were analyzed for TL and TAG concentrations. Linear regressions of TAG concentrations as a function of TL were made to determine appropriate prediction equations for estimation of TAG concentrations. Over the entire range of TL (3 to 35% wet weight) and TAG, the  $R^2$  for linear regression was 0.99 and was described by the equation  $\text{TAG} = -2.83 + (1.04 \times \text{TL})$  (Figure A1A). It became obvious, however, that this does not adequately describe the relationship at TL concentrations of less than 5% wet weight (Figure A1B). The linear equation best describing the relationship of TAG to TL in that range was  $\text{TAG} = -0.70 + (0.44 \times \text{TL})$  and the  $R^2$  was only 0.55 for 181 observations. For the above reasons, a nonlinear curve fit was employed across the entire data set to describe the relationship (Figure A1C). The result of the nonlinear fitting derived the equation  $\text{TAG} = -2.88 + (1.05 \times \text{TL}) + (-3.91\text{E-}4 \times \text{TL}^2)$  and resulted in an  $R^2$  of 0.98.

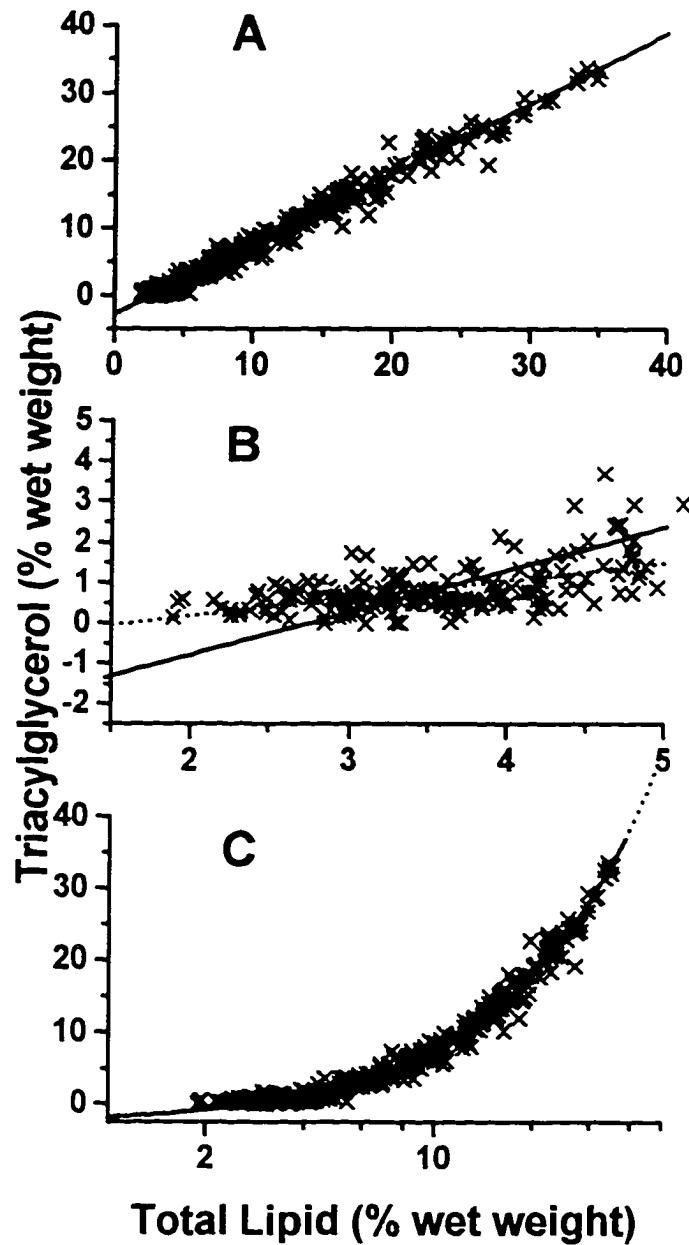


Figure A1. Relationships of liver triacylglycerol (TAG) to total lipid (TL) in liver samples collected during the course of the described experiment. **A. Linear regression of TAG as a function of TL in all samples (x).** N = 464.  $R^2 = 0.99$ . TAG =  $-2.83 + (1.04 \times \text{TL})$  (solid line). **B. Linear regression TAG as a function of TL in samples with less than 5% TL (x).** N = 181.  $R^2 = 0.55$ . TAG =  $-0.70 + (0.44 \times \text{TL})$  (dotted line). Solid line is regression from A. **C. Nonlinear regression TAG as a function of TL in all samples (x, log scale for TL).** N = 464.  $R^2 = 0.98$ . TAG =  $-2.88 + (1.05 \times \text{TL}) + (3.91\text{E-}4 \times \text{TL}^2)$  (solid line).

Above TL concentrations of 5% wet weight of livers, the prediction equations provide a good estimate of TAG concentration. The quantification of TAG involves, first the separation of TL and then subsequent enzymatic or chemical quantification. The use of equations for estimation of TAG content will decrease greatly the amount of time spent on analysis and provide accurate and timely measure of TAG for concentrations most critical during the development of fatty liver.

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