Glucose kinetics and hepatic gluconeogenesis in ketotic and fasted steers

Robert Randall Lyle

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GLUCOSE KINETICS AND HEPATIC GLUCONEOGENESIS IN KETOTIC AND FASTED STEERS

Iowa State University

Ph.D. 1983

University Microfilms International 300 N. Zeeb Road, Ann Arbor, MI 48106
Glucose kinetics and hepatic gluconeogenesis in ketotic and fasted steers

by

Robert Randall Lyle

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Department: Animal Science
Major: Nutritional Physiology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

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For the Graduate College

Iowa State University
Ames, Iowa

1983
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\[
SRA_t = 0.00413e^{-0.41195t} + 0.00234e^{-0.05361t} + 0.0094e^{-0.00690t}
\]

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INTRODUCTION

Glucose metabolism in ruminants differs in many respects from that in other mammals. In non-ruminants, dietary carbohydrates are digested to glucose and other monosaccharides, absorbed into portal blood, thereby providing a direct source of carbohydrates for metabolism. In ruminants, however, most of the dietary carbohydrates are fermented to volatile fatty acids in the rumen (21). Acetic, propionic, and butyric acids are the major volatile fatty acids formed in the rumen, and of these, propionate is the only gluconeogenic precursor. Acetate and butyrate are exclusively ketogenic. When ruminants consume diets based on forages, less than 10% of the glucose utilized is absorbed from the digestive tract (4, 105).

In ruminants, as in other mammals, glucose serves two major functions in metabolism. It provides components for synthesis of nucleic acids, proteins, and lipids, which are used to construct complex macromolecules of cells. In addition, glucose is utilized to provide the energy required for the synthesis of macromolecules, and it also serves as an energy substrate for functioning of the central nervous system (84).

Ruminants are fully capable of maintaining glucose
homeostasis during most physiological states; however, during periods of high production, such as lactation in the dairy cow or pregnancy in the ewe, glucose homeostasis may not be maintained. Because the gluconeogenic process must provide up to 90% of the glucose utilized by ruminants (84, 88), it is evident that any failure in processes regulating gluconeogenesis could cause severe physiological perturbations.

Bovine lactation ketosis is thought to be related to or caused by a breakdown in processes regulating glucose homeostasis, thereby allowing development of hypoglycemia and ketonemia. During later stages of lactation, cows are seldom ketotic; however, during early lactation when milk production is high, cows are particularly susceptible to carbohydrate insufficiency and adipose tissue mobilization. It seems that high-producing cows have difficulty adjusting feed intake to milk output during early lactation and are usually, therefore, in negative energy and protein balance (69).

Krebs (77) described how selective breeding for increased milk production has produced cows capable of producing much more milk than is required for rearing a calf. A typical dairy cow now is capable of producing
7500 kg of milk during a 305 day lactation, when only a few hundred kg is sufficient for rearing a calf. Therefore, greatly increased milk production presents a challenging physiological stress that can disrupt homeostatic mechanisms involved in regulation of metabolism.

In lactation ketosis, carbohydrate insufficiency and adipose tissue mobilization probably originate as the result of interplay between at least three factors. The first factor is the priority accorded to the lactating mammary gland for nutrients, particularly glucose. The second is that in early lactation, voluntary food intake may be limited, causing an insufficiency in energy for maintenance and production. The third is that the endocrine status of the cow is oriented to promote lipid mobilization (7). It seems that these three factors are primarily responsible for development of spontaneous lactation ketosis.

The studies reported in this dissertation were undertaken to determine the efficacy of using dietary 1,3-butanediol, which causes ketonemia, and injections of phlorizin, which causes glucosuria, to induce an experimental ketosis in steers. Subsequently, effects of ketonemia and glucosuria on kinetics of glucose metabolism and on in vitro hepatic gluconeogenesis and ketogenesis
were determined. In addition, effects of fasting on these same variables were studied. Finally, some effects of Ca\textsuperscript{++}-free incubation media and in vitro additions of ketone bodies and long-chain fatty acids on in vitro hepatic gluconeogenesis were determined.
REVIEW OF LITERATURE

The purpose of this review of literature is to provide the reader, in a concise manner, with a good overview of the information available regarding: (a) etiology of bovine lactation ketosis, (b) metabolic effects of ketosis, (c) gluconeogenic precursors in the ruminant, (d) kinetics of glucose metabolism, (d) the need for an experimental ketosis, (e) metabolic effects of fasting, (f) metabolic effects of phlorizin, and (g) metabolic effects of 1,3-butane diol.

**Etiology of bovine lactation ketosis**

Bovine lactation ketosis is a metabolic disorder that affects cows during the first few weeks of lactation. Ketosis is characterized by ketonemia, hypoglycemia, depletion of liver glycogen, hypoinsulinemia, hypophagia, fatty liver, and decreased milk production. If ketosis occurs spontaneously, i.e., is not accompanied by some other physiological abnormality, it is termed primary ketosis. If ketosis develops as a result of physiological perturbations associated with some other disorder, it is termed secondary ketosis. Primary ketosis is observed most frequently in high-producing cows that are either
over-conditioned (fat) or under-nourished. Secondary ketosis frequently occurs in cows suffering from simple indigestion, milk fever, metritis, mastitis, or displaced abomasum. Whether ketosis is primary or secondary, ketonemia and hypoglycemia always occurs.

Lactation ketosis is rarely fatal, because there is usually not complete inappetance and ketosis usually remits when milk production has decreased substantially. But the more advanced ketosis becomes, the more difficult it is to reverse, because of hepatic changes probably related to fat deposition, which alter normal liver function (121).

Despite extensive research, the mechanisms responsible for development of spontaneous ketosis in cows remain unknown. It is doubtful that ketosis is caused by abnormal fermentation in the rumen. Radloff and Schultz (107) reported that concentrations and proportions of volatile fatty acids in the rumen of cows during the early stages of ketosis were not different from those in normal cows.

The most accepted theory regarding causes of ketosis involves development of a carbohydrate insufficiency or "glucose shortage" in cows during the period of negative
energy balance in early lactation (8, 121). Bergman (21) stated that hypoglycemia is probably the major factor involved in the onset and development of early clinical aspects of ketosis. In an eloquent review of the literature on bovine ketosis, Baird (8) states, "the key factor in rendering dairy cows susceptible to spontaneous ketosis is the metabolic priority given to the demands of milk production at a time when appetite is limited". In lactating cows, the mammary gland, rather than other body tissues, preferentially receives nutrients from blood as a result of the pattern of hormone secretions during the periparturient period (8). During lactation, therefore, the gluconeogenic capacity of the liver should increase to meet the needs of the cow for glucose for maintenance and production.

A proposed mechanism by which carbohydrate insufficiency or "glucose shortage" could cause ketosis is as follows. First, high levels of milk production and the secretion of large quantities of lactose results in carbohydrate stress, which causes hypoglycemia in the cow. The supply of glucose may not meet the demand for glucose, perhaps as a result of either inadequate availability of gluconeogenic precursors or a limited rate of gluconeogenesis. Second, hypoglycemia may cause hypo-
insulinemia, which subsequently would allow greater mobilization of fatty acids from adipose tissue for use as energy substrates, thereby increasing influx of free fatty acids (FFA) into the liver. Third, in the liver, FFA are either reesterified into triglyceride for storage and incorporation into very low density lipoproteins (VLDL) or are oxidized to carbon dioxide and ketone bodies (KB). Factors regulating the metabolism of FFA by liver have not been determined fully. Fourth, an influx of large quantities of FFA into liver causes a high rate of hepatic ketogenesis and the concurrent hypoinsulinemia may limit oxidation of KB by peripheral tissues, thereby causing ketonemia and the ensuing clinical signs of ketosis. Fifth, ketonemia usually is accompanied by hypophagia, which causes decreased milk yield.

Kronfeld (79), in a recent review, offers a "heuristic" approach to the problem of ketosis in dairy cows and states that a major determinant of development of spontaneous ketosis is the ratio of glucogenic to lipogenic nutrients in the diet. Kronfeld states that an excess of glucogenic nutrients relative to lipogenic nutrients is the cause of spontaneous ketosis. The antithesis of Kronfeld is based on the following: (1) milk volume is determined by the availability of glucose for
uptake by the mammary gland, thereby creating a demand for lipogenic nutrients; (2) because diets fed to ruminants usually have a low fat content, body fat is mobilized to meet the needs of the mammary gland for synthesis of milk fat; (3) the outcome is that the increased plasma FFA concentration causes a proportionate increase in hepatic uptake of FFA, and hence, increased hepatic ketogenesis. Therefore, in attempts to explain the causes of ketosis, the lipid shortage theory of Kronfeld is in direct opposition to the glucose shortage theories of Baird (8) and Schultz (121).

The role of glucose in regulating mobilization of fatty acids from adipose tissue is especially important and concentrations of plasma glucose and FFA are correlated negatively to each other (110). Plasma FFA concentration is a sensitive index of the nutritional status of an animal and is correlated negatively to feed intake (108) and correlated positively to blood KB concentration (110).

The liver is a major utilizor of FFA, with about 25% of the total FFA mobilized from adipose tissue being taken up regardless of FFA concentrations in plasma (23). Thus, when more fatty acids are released from adipose tissue, more will be taken up by the liver. Liver and rumen
epithelium are the principal sites of ketogenesis in the ruminant (20). In liver, the principal precursor of KB is FFA (86) and in rumen epithelium, the principal precursor is butyrate (140). Ketone bodies should not be considered as metabolic waste products, because they serve as fuel for respiration in animal tissues and play an important role in maintaining caloric homeostasis. Only during periods of excessive ketogenesis do ketone bodies present a physiological hazard to cows.

Experiments conducted with fasted cows during various stages of lactation demonstrate the priority given to synthesis of milk in early lactation. Baird (6) showed that, from about 14 to 35 days post-partum, cows will attempt to maintain milk yield during food deprivation, and as a result, will become ketotic. Baird et al. (10) showed that, during later stages of lactation, milk production decreases rapidly during food deprivation and ketosis is avoided. These reports support the observation that dairy cows are most susceptible to ketosis between 10 and 35 days post-partum and that ketosis often is not observed beyond 75 days post-partum (120).

Krebs (76) described two types of bovine ketosis. The first type, physiological ketosis, occurs during
starvation or when a low carbohydrate diet is consumed and can be considered a normal process for supplying tissues with a readily utilizable fuel for respiration. The second type, pathological ketosis, often is seen in high-producing cows and is characterized by severe ketonemia and occurs when the quantity of ketone bodies formed grossly exceeds possible utilization. Development of pathological ketosis seems to occur only when demands on the gluconeogenic pathway are great. It is apparent that these two types of ketosis are different, yet there is no precise boundary distinguishing one from the other and they cannot be considered totally different entities.

Brockman (32) aptly discussed the involvement of insulin and glucagon in the development of ruminant ketosis and reported that the endocrine system, especially the pancreas, probably is involved intimately in the development of ketosis. Low concentrations of insulin in plasma may cause ketosis by triggering release of fatty acids from adipose tissue (32), by promoting maximal rates of hepatic ketogenesis (32), and by impairing utilization of KB by peripheral tissues (15). Concentrations of insulin in plasma are lower during early lactation than at any other time of the lactation cycle and this often occurs concurrently with ketonemia (62).
The relationship between glucagon and bovine ketosis is not known fully, however, it has been reported that high concentrations of glucagon may be slightly lipolytic and ketogenic in sheep (31). But another report (8) indicated that, compared with nonruminants, the ketogenic effect of glucagon in ruminants is very low. Studies with sheep (31) revealed that insulin can negate the ketogenic effect of glucagon.

Based on available information, it seems that spontaneous lactation ketosis is caused, at least partly, by an inadequate supply of dietary gluconeogenic precursors during the early stages of lactation when milk production is great and when cows are in negative energy balance. Although hormonal imbalances may be associated closely with ketosis, they are secondary to the inability of dietary gluconeogenic substrates to meet the nutritional and energy demands imposed by lactation and may also be secondary to the development of ketosis.

**Metabolic effects of ketosis**

Bovine lactation ketosis is characterized by several changes in blood, such as hypoglycemia (6, 20, 78), hyperketonemia (6, 13, 60), hypoinsulinemia (32, 62, 123), and increased FFA (6). Ketosis also involves changes in
concentrations of metabolites in liver, such as decreased glycogen (13, 60), increased triglyceride (20, 60), and increased ketone bodies (13, 60). Baird et al. (10) and Baird and Heitzman (9) reported that concentrations of intermediates associated with the tricarboxylic acid cycle and the Embden-Meyerhof pathway were less in liver of ketotic cows than in liver of normal cows.

It is possible that, during ketosis, hypoglycemia is the primary deviation from the normal physiological state and that all other changes in blood and liver metabolite concentrations are secondary to hypoglycemia. This possibility was discussed previously in the section dealing with "Etiology of Bovine Lactation Ketosis".

In ruminants, gluconeogenesis is essential for maintaining glucose homeostasis both during periods of feeding and food deprivation (88). In nonruminants, however, gluconeogenesis is of value in maintaining glucose homeostasis only during periods of food deprivation (16), and the mechanisms that regulate gluconeogenesis in non-ruminants have been well elucidated (50, 104, 124). The patterns of adaptation of key gluconeogenic enzymes in ruminant liver, however, are different from those in non-ruminants, indicating that the mechanisms that control gluconeogenesis in ruminants are unique to the species (16).
Flux of metabolites through the gluconeogenic pathway is mediated by certain key enzymes, namely pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-diphosphatase (FDPase), glucose-6-phosphatase, and propionyl-CoA carboxylase. Propionyl-CoA carboxylase, PEPCK, and PC are likely to be of importance in hepatic metabolism in ruminants in two ways. First, their activities may determine the overall rate of hepatic gluconeogenesis (21). Second, the relative activities of these three enzymes may be of significance in determining steady-state concentrations of intermediates of the tricarboxylic acid cycle (76).

Mills (101) reported that, in lactating cows with experimental ketosis, gluconeogenic capacity of liver was much less than when cows were not lactating or when cows were lactating and normal. Mills' findings, however, have not been substantiated by other reports. The effects of bovine ketosis on activities of the key gluconeogenic enzymes are not known fully, however, it seems that the gluconeogenic capacity of liver is not altered during ketosis. No distinct differences in the activities of PC, PEPCK, or FDPase were noted between normal and ketotic cows (13). But Ballard et al. (16) reported that activity of PC was greater in liver of ketotic cows than in
liver of normal cows. Baird and Heitzman (9) and Ballard et al. (16) found that activity of PEPCK in liver was not affected by ketosis and Hibbitt and Baird (60) reported that the activity of FDPase in liver of spontaneously ketotic cows was not different from that in normal lactating cows. Activity of hepatic glucose-6-phosphatase was not affected by ketosis (56). Although it is not a gluconeogenic enzyme, pyruvate kinase controls the glycolytic pathway at the point between phosphoenolpyruvate and pyruvate and variations in its activity probably play a key role in the regulation of gluconeogenesis. Activity of pyruvate kinase was only 60% of normal in liver of ketotic cows (56), suggesting that glucose was being spared from oxidation.

Propionate derived from rumen fermentation is considered to be the major gluconeogenic substrate in fed ruminants. Activity of propionyl-CoA carboxylase, which regulates the conversion of propionate to tricarboxylic acid cycle intermediates, was not different from normal when cows were ketotic (13). Mathias and Elliot (92) reported that conversion of propionate to tricarboxylic acid cycle intermediates in liver homeogenates from cows was not affected by ketosis.

Based on available information regarding activities
of enzymes involved with gluconeogenesis, it seems that the gluconeogenic capacity of liver is not impaired during ketosis. In addition, in vivo experiments indicated that glucose irreversible loss was not affected during the early stages of spontaneous ketosis (80, 81, 83). Therefore, it seems that the "glucose shortage" that is implicated in the development of ketosis can be attributed to either outflow of glucose exceeding inflow, or to a limitation in the availability of gluconeogenic substrates, rather than to a limitation in the capacity of liver to synthesize glucose. Atwal and Sauer (5) reported that precursor availability was the major factor limiting hepatic gluconeogenesis in ruminant liver and that the availability of glucogenic precursors was determined by feed intake and by rates of mobilization of amino acids from muscle proteins and of glycerol from adipose tissue. The hypophagia that typically accompanies ketosis (120) undoubtedly limits availability of glucogenic precursors for hepatic gluconeogenesis.

Gluconeogenic precursors in the ruminant

Because most of the glucose in diets of ruminants is fermented to volatile fatty acids in the rumen and because little glucose is absorbed from the intestinal tract, it
is apparent that gluconeogenesis is of vital importance in maintaining glucose homeostasis. Rates of hepatic glucose production in pregnant and non-pregnant sheep were, on the average, 85% of the total glucose turnover (21), thereby indicating the importance of the liver in supplying glucose to the ruminant.

The four groups of metabolites that serve as significant glucogenic substrates in ruminants are: (1) propionate, (2) glycerol, (3) amino acids, and (4) lactate and pyruvate (51, 74). The relative importance of each group still is not known. Obviously, type of diet consumed, feeding regime, and physiological status of the animal would greatly influence relative contributions of each compound to gluconeogenesis.

Propionate is the major gluconeogenic volatile fatty acid produced by rumen fermentation (24, 85, 118). The conversion of propionate to glucose occurs almost entirely in liver (21) and about 90% of the propionate in portal blood is removed in every circulation through the liver (26, 39). Estimates of the amount of absorbed propionate converted to glucose are variable and range from 19 to 60% in mature sheep (21, 24, 67, 85, 129, 138). Estimates of the amount of glucose derived from propionate are
variable and range from 27 to 55% (24, 68, 87). On a quantitative basis, propionate is probably the most important glucogenic precursor in the fed ruminant.

Glycerol is a moiety of triglycerides and, therefore, most glycerol in the body exists in combination with long-chain fatty acids. Under normal conditions, blood contains only small amounts of free glycerol. In fed animals, gluconeogenesis from glycerol is small and probably accounts for less than 5% of the total glucose produced (25). In the undernourished animal or in heavily lactating dairy cows, large amounts of glycerol are released from adipose tissue. Under such conditions, glycerol can be a quantitatively important glucose precursor (21). In experiments with fasted and ketotic sheep, glycerol accounted for nearly 30% of the glucose production (25).

The contribution of amino acids to gluconeogenesis is variable and is influenced by the nutritional and physiological status of the animal (21). Amino acids derived from muscle protein are important substrates for gluconeogenesis during fasting (37). Almost all amino acids are glucogenic, with lysine, leucine, and taurine being exceptions (75). Any stored amino acids are almost exclusively in muscle and these, along with dietary amino
acids, are utilized by the liver and kidneys as glucose precursors (21).

From studies with sheep, Ford and Reilly (53) and Reilly and Ford (111) estimated that incorporation of amino acids into glucose accounted for 13 to 30% of glucose turnover. Hunter and Millson (63) reported that 12% of milk lactose was derived from amino acids in lactating cows. Black et al. (28) and Egan and Black (49), using lactating cows and goats, estimated that 30 to 50% of glucose turnover may arise from amino acids, of which alanine and glutamate each represent 6 to 8% of glucose turnover. Wolff and Bergman (139) reported that sheep derived at least 11% of their total glucose from the composite of alanine, glutamine, glutamate, glycine, and serine. As a maximum based on net hepatic amino acid uptake, 30% of the glucose could have been derived from the total of all glucogenic amino acids.

Pyruvate is the end-product of glycolysis and if cells of actively working muscle become anaerobic, pyruvate is reduced to lactate. Both pyruvate and lactate are gluconeogenic precursors. When lactate is produced in muscle, it is carried by blood to more aerobic organs, such as heart, kidneys, or liver for further metabolism. In
liver and kidney, lactate either may be oxidized or converted to fatty acids or glucose.

If lactate is converted to glucose, the hexose then is released into the blood and may return to muscle for synthesis of glycogen or oxidation (84). This cyclic process is referred to as the Cori cycle (40) and is of significance only when the supply of glucose precursors, other than lactate from glucose, is limited. In fed ruminants, where the supply of glucose precursors is large with respect to the amount actually required, the passage of glucose carbon through lactate and back to glucose occurs mainly due to a dilution of lactate in gluconeogenic precursor pools (84).

It is important to note that operation of the Cori cycle will not result in a net synthesis of glucose, because lactate itself is derived from glucose. Lactate originating from microbial fermentation in the rumen, however, can result in a net synthesis of glucose, but ruminal lactate is of quantitative significance only when cattle are fed high-concentrate diets. Studies using fed sheep (2, 23) indicated that lactate accounted for a maximum of 4 to 10% of the glucose turnover. In experiments with starved sheep (2), 15% of the glucose turnover originated from lactate.
Kinetics of glucose metabolism

Numerous investigations using radioisotope dilution techniques have been made of various parameters of kinetics of glucose metabolism in ruminants. The most frequently reported variables dealing with the kinetics of glucose metabolism are irreversible loss (also known as entry rate), total entry rate, recycling, pool size, and glucose space (137).

Glucose irreversible loss is the rate at which glucose carbon leaves the sampled compartment never to return (137). If an animal is in steady state in regard to glucose metabolism, then the rate at which glucose enters the sampled compartment equals the rate at which glucose leaves the sampled compartment, therefore, irreversible loss is an estimate of the net rate at which new glucose enters the glucose pool. Because, as discussed previously in "INTRODUCTION", the ruminant must synthesize up to 90% of the glucose utilized, irreversible loss also can be regarded as an estimate of the net incorporation of glucogenic substrates into glucose. Irreversible loss has been determined by using single injections of [U-14C]glucose (3, 83, 84) or by using primed-continuous infusions of [U-14C]glucose
Young et al. (143), after measuring kinetics of glucose metabolism in calves under standardized conditions, reported that values obtained for glucose irreversible loss were similar when determined either by single injections or by primed-continuous infusions of tracer.

Glucose total entry rate is the rate of entry of all glucose carbon into the sampled compartment (137), regardless of whether the glucose is absorbed from the intestinal tract, is synthesized from exogenous or endogenous precursors, is released by hydrolysis of glycogen, or is recycled from another compartment.

Glucose recycling is the rate of return of glucose carbon that temporarily left the sampled compartment (137) and recycling occurs by both physical and chemical means. The difference between total entry rate and irreversible loss is regarded as the rate of recycling of glucose carbon between the sampled and other peripheral compartments.

Glucose pool size is the quantity of glucose carbon present in the sampled compartment (137). Pool size can be regarded as the quantity of body glucose with which injected isotopically-labeled glucose mixes.
Glucose space is the volume of body water in which the glucose pool is distributed (137). Glucose space is expressed as a percent of body weight.

The single-injection technique for measuring the kinetics of glucose metabolism allows for determination of each of the variables mentioned previously, however the primed-continuous infusion technique only allows for determination of irreversible loss. But because irreversible loss is considered to be the most important variable dealing with kinetics of glucose metabolism, use of the primed-continuous infusion technique is warranted. Because a plateau is determined for blood glucose specific radioactivity during primed-continuous infusions, estimates can be made to determine whether the animal is in steady-state in regard to glucose metabolism. However, steady-state conditions cannot be verified when the single-injection technique is used because, when using this technique, blood glucose specific radioactivity continually changes following injection of tracer.

Few studies have dealt with changes in the kinetics of glucose metabolism caused by lactation ketosis, probably because of the high costs associated with such
experiments and because of the lack of a predictable supply of clinically ketotic cows. Reports by Kronfeld et al. (81, 83) and Kronfeld and Raggi (80) indicated that glucose irreversible loss and glucose pool size were not different from normal during the early stages of spontaneous ketosis. Glucose space, however, was greater in ketotic cows than in normal cows (78, 83), which could explain how hypoglycemia could occur when glucose irreversible loss and pool size remained normal. Although values for glucose irreversible loss were not affected during the early stages of spontaneous ketosis, values were decreased during advanced stages of ketosis when cows were hypophagic (6, 78).

Effects of fasting on kinetics of glucose metabolism are well-documented, as evidenced by studies which verify that glucose irreversible loss is decreased during fasting in sheep (128), steers (143), and cows (78, 80, 81). It seems, therefore, that during food deprivation, the availability of endogenous glucogenic precursors is not sufficient to maintain normal rates of gluconeogenesis.

The need for an experimental ketosis

A major problem in the study of bovine lactation ketosis is the lack of a consistent and predictable supply of ketotic cows for use in controlled experiments.
Most studies of ketosis have involved analyses of blood collected from ketotic cows on dairy farms. But the analysis of blood provides information only about secondary changes arising from the primary changes in individual organs and tissues of ketotic cows. For researchers to conduct experiments using body tissues, clinically ketotic cows must be available for use at laboratories suitably equipped for the surgical removal of tissue samples and subsequent study of metabolic capacities of the tissues. In addition, experiments that involve use of radioactive tracers in ketotic cows preclude on-farm experiments, thereby requiring that tracer experiments be conducted in suitable research facilities. For these reasons, it would be very beneficial if a suitable ketosis model could be developed, that would eliminate several problems involved in conducting metabolic experiments with ketotic cows located on dairy farms.

To provide a dependable supply of ketotic cows for use in controlled experiments, attempts have been made to induce experimental ketosis. Indeed, some forms of ketosis in cows have been produced by reducing feed intake (12, 33, 52) or by using a combination of reduced feed intake and injections of thyroxine (70, 71).
Ketosis, accompanied by the clinical symptoms of acetonemia, was induced in cows by a combination of high-protein feeding and injections of L-thyroxine (58, 59). Burtis et al. (34) used a combination of starvation, hormone, and phlorizin treatments to cause ketosis in ewes.

Both use of fasting, either alone or in combination with hormone treatments, and use of lactating cows to provide ketotic animals for use in ketosis research create problems. When lactating cows are fasted or feed-restricted, milk production usually declines immediately, thereby decreasing or eliminating the causative stress. Furthermore, it seems that ketosis induced by starvation is not entirely typical of spontaneous ketosis. Kronfeld (78) reported that plasma acetate and liver ketone body concentrations, mammary acetoacetate release, glucose space, and glucose transport rates were greater during spontaneous ketosis than during starvation ketosis. In addition, it generally is thought that ketonemia causes hypophagia during spontaneous ketosis. But an obvious contradiction occurs when ketosis is induced by fasting, because, in this situation, reduced feed intake precedes ketonemia.

Use of lactating cows to study ketosis is often
impractical, because of the high expenses incurred. High-producing dairy cows and the milk they produce are valuable and when kinetics of metabolites are studied, use of radioactive tracers necessitates disposal of both the cow and the milk. Therefore, it would be advantageous if a suitable ketosis model, other than the feed-restricted cow, was available for use in ketosis research.

**Metabolic effects of fasting**

In fasting ruminants, as in other fasted animals, the major source of fuel for cellular metabolism is the triglycerides of adipose tissue. Through the action of lipolytic enzymes and fatty acid-mobilizing hormones, fatty acids are hydrolyzed from triglyceride molecules and the FFA are transported via blood as a complex with plasma albumin to the liver and to other tissues for use as energy substrates. Studies indicate that negative energy balances imposed on dairy cows by fasting cause increased concentrations of FFA in plasma (10, 12, 17, 112). Negative energy balances imposed by fasting not only cause increases in plasma FFA but also cause increases in concentrations of blood ketone bodies (10, 12, 17, 112), liver ketone bodies (12), liver
triglycerides (109), and hepatic outputs of ketone bodies (12). Baird et al. (10) reported that fasting caused marked reductions in blood glucose concentrations in lactating cows, however, in a later study with non-lactating cows (12), fasting did not cause marked reductions in blood glucose concentrations.

When lactating cows were fasted for only 24 hours, extensive changes in blood metabolite concentrations were noted even though there still was a considerable quantity of utilizable food remaining in the rumen (10). It seems, therefore, that metabolic consequences to fasting are rapid, with the purpose of providing body tissues with an energy substrate. Robertson et al. (112) and Baird et al. (10) reported that mature cows in the early stages of lactation developed severe ketonemia when fasted for 4 to 6 days. When cows were fasted in later stages of lactation, however, susceptibility to food-deprivation ketosis was not as great. When non-lactating cows were fasted for 6 days (12), ketonemia occurred, but severity of the ketonemia was no greater than that normally seen in healthy, fed cows during early lactation.

It is known that fasting increases hepatic gluconeogenic capacity in non-ruminants (119); however, the
effects of fasting on hepatic gluconeogenesis are not fully known for ruminants. Because gluconeogenesis is a continual process in ruminants (88), it is possible that fasting does not induce an increase in the process. Lindsay (87) indicated there was a decrease in the rate of gluconeogenesis when cows were fasted. Baird and Young (14) reported that during fasting of lactating cows, the activity of propionyl-CoA carboxylase decreased, but no effects on activities of PC or PEPCK were noted. In other reports, however, fasting caused a decrease in PEPCK activity (10, 36). Baird et al. (10) found that when lactating cows were fasted, activity of pyruvate kinase decreased and activity of glucose-6-phosphatase was not affected.

Changes in blood and liver metabolite concentrations caused by fasting are similar to those observed in cows having ketosis, which suggests that the two conditions are similar. It is likely that the hypophagia that usually accompanies ketosis is largely responsible for physiological similarities of ketosis and fasting. If ketotic cows did not become hypophagic, changes in blood and liver metabolite concentrations probably would not be as great. Caution must be exercised, therefore, when evaluating effects of ketosis on intermediary
metabolism, because effects of ketonemia-hypoglycemia and hypophagia usually are confounded.

**Metabolic effects of phlorizin**

In mammalian kidney, filtered glucose is almost completely reabsorbed in the proximal convoluted tubule by an active mechanism with a maximum transport rate (27). Von Mering (135, 136) was the first to report that phlorizin (phloretin-2-β-glucoside) caused excretion of large amounts of glucose in the urine. The glucosuria induced by phlorizin (P) results from an inhibition of renal tubular reabsorption of filtered glucose (61, 66, 93, 106). The interaction of P with proximal tubule glucose transport involves both competitive and non-competitive inhibition (30). Phlorizin also has been shown to inhibit glucose transport across intestinal brush border in vitro (1).

It seems that P acts by competing with glucose for the membrane receptor site that normally binds glucose, thereby preventing the first step in the series of reactions that leads to transport of glucose across cell membranes in the renal tubule (46). But P itself is not translocated across the brush border membrane by the glucose transporter (126, 130). Diedrich (46) did
not determine whether the glucose moiety of P was solely responsible for the inhibitory effects on glucose transport. It seems that some feature of the aglucone moiety of P facilitates interaction of the glucosidic portion of the molecule with the glucose receptor and, as a result, stability of the complex is much greater than that formed with the free glucose molecule.

The ability of P to depress the transfer maximum for glucose suggests that P has a much greater affinity for the transport receptor site (46). The affinity of P for the glucose transport site in both intestine (1) and kidney (38) has been estimated to be at least 1000 times greater than that shown by glucose.

Although P blocks entry of monosaccharides into cells, it does not affect energy yielding processes of cells (46). For example, P inhibits penetration of hexoses through cell membranes of tissues in which transport of glucose is not energy dependent (18, 43, 102). Newey et al. (103) reported that P inhibits intestinal transport of glucose at concentrations that do not affect the intracellular metabolism of the hexose.

Other reports, however, suggest that P may cause physiological perturbations other than glucosuria. Shapiro (125) reported that P decreased glycolysis and
the aerobic metabolism of pyruvate and citrate in kidney mince. Lotspeich and Keller (89) observed that not only oxidation of citrate but also oxidation of all tri-carboxylic acid cycle intermediates and of β-hydroxybutyrate and glutamate were depressed in kidney upon addition of P. Horsburgh et al. (61) stated that metabolism of glucose by kidney, expressed as the rate of carbon dioxide production, was variably altered by P. Eboue-Bonis and Clauser (48) reported that P depressed biosynthesis of glycogen in isolated rat diaphragm. Other reports indicated that P depressed phosphorylation reactions in mammalian tissues (42, 90, 113). Whether P affects activities of these metabolic pathways in vivo is not known.

Several reports indicate that activities of some enzymes are sensitive to P. For example, hexokinase (42), phosphorylase (41), and phosphatase (89, 93) are inhibited by P in vitro. Phlorizin also inhibited activity of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and alkaline phosphatase in kidney in vivo, however, activities of these enzymes, when from a purified, commercial source, were not influenced by P in vitro (144).
Most early work with P involved experimentation with dogs, however, P has been used more recently with ruminants (34, 65, 142). The effects of P in ruminants are similar to those observed in nonruminants. Phlorizin induces glucosuria in goats (122), sheep (55), and cattle (127, 142). Nonpregnant ewes treated with P developed ketonemia and hypoglycemia (34, 65) typical of ovine pregnancy toxemia; however, despite P-induced changes in blood metabolites, clinical signs of ketosis did not develop. Other reports indicated that P caused hypoglycemia when given to fasted sheep (34, 35, 65) and fasted goats (55). Young et al. (142) were the first to report results of dose response studies with P in the bovine and they found that glucosuria in steers was maximal when the P dose was 2 g per day subcutaneously.

There are only a few studies involving effects of P on in vivo kinetics of glucose metabolism. Young et al. (142) reported that treatment of steers with P caused glucose irreversible loss to increase but did not affect pool size or total entry rate of glucose. Kolodny et al. (73) reported that P caused a rapid increase in hepatic glucose output in normal dogs, but not in functionally nephrectomized dogs, indicating
that the P-induced increase in hepatic glucose output was dependent on loss of glucose from the body via the urine. The mechanisms by which P causes increases in glucose irreversible loss and hepatic glucose output have not been determined, but it was suggested that low concentrations of glucose in blood perfusing the liver cannot account for the P effect (73).

Metabolic effects of 1,3-butanediol

The compound 1,3-butanediol (BD) is a commercially-available, petroleum-derived glycol that is odorless and colorless and has been used as an energy source in the diets of rats (94, 95, 100, 132, 134), pigs (115, 117), chickens (44, 45, 64), cattle (29, 57, 141), dogs (114), and humans (72). The metabolizable energy value of BD is approximately 6.5 kcal/g (100), therefore, the caloric density of BD is greater than that of carbohydrates but less than that of fats. Dylmsza and Miller (47), Mehlman et al. (94), and Stoewsand et al. (131) reported that rats readily consumed BD when it was fed at 20% of the diet. Hess and Young (57) and Bonner et al. (29) reported that cattle consumed BD without toxic effects when it was fed at 4% of the diet,
however, when cattle were fed BD at 6% of the diet, hyperactivity and reduced feed efficiency were observed.

Bonner et al. (29) and Hess and Young (57) reported that dietary BD did not affect pH or volatile fatty acid concentrations and ratios of rumen contents in cattle, suggesting that BD did not affect microbial activity in the rumen. Hess and Young (57) reported that BD, when mixed with high-concentrate diets, alleviated milk fat depression in dairy cows.

In rats (54, 95, 97, 133), chicks (64), and dogs (90), BD is oxidized by the liver to β-hydroxybutyric acid, which subsequently is oxidized further in peripheral tissues. Mills (101) reported that the liver is at least partly responsible for oxidation of BD to β-hydroxybutyrate in steers. In addition, studies by Hess and Young (57) and Bonner et al. (29) indicated that concentrations of acetoacetate and β-hydroxybutyrate in blood were greater than control values when BD was fed to cattle, suggesting that BD is oxidized to β-hydroxybutyrate in ruminants. Therefore, BD seems to be an effective ketogenic agent when fed to either ruminants or non-ruminants.

It is not conclusively known whether dietary BD affects concentrations of glucose in blood. Bonner et al.
(29) and Hess and Young (57) reported that BD did not affect blood glucose concentrations in cattle and Rosebrough et al. (117) reported similar findings from sows. When BD was fed to rats (96, 97) and dogs (114), however, blood glucose concentrations were lower than control values. Mehlman et al. (96) attributed low blood glucose concentrations of BD-fed rats to a blocking of gluconeogenesis in kidney at the conversion of 3-phosphoglycerate to glyceraldehyde-3-phosphate. No other reports have indicated that BD affects gluconeogenesis. In the reports of Mehlman et al. (96, 97), BD replaced a portion of the carbohydrate in diets fed to rats, therefore, it is not possible to attribute lower blood glucose concentrations in these animals directly to BD.

Dietary BD possibly could cause a decrease in blood glucose concentrations, because BD is a ketogenic compound and ketone bodies stimulate release of insulin in sheep (32). It seems, however, that the glucoregulatory mechanisms of the body would prevent any long-term deviations from physiological concentrations of blood glucose when BD is fed.

Any effects of dietary BD on kinetics of glucose metabolism in ruminants are not known. In work with
non-ruminants, Romsos and Leveille (114) reported that BD did not affect glucose irreversible loss or glucose pool size.

Effects of dietary BD on intermediary metabolism in ruminants have not been determined. Romsos et al. (116) and Mehlman et al. (96, 97, 98) studied effects of dietary BD on enzyme activities in rat liver and adipose tissue. Rates of in vitro fatty acid synthesis of liver were markedly decreased, however, rates of fatty acid synthesis of adipose tissue were not affected when BD was fed to rats (116). The marked decrease in hepatic fatty acid synthesis in rats fed BD may have been caused by an increase in the cytoplasmic NADH/NAD$^+$ ratio after BD oxidation. An increase in the NADH/NAD$^+$ ratio would diminish conversion of glucose to fatty acids, possibly at the level of glyceraldehyde-3-phosphate dehydrogenase and(or) lactate dehydrogenase (116).

The capacity of the tissues to synthesize fatty acids is usually reflected by activities of hepatic and adipose tissue malic enzyme. Romsos et al. (116) reported that activity of malic enzyme was decreased in both liver and adipose tissue only when high levels (20% of the diet) of BD were fed to rats. Mehlman et al.
(96), however, reported no effects on malic enzyme activities in liver and adipose tissue when rats were fed diets containing 18% BD. Activities of PC and PEPCK in liver were slightly greater in rats fed BD than in rats not fed BD (96, 97, 98). Therefore, it seems that in addition to causing ketonemia-ketonuria, BD may, in some cases, cause other physiological abnormalities when fed to animals at moderate to high levels, however, it is apparent that BD, in most cases, is readily oxidized by body tissues and is not toxic.

**Summary of literature**

Ruminants are dependent on the process of gluconeogenesis to maintain a supply of glucose for various metabolic needs. During periods of high production, such as lactation, the requirement for glucose is great and often the supply of glucose does not meet the demand. For this reason, high-producing dairy cows, during early lactation, experience hypoglycemia which, along with hormonal signals, causes the mobilization of fatty acids from adipose tissue to meet the energy deficit imposed by lactation. The capacity of the liver to completely oxidize FFA is sometimes exceeded during early lactation, and hepatic ketogenesis increases
greatly, thereby causing ketonemia. Bovine lactation ketosis, therefore, is characterized primarily by hypoglycemia and ketonemia. Advances in understanding ketosis have been hindered because of the lack of a reliable source of ketotic cows. Development of a valid ketosis model would allow researchers to evaluate effects of ketonemia on carbohydrate, lipid, and hormonal interactions in the bovine.

Explanation of dissertation format

This dissertation is presented in the alternate format, as outlined in the Iowa State University Graduate College Thesis Manual. By using the alternate format, the dissertation can be written in sections, each independent, and in a form suitable for publication in a professional journal, thereby easing the arduous task of manuscript preparation, which most graduates encounter after writing a dissertation.

The results of the research that I performed to partly fulfill requirements for the Ph.D. degree are presented in three separate papers, each with its own abstract, introduction, conclusion, and bibliography. Although these papers are presented separately, the subject matter in them is closely related, allowing for a general, integrated discussion at the conclusion.
REFERENCES CITED


SECTION I. GLUCOSE KINETICS AND PLASMA METABOLITES DURING AN EXPERIMENTAL KETOSIS PRODUCED IN STEERS BY 1,3-BUTANEDIOL AND PHLORIZIN
ABSTRACT

Both 1,3-butanediol, which causes ketonemia, and phlorizin, which induces glucosuria, were given to steers in an attempt to simulate lactation ketosis and to determine effects of ketonemia and glucosuria on plasma metabolite concentrations and on kinetics of glucose metabolism. Four steers received four treatments (control; 1,3-butanediol mixed with the control ration; control plus phlorizin given by subcutaneous injection; and control plus 1,3-butanediol and phlorizin) in a Latin square design. Each treatment lasted 14 days. All steers received a 30% grain, 70% forage ration in equal portions at 2-h intervals. Plasma and urine metabolite concentrations and kinetics of glucose metabolism were measured on each of the last 3 days of each treatment period. Carbon-14 glucose, given by single injection, was used to measure glucose kinetics. Compared with controls, 1,3-butanediol increased glucose pool size. Phlorizin caused glucosuria, decreased plasma glucose, increased plasma free fatty acids, increased glucose irreversible loss, decreased glucose total entry rate, and decreased glucose recycling. Treatment with 1,3-butanediol plus phlorizin caused glucosuria and ketonuria, decreased plasma glucose, increased blood ketone
bodies, increased plasma free fatty acids, increased glucose irreversible loss, and increased glucose pool size.
INTRODUCTION

Lactation ketosis is characterized by increases in blood, urine, and milk ketone body concentrations, increases in plasma free fatty acid (FFA) concentrations, and decreases in blood glucose concentrations. It generally is agreed that a ketotic state is attained when total blood ketones (acetoacetate and $\beta$-hydroxybutyrate) exceed 10 mg/dl and blood glucose decreases to 40 mg/dl or less.

A major problem in studying bovine lactation ketosis is lack of a consistent and predictable supply of clinically ketotic cows. This problem is compounded by high costs of conducting metabolic experiments with lactating cows. Our primary objective was to develop a model of lactation ketosis that would be less expensive than high-producing cows for studying ketosis. For a model to be valid, the induction protocol would have to: (a) cause a glucose excretion that would simulate the lactose removal associated with lactation, (b) reduce plasma glucose concentration, (c) produce ketonemia and ketonuria, (d) increase plasma FFA concentrations, and (e) maintain feed intake until a spontaneous decrease occurs. In an attempt to satisfy these criteria, 1,3-butanediol (BD) was fed to steers to cause ketonemia and ketonuria, and phlorizin
was injected to cause glucosuria and, hopefully, a subsequent reduction in plasma glucose concentration. Our second objective was to observe effects of glucosuria and ketonemia on the kinetics of glucose metabolism in steers.

Butanediol is an odorless glycol that has been used as an energy source in diets of rats (17), pigs (22), humans (12), and cattle (9, 31). Rats oxidize BD to β-hydroxybutyric acid (BHBA) in the liver (27). BD is an effective ketogenic agent when fed to ruminants (2), and in vitro studies (19) indicate that the liver may be partly responsible for oxidation of BD to BHBA in steers. Bonner et al. (2) reported that rumen pH, volatile fatty acid ratios, and blood glucose concentrations were not affected in steers fed BD at amounts up to 8% (839 g) of the daily ration, but blood ketone bodies were increased, suggesting that BD is converted to BHBA in ruminants. The absence of an effect of BD on volatile fatty acid ratios and rumen pH in cattle suggests that BD is not metabolized by rumen microorganisms.

Von Mering (28) reported that phlorizin (P) caused excretion of glucose in urine. The copious glucosuria results from both competitive and noncompetitive inhibition of renal tubular reabsorption of glucose (3, 11).

Glucose is essential in ruminants because it is
needed by neural tissue, mammary gland, muscle, and the fetus and for synthesis of triglycerides. In ruminants, gluconeogenesis must provide at least 90% of the necessary glucose (32). In lactating cows, glucose required by the mammary gland for lactation, coupled with glucose required by other body tissues, places a tremendous challenge upon the capacity of the gluconeogenic pathway to provide sufficient quantities of glucose to maintain glucose homeostasis.
EXPERIMENTAL PROCEDURES

One milking Shorthorn and three Holstein steers, with initial weights averaging 190 kg, were housed in individual pens and received four treatments in a 4 x 4 Latin square design. Each treatment period lasted 14 days. Because only two automatic feeders were available, two steers completed the trial before the remaining two began. The control ration was 30% concentrate and 70% forage, consisting of 1470 g ground corn plus minerals (5% monosodium phosphate and 3% trace-mineral salt) and 3140 g chopped alfalfa hay daily. The ration was divided into 12 equal portions and fed at 2-h intervals. Water was available at all times. Steers gained 300 to 500 g/day. Previous work indicated that steers fed according to this regime are in metabolic steady state because no diurnal changes in kinetics of glucose metabolism occurred (1).

The control ration (C) was given alone, with BD\textsuperscript{1}, with P\textsuperscript{2}, and with BD plus P (Table 1). BD was mixed with the grain portion of the ration. BD dosage was determined during the first 7 days of the 14-day treatment periods.

\textsuperscript{1}Butanediol was a gift from the Celanese Chemical Co., New York, NY.

\textsuperscript{2}Phlorizin was purchased from Sigma Chemical Co., St. Louis, MO.
Table 1. Treatments used in the 4 x 4 Latin square

<table>
<thead>
<tr>
<th>Treatment abbreviation</th>
<th>Treatment composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>Control ration</td>
</tr>
<tr>
<td>BD</td>
<td>Control ration + 1,3-butanediol</td>
</tr>
<tr>
<td>P</td>
<td>Control ration + phlorizin</td>
</tr>
<tr>
<td>BDP</td>
<td>Control ration + 1,3-butanediol + phlorizin</td>
</tr>
</tbody>
</table>

At low BD intake (300 g/day), steers readily consumed their rations. BD feeding was increased daily until steers began refusing small amounts of feed. The amount of BD required to induce this mild hypophagia was noted, and amounts subsequently fed were reduced by 10% to insure that steers consumed all of their ration. Some steers consumed more BD than others without becoming hypophagic. Daily BD intake ranged from 450 to 850 g/day per steer. P was dissolved in propylene glycol (.25 g/ml), and 1 g of P was given subcutaneously twice daily for the last 7 days of the treatment period. The P dosage was based upon earlier results (33).

Concentrations of plasma glucose\(^3\), blood acetoacetate (ACAC) (18), and BHBA (30) were determined for samples

\(^3\)Glucostat, Worthington Biochemicals, Freehold, NJ.
obtained on the last 3 days of each treatment period. Plasma FFA concentrations were determined by a modification (unpublished) of the procedure of Ho (10). Urine glucose and ketone body excretion rates were determined by collecting urine from steers in metabolism crates, using NaF as the urine preservative, over a 12- to 18-h period. A representative sample was assayed for glucose and ketone body concentrations (18, 30), and hourly excretion rates were calculated. Differences between treatment means were tested by using orthogonal comparisons (8, p. 237).

Kinetics of glucose metabolism were measured on the last 3 days of each treatment period by giving each steer a single injection of [U-\(^{14}\text{C}\)]glucose each day and observing the decline in plasma glucose specific radioactivity (SRA) for the next 8 h. Steers were accustomed to experimental conditions. Catheters were placed in both jugular veins the day before \(^{14}\text{C}\)]glucose injections began; one catheter was used for withdrawals and the other for injections. A 300-\(\mu\text{Ci}\) dose of [U-\(^{14}\text{C}\)]glucose, checked for radiochemical purity (20), in 18 ml of sterile .15 M NaCl was injected through a jugular catheter over a 20-sec interval, after which, the catheter was flushed with 50 ml of sterile .15 M NaCl. Blood samples were withdrawn at 2,

Plasma glucose SRA was determined (20). The line of best fit for the decline of plasma glucose SRA, as a fraction of dose, after injection of tracer was calculated by using the nonlinear regression procedure (NLIN) developed by Goodnight (8, p. 317). An exponential equation with three terms was necessary to fit the data. Subsequently, glucose irreversible loss, pool size, total entry rate, recycling, and glucose space, as defined by White et al. (29), were calculated by using coefficients and exponents generated by the NLIN procedure.

For data presented in (21), liver samples were taken by puncture biopsy from each steer on the day following glucose kinetics experiments. One steer died as a result of its third biopsy; therefore, only three steers received treatment BD.
RESULTS AND DISCUSSION

**Metabolite concentrations in plasma**

Treatment means for urinary glucose excretion rates and for plasma glucose and FFA concentrations are in Table 2. When steers received C or BD, they did not excrete measurable glucose in urine, but when P or BDP was given, much glucose was excreted. Young et al. (33) reported similar results. Glucosuria has been reported also in steers (25) and goats (23, 24) treated with P.

Plasma glucose concentrations were greater during treatments C and BD than during treatments P and BDP, indicating that subcutaneous injections of P effectively caused glucosuria and a decrease in plasma glucose concentrations. Others have indicated that P caused hypoglycemia when given to fasted sheep (4, 5) and goats (7). Schultz et al. (23, 24), however, observed no changes in blood glucose concentrations when fed goats received P. Our lack of an effect of dietary BD on plasma glucose concentrations in steers is in agreement with Bonner et al. (2) and Hess and Young (9).

Plasma FFA concentrations seemed to increase over controls when steers received BD, but the 2.6-fold increase was not statistically significant (Table 2). When steers
Table 2. Effects of feeding 1,3-butanediol, injecting phlorizin, and a combination thereof on urinary glucose excretion and plasma glucose and plasma free fatty acid concentrations in steers

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment</th>
<th>1,3-Butanediol</th>
<th>Phlorizin + Phlorizin</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary glucose excretion (g/day)</td>
<td>Control</td>
<td>0</td>
<td>200</td>
<td>248</td>
</tr>
<tr>
<td></td>
<td>1,3-Butanediol</td>
<td>0</td>
<td>200</td>
<td>248</td>
</tr>
<tr>
<td></td>
<td>Phlorizin + Phlorizin</td>
<td>248</td>
<td>248</td>
<td>248</td>
</tr>
<tr>
<td></td>
<td>Standard error</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma glucose (mg/dl)</td>
<td>93.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>94.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>73.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>69.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma free fatty acids (μM)</td>
<td>30&lt;sup&gt;c&lt;/sup&gt;</td>
<td>79&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>103&lt;sup&gt;d&lt;/sup&gt;</td>
<td>121&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> Means in rows with different superscript letters differ (P<.01).
<sup>c,d</sup> Means in rows with different superscript letters differ (P<.05).
were given P or BDP, plasma FFA concentrations increased significantly compared with C. Increased plasma FFA concentrations in ruminants receiving P have been reported (4, 5). A possible explanation for increases in plasma FFA induced by P is that urinary glucose excretion causes carbohydrate insufficiency, resulting in increased FFA mobilization from adipose tissue, thereby providing an alternative substrate for oxidation. This mechanism for FFA mobilization from adipose tissue probably would be mediated by a decrease in plasma insulin or an increase in glucagon or growth hormone concentrations, but as reported in an accompanying paper (6), concentrations of these hormones in plasma were not affected significantly by BD, P, or BDP treatments.

Treatment means for blood BHBA and ACAC concentrations, BHBA to ACAC ratios, and urinary ketone body excretion rates are in Table 3. Blood BHBA and ACAC concentrations for treatments BD and P were not increased significantly from values for C. When steers received BDP, however, BHBA and ACAC concentrations increased significantly when compared with other treatments. Earlier reports indicated that P caused hyperketonemia when given to fasted sheep (4, 5, 7). However, Schultz
Table 3. Effects of feeding 1,3-butanediol, injecting phlorizin, and a combination thereof on blood ketone body concentrations, blood ketone body ratios, and urinary ketone body excretion in steers

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment</th>
<th>Control</th>
<th>1,3-Butanediol</th>
<th>Phlorizin</th>
<th>1,3-Butanediol + Phlorizin</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood β-hydroxybutyrate</td>
<td></td>
<td>2.5^a</td>
<td>5.8^a</td>
<td>4.1^a</td>
<td>15.6^b</td>
<td>.9</td>
</tr>
<tr>
<td>(mg/dl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood acetoacetate</td>
<td></td>
<td>.1^a</td>
<td>.6^a</td>
<td>.2^a</td>
<td>1.5^b</td>
<td>.1</td>
</tr>
<tr>
<td>(mg/dl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood β-hydroxybutyrate:</td>
<td></td>
<td>31^a</td>
<td>11^b</td>
<td>21^c</td>
<td>13^b</td>
<td>1.3</td>
</tr>
<tr>
<td>acetoacetate ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary ketone body</td>
<td></td>
<td>0^a</td>
<td>1.3^a</td>
<td>.1^a</td>
<td>9.5^b</td>
<td>1.4</td>
</tr>
<tr>
<td>excretion (g/day)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a,b,c</td>
<td>Means in rows with different superscript letters differ (P&lt;.05).</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d</td>
<td>Sum of ACAC plus BHBA.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Sum of ACAC plus BHBA.
et al. (23, 24) observed no changes in blood ketone body concentrations when P was given to fed goats. Bonner et al. (2) and Hess and Young (9) reported that feeding BD to cows and steers significantly increased total blood ketone body concentrations 3- to 10-fold.

The ratio of BHBA to ACAC in blood was significantly less when steers received treatment P than when steers received C (Table 3). Further reduction in the ratio was noted when treatments BD and BDP were given. The effect of P on blood ketone body ratios has not been reported previously. The reduction in BHBA to ACAC ratios may signify a change in the oxidation:reduction potential in tissues that metabolize BD. Tate et al. (27) reported that rats converted BD to BHBA by alcohol dehydrogenase in liver cytosol.

When steers received treatment C, ketone bodies were not detectable in urine (Table 3). When treatments BD and P were given, values increased to detectable amounts, but the increases were not significant. When steers received BDP, ketone body excretion increased significantly over other treatments. Changes in urinary ketone body excretion paralleled changes in ketone body concentrations in blood, and significant increases occurred only when BDP was given. In contrast to data presented from our study,
Bonner et al. (2) and Hess and Young (9) reported that feeding BD caused marked ketonuria in steers, but they also reported greater concentrations of blood ketone bodies than reported here, which may explain the discrepancy.

**Kinetics of glucose metabolism**

A typical curve for the decline in plasma glucose SRA, as a fraction of dose, versus time after injection of [U-\(^{14}\)C]glucose is in Figure 1. Both residual sums of squares generated by the NLIN iterative procedures and systematic deviations between observed and predicted points were minimized by using an exponential equation containing three terms. Reports indicate that curves generated by the decline in plasma glucose SRA as a function of time are best described by equations having three exponential components (15, 26).

To accurately determine the area subtended by SRA decay curves, Kronfeld and Ramberg (15) state that plasma glucose SRA should be measured from as soon as possible after administration of tracer until the final slope of the SRA decay curve is observed. To verify that a 480-min observation period after [U-\(^{14}\)C]glucose injection was sufficient, slopes of SRA decay curve segments between 240 and 360 min and between 360 and 480 min (see Figure 1) were
Figure 1. Plasma glucose specific radioactivity decay curve after intravenous injection of [U-$^{14}$C] glucose in a steer. Data points represent observed values. The equation for the line of best fit to the data is $SRA_{t} = .00413e^{-.41195t} + .00234e^{-.03361t} + .0094e^{.00690t}$. 
compared by using a paired comparison t-test (8, p. 425). Results are in Table 4. Comparisons between slopes of these curve segments also were made on SRA decay curves from each animal within treatment. In no instance were slopes of the two SRA decay curve segments significantly different. Because the slope of these SRA decay curves did not change after 240 min, an observation period of 480 min is considered sufficient.

Treatment means of variables dealing with kinetics of glucose metabolism are in Table 5. Glucose irreversible loss (IL) is the rate at which glucose carbon leaves the sampled compartment (i.e., plasma) never to return. Because steers were fed 12 times daily, the supply of digestible and fermentable substrate was continuous, and steers were in steady state in regard to glucose metabolism. Therefore, glucose IL is an approximate measure of the net amount of glucogenic substrate utilized. Glucose IL was similar for C and BD treatments. Values increased significantly when P and BDP were given. No previous data are available regarding the effect of BD on glucose IL in ruminants. However, Young et al. (33) reported that P caused an increase in glucose IL in steers. In our study, the average P-induced increase in glucose IL of 215 g/day parallels the average P-induced increase in urinary glucose
Table 4. Slopes of plasma glucose specific radioactivity decay curve segments between 240 and 360 min and 360 and 480 min after \([U-^{13}C]\)glucose injection

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Slope A (240-360 min)</th>
<th>Slope B (360-480 min)</th>
<th>Difference (Slope A - Slope B)</th>
<th>T Value</th>
<th>PR&gt;T°b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-.0062</td>
<td>-.0065</td>
<td>.0003</td>
<td>.26</td>
<td>.80</td>
</tr>
<tr>
<td>1,3-Butanediol</td>
<td>-.0058</td>
<td>-.0059</td>
<td>.0001</td>
<td>.03</td>
<td>.98</td>
</tr>
<tr>
<td>Phlorizin</td>
<td>-.0072</td>
<td>-.0069</td>
<td>-.0003</td>
<td>-.37</td>
<td>.72</td>
</tr>
<tr>
<td>1,3-Butanediol + Phlorizin</td>
<td>-.0078</td>
<td>-.0072</td>
<td>-.0006</td>
<td>-1.14</td>
<td>.28</td>
</tr>
</tbody>
</table>

*aEach mean is the average of 9 to 12 values.

°bProbability that mean difference is significantly different from zero.
Table 5. Effects of feeding 1,3-butanediol, injecting phlorizin, and a combination thereof on kinetics of glucose metabolism in steers

<table>
<thead>
<tr>
<th>Glucose kinetic variable</th>
<th>Treatment</th>
<th>Control</th>
<th>1,3-Butanediol</th>
<th>Phlorizin</th>
<th>1,3-Butanediol + Phlorizin</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irreversible loss (g/day)</td>
<td></td>
<td>410^a</td>
<td>434^a</td>
<td>575^b</td>
<td>671^b</td>
<td>22</td>
</tr>
<tr>
<td>Total entry rate (g/day)</td>
<td></td>
<td>4900^c</td>
<td>5448^c</td>
<td>2859^d</td>
<td>3633^c,d</td>
<td>263</td>
</tr>
<tr>
<td>Recycling (g/day)</td>
<td></td>
<td>4489^c</td>
<td>5014^c</td>
<td>2284^d</td>
<td>2962^c,d</td>
<td>256</td>
</tr>
<tr>
<td>Pool size (g)</td>
<td></td>
<td>12.1^c</td>
<td>15.4^d</td>
<td>14.2^c,d</td>
<td>14.9^d</td>
<td>.4</td>
</tr>
<tr>
<td>Glucose space (% body wt)</td>
<td></td>
<td>6.4^c</td>
<td>7.8^c</td>
<td>9.6^d</td>
<td>10.7^d</td>
<td>.4</td>
</tr>
</tbody>
</table>

^a,b^ Means in rows with different superscript letters differ (P<.01).

^c,d^ Means in rows with different superscript letters differ (P<.05).
excretion of 225 g/day (Table 2). Because feed intake was constant across all treatments, the mechanism by which glucose IL increased in response to P may involve an increase in utilization of endogenous glucogenic substrates or possibly an increase in the efficiency of utilization of glucogenic substrates of dietary origin, or a combination of the two. In vitro gluconeogenic capacity of liver slices prepared from these steers was greater when P and BDP treatments were given (21), which may explain the P-induced increase in glucose IL.

Glucose total entry rate (TER) is the rate at which glucose carbon enters the sampled compartment. Glucose TER for C and BD treatments was not significantly different (Table 5). Values decreased during P and BDP treatments, but the decrease was only significant for P. No previously reported data are available regarding the effect of BD on glucose TER.

Glucose recycling, occurring by both physical and chemical means, is the rate of return of glucose carbon that temporarily leaves the sampled compartment and is the difference between TER and IL. Glucose recycling rates were similar for the C and BD treatments (Table 5). Values decreased for P and BDP treatments, but the decrease was significant only for P.
Young et al. (34) reported much smaller values for glucose TER and recycling than those reported here. Glucose TER and recycling values are determined largely by the first derivative of the SRA decay curve at the time of injection. As the value of the first derivative increases, values for TER and recycling increase. Our SRA decay curves had greater slopes and Y intercepts (i.e., greater first derivatives) than those of Young et al., because our first blood samples were drawn at 2 min after tracer injection and the first samples of Young et al. (34) were taken 5 min after tracer injection. Therefore, the magnitude of glucose TER and recycling is largely influenced by the sampling interval immediately after tracer injection.

Glucose pool size is the amount of glucose in the sampled compartment. Glucose pool sizes for treatments C and P were similar, however, when steers received BD or BDP, glucose pool sizes were larger than those of controls (Table 5). Young et al. (33) reported that glucose pool size was not affected by P in steers. No previous data are available regarding BD effects on glucose pool size in ruminants, but data presented herein indicate a modest, yet statistically significant, increase in glucose pool size when steers were fed BD. It is possible that ketone bodies
derived from BD metabolism may have served as oxidative fuels, thereby sparing glucose from oxidation.

Glucose space, or volume of distribution, expressed as a percentage of body weight, was less for treatment C and BD than for treatments P or BDP (Table 5). Kronfeld et al. (16) and Kronfeld (13) reported an increase in glucose space when cows became ketotic. In our study, the P-induced decrease in plasma glucose concentrations may be a result of the increase in glucose space, to the increase in urinary excretion of glucose, or to a combination of the two. Similar mechanisms regarding reductions in plasma glucose concentrations in ketotic cows have been proposed by Kronfeld and Raggi (14), who, after experiments with fasted and insulin-treated cows, suggested two types of hypoglycemia. The first type, fasting hypoglycemia, develops when glucose utilization or outflow exceeds glucose formation or inflow; the second type, insulin-induced hypoglycemia, develops when glucose space increases as a result of increased cell membrane permeability to glucose. The concomitant increase in glucose pool size (Table 5) and decrease in plasma glucose concentration (Table 2) when BDP was given may be a result of the P-induced increase in glucose space.
GENERAL DISCUSSION

When steers received both 1,3-butanediol and phlorizin, they exhibited changes in blood glucose, ketone body, and FFA concentrations similar to changes observed when cows are in early stages of ketosis. Phlorizin-induced glucosuria seemed to increase the rate of incorporation of glucogenic substrates into glucose, which simulates the increased glucose requirement imposed by lactation. Effects of 1,3-butanediol and phlorizin on plasma metabolites and kinetics of glucose metabolism might have been more marked if treatment periods had been longer.

This model shows potential to provide a consistent and predictable supply of relatively inexpensive ketotic cattle, allowing the study of carbohydrate, lipid, and hormone interactions in ketotic states. It should be especially valuable for studying interactions of varying glucose excretion rates and varying inputs of ketone bodies in cattle. One disadvantage, however, is the high cost of phlorizin for inducing glucosuria.
ACKNOWLEDGEMENTS

Corey Michelle, Rick Deerfield, Craig Umstead, and Leslie Kelch are acknowledged gratefully for their competent laboratory assistance. Thanks are expressed to Linda Erickson for competent secretarial services.
REFERENCES CITED


SECTION II.  PLASMA AND LIVER METABOLITES AND GLUCOSE KINETICS AS AFFECTED BY PROLONGED KETONEMIA-GLUCOSURIA AND FASTING IN STEERS
ABSTRACT

Both 1,3-butanediol, which causes ketonemia, and phlorizin, which causes glucosuria, were given to four steers for 28 days to determine effects of prolonged ketonemia and glucosuria on metabolites in blood and liver and on kinetics of glucose metabolism. Ketonemia and glucosuria in steers simulates the physiological situation in ketotic, lactating cows. In addition, effects of fasting on blood and liver metabolites and on glucose kinetics were determined. Treatments were: control (1476 g corn, 3144 g alfalfa hay daily); control with butanediol plus phlorizin; and fasting for 9 days. Butanediol was mixed with the corn and phlorizin was injected twice daily. Glucose kinetics were measured by primed-continuous infusions of [U-\(^{13}\)C]glucose. Butanediol plus phlorizin caused glucosuria, ketonuria, and ketonemia, but did not affect plasma insulin, glucagon or growth hormone. Fasting caused hypoinsulinemia but did not affect glucagon or growth hormone. Butanediol plus phlorizin and fasting caused 18% and 19% reductions in plasma glucose and a 2.5- and 6-fold increase in plasma FFA. Butanediol plus phlorizin did not affect liver triglyceride or glycogen, but did increase liver ketone bodies. Fasting did not affect liver triglyceride
or ketone bodies, but did decrease liver glycogen by 60%. During control, butanediol plus phlorizin treatment, and fasting, glucose irreversible loss averaged 371, 541, and 182 g/day. Effects of ketonemia-glucosuria on blood and liver metabolite concentrations were no greater after 28 days than after 14 days. Steers given butanediol plus phlorizin did not show all the signs of lactation ketosis, but the treatment offers promise for studying causes and interactions of ketosis.
INTRODUCTION

Bovine lactation ketosis is characterized by hypoglycemia (23), hyperketonemia (4), and hypoinsulinemia (17). There are also changes in liver metabolite concentrations, such as decreases in glycogen (4) and increases in triglyceride (6) and ketone bodies (4). The difficulties in conducting research with spontaneously ketotic cows have hindered advances in understanding ketosis. To partly alleviate the problems, workers have attempted to induce ketosis in ruminants by starvation (3, 9, 14), starvation plus thyroxine treatment (19, 20), and a combination of starvation, hormone, and phlorizin treatments (10). Ketosis induced by starvation, however, is not typical of spontaneous ketosis (23).

We reported (11, 28, 32) an attempt to create a ketosis model by using maintenance-fed steers receiving 1,3-butanediol (BD), a ketogenic substrate, and phlorizin (P), a compound which causes glucosuria. When BD was fed for 14 days and P was injected on the last 7 days of BD treatment, blood glucose decreased and blood ketone bodies and free fatty acids (FFA) increased (28). However, liver glycogen decreased and liver triglyceride (TG) increased only slightly (32) and plasma insulin, glucagon, and growth
hormone changed only slightly (11). These changes in blood and liver metabolites in the steers were in the same directions as those in cows during the onset of lactation ketosis, but were not as marked.

For the BDP model of ketosis to be valid, we believe that greater decreases in liver glycogen and plasma glucose and insulin, along with greater increases in plasma FFA and liver TG concentrations are needed. Therefore, we attempted to cause greater changes in these metabolites by increasing the length of BDP treatment to 28 days.

In addition to extending BDP treatment, steers were fasted for 9 days to determine if the nutritional stress imposed by BDP treatment was as great as that imposed by food deprivation. We also wanted to determine if steers could maintain glucose homeostasis during a prolonged fast. The trial reported herein, therefore, allowed observations during three physiological states: normal, ketonemia-glucosuria (experimental ketosis), and starvation.
EXPERIMENTAL PROCEDURES

Two Brown Swiss and two Holstein steers, with initial weights averaging 226 kg, were housed in individual pens. The trial consisted of four periods and during each period all steers were receiving the same treatment. The ration was 30% concentrate and 70% forage, consisting of 1470 g ground corn plus minerals (5% monosodium phosphate, 3% trace-mineral salt) and 3140 g chopped alfalfa hay each day. The ration was divided into 12 equal portions and fed at 2-h intervals to maintain a steady metabolic state. Water was always available.

Periods 1 and 3 were control periods; period 2 was control plus BD\(^1\) and P\(^2\) (BDP); and period 4 was fasting for 9 days (Table 1). In period 2, BD was mixed with the grain portion of the ration. During the first three days of BD feeding, BD intake was increased from 300 g/day to 500 g/day and this amount was fed thereafter. Phlorizin was dissolved in propylene glycol (.25 g/ml) and 1 g of P was injected subcutaneously twice daily during period 2. Period 3 was to prevent possible carryover effects of BDP into the fasting period.

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\(^1\)Butanediol was a gift from the Celanese Chemical Co., New York, NY.

\(^2\)Phlorizin was purchased from Fluka Chemical Corp., Hauppauge, NY.
Table 1. Experimental design and treatments

<table>
<thead>
<tr>
<th>Period of trial&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Length of period (days)</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>control ration (control I)</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td>control ration + BD + P (BDP)</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>control ration (control II)</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>fasted</td>
</tr>
</tbody>
</table>

<sup>a</sup>During a given period, all steers received the same treatment. Periods were in chronological order.

Concentrations of glucose<sup>3</sup>, FFA (37), insulin (41), glucagon (16), and growth hormone (42) in plasma and β-hydroxybutyrate (46) and acetoacetate (30) in blood were determined at the end of the first control period, at the middle (day 14) and end (day 28) of the BDP period, at the end of the second control period, and at the end of the fasting period (Table 1). On these same days, excretion rates of glucose<sup>3</sup> and ketone bodies (30, 46) in urine also were determined. Plasma glucose, FFA, and hormones for each steer were determined in nine samples taken during an 8-h period (6 a.m. to 2 p.m.). Fluctuations in blood metabolite concentrations induced by feeding should have been minimized by the 2-h feeding intervals. Liver tissue

<sup>3</sup>Glucostat, Worthington Biochemicals, Freehold, NJ.
was obtained by puncture biopsy (18) in each period of the trial on the days following blood and urine sampling. Concentrations of glycogen (21), triglyceride (13), acetoacetate (30), and β-hydroxybutyrate (46) in liver were determined.

Glucose irreversible loss (44) and transfer quotients for blood glucose to blood CO₂ were determined at the end of the first control period, at the middle (day 14) and end (day 28) of the BDP period, and at the end of the fasting period (Table 1) by using [U-¹⁴C]glucose in the primed-continuous infusion technique (39). Kinetics of glucose metabolism and concentrations of metabolites in blood and urine were determined concurrently on the same days. Steers were accustomed to experimental conditions. Catheters were placed in both jugular veins, one catheter for withdrawals and the other for infusions. A primer dose of 30 μCi [U-¹⁴C]glucose in 20 ml of sterile .15 M NaCl was injected, followed by infusion of .5 μCi of [U-¹⁴C]-glucose per ml of .15 M NaCl per min for 8 h. Blood samples were withdrawn immediately before administration of tracer and every 30 min during the last 6 h of the infusion. Specific radioactivity (SRA) of plasma glucose (31) and blood CO₂ (35) were determined.
Differences between treatment means for all variables were tested by using orthogonal comparisons (15, p. 237).
RESULTS AND DISCUSSION

**Plasma metabolite concentrations**

Glucose excretion in urine and concentrations of glucose, FFA, insulin, glucagon, and growth hormone in plasma are in Table 2. In the control and fasting periods, steers did not excrete glucose in urine, however, when BDP was the treatment, glucosuria was evident. Excretion of glucose is not caused by BD (28), therefore, glucosuria during BDP treatment can be attributed solely to P. In previous reports (28, 47), glucosuria induced in steers by P was similar to that reported here.

Concentrations of glucose in plasma decreased from controls by 18% when steers received BDP, however, the steers probably were not as hypoglycemic as clinically ketotic cows. Bergman (6) reported that plasma glucose concentrations were less than 55 mg/dl during ketosis and Kronfeld (23) found plasma glucose concentrations as low as 27 mg/dl in spontaneously ketotic cows. Data presented in (28) indicated that BD had no effect on plasma glucose concentrations, and that P, when given alone or in combination with BD, caused plasma glucose concentrations to decrease. Data in Table 2 indicate that maximal decreases and increases in plasma glucose concentrations and urinary
Table 2. Effects of 1,3-butanediol and phlorizin treatment and fasting on urinary glucose excretion and on plasma glucose, free fatty acid, insulin, glucagon, and growth hormone concentrations in steers

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control I</th>
<th>BDP (day 14)</th>
<th>BDP (day 28)</th>
<th>Control II</th>
<th>Fasted</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary glucose excretion (g/day)</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>269&lt;sup&gt;b&lt;/sup&gt;</td>
<td>255&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35</td>
</tr>
<tr>
<td>Plasma glucose (mg/dl)</td>
<td>77.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>66.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>80.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>65.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>.8</td>
</tr>
<tr>
<td>Plasma FFA (mM)</td>
<td>.20&lt;sup&gt;c&lt;/sup&gt;</td>
<td>.32&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>.42&lt;sup&gt;d&lt;/sup&gt;</td>
<td>.14&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.03&lt;sup&gt;e&lt;/sup&gt;</td>
<td>.03</td>
</tr>
<tr>
<td>Plasma insulin (ng/ml)</td>
<td>.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>.32&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>.02</td>
</tr>
<tr>
<td>Plasma glucagon (pg/ml)</td>
<td>115&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>136&lt;sup&gt;a&lt;/sup&gt;</td>
<td>110&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>101&lt;sup&gt;b&lt;/sup&gt;</td>
<td>98&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.87</td>
</tr>
<tr>
<td>Insulin:glucagon molar ratio</td>
<td>2.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.68&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>.55&lt;sup&gt;b&lt;/sup&gt;</td>
<td>.07</td>
</tr>
<tr>
<td>Plasma growth hormone (ng/ml)</td>
<td>10.4&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>8.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.3&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>13.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.8&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>.56</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Means in rows with different superscript letters differ (P<.05).

<sup>c,d,e</sup>Means in rows with different superscript letters differ (P<.07).
glucose excretion occurred by day 14 of BDP treatment and
continued treatment with BDP did not cause further signifi-
cant changes.

During fasting, concentrations of glucose in plasma
decreased by 19%, when compared with controls, and were not
different from those when steers were receiving BDP.
These observations were made on the eighth day of the fast,
therefore, the steers probably had been postabsorptive for
3 to 4 days, suggesting that glucose sparing mechanisms
and utilization of endogenous substrates for gluconeo-
genesis prevented marked hypoglycemia. When nonlactating
cows were fasted for 6 days (3) and when steers were
fasted for 7 days (48), plasma glucose concentrations
decreased by 10 and 24%.

Concentrations of FFA in plasma (Table 2) averaged
0.17 mM during the control periods. On days 14 and 28 of
BDP treatment, plasma FFA concentrations were 1.9- and
2.5-fold greater than the average during the control
periods. In an earlier report (28), short-term treatment
with BDP caused plasma FFA to increase by 4-fold in steers.
When the steers were fasted in this trial, plasma FFA
concentrations were over 5-fold greater than the average
during the control periods. DiMarco et al. (12) fasted
steers for 9 days and reported increases in plasma FFA concentrations similar to those reported here.

Concentrations of insulin in plasma were somewhat less during BDP treatment than during the control periods (Table 2), however, the decreases were not significant. deBoer et al. (11) reported similar nonsignificant decreases when steers received BDP for shorter periods. During BDP treatment, the decrease in plasma glucose may have caused the small decrease in plasma insulin; however, in cows the correlation between plasma glucose and insulin concentrations usually is low (36). Ketone bodies stimulate insulin secretion (45), however, during BDP treatment, when ketonemia occurred (Table 3), plasma insulin concentrations were less than during the control periods. It is possible that ketonemia in the steers was not severe enough to stimulate insulin secretion.

In ruminants, FFA concentrations increase in response to decreased concentrations of insulin or increased concentrations of glucagon and growth hormone in plasma (33). The marginal increases in plasma FFA during BDP treatments (Table 2) may have been mediated by the slight decrease in plasma insulin.

When steers were fasted, plasma insulin concentrations (Table 2) were only 25% of the average during BDP
treatment, yet plasma glucose during fasting was not different from values during BDP treatment, indicating that hypoinsulinemia was not mediated by hypoglycemia. During fasting, ruminal volatile fatty acid production undoubtedly was diminished. It is known that insulin secretion in ruminants is stimulated by propionate, butyrate, and valerate (8), therefore, the fasting-induced hypoinsulinemia may have been caused by low concentrations of volatile fatty acids in blood. Brockman (8) reported that plasma insulin decreased when ruminants were fasted. Data in Table 2 suggest that, during fasting, hypoinsulinemia may have contributed to the marked increases in plasma FFA.

Concentrations of glucagon in plasma were not affected significantly by BDP treatment or fasting (Table 2), however, concentrations on day 14 of BDP were greater than those during periods 3 (control II) and 4 (fasting). Short-term BDP treatment in steers did not affect plasma glucagon concentrations in an earlier report (11). Brockman (8) reported that plasma glucagon concentrations decreased when ruminants were fasted. The marked decrease in the insulin to glucagon ratio during fasting (Table 2) results from a major decrease in insulin, not an increase in glucagon.
Growth hormone concentrations (Table 2) did not seem to be markedly affected by BDP treatment or fasting, however, concentrations on day 14 of BDP treatment were less than concentrations during period 3 (control II). Short-term BDP treatment (11) in our earlier experiment and fasting (43) did not affect concentrations of growth hormone in plasma of steers.

Concentrations and ratios of ketone bodies in blood and urine of steers are in Table 3. Blood β-hydroxybutyrate (BHBA) and acetoacetate (ACAC) concentrations increased 7- and 8-fold when BDP was given. Similar increases in blood ketone bodies in steers given BDP for shorter periods were reported earlier (28). Ketonemia and ketonuria during BDP treatment cannot be attributed solely to either BD or P, because neither of the conditions is effectively produced unless BD and P are given in combination (28). Although the BDP-induced increases in blood ketone bodies were marked in these steers, ketonemia was not as severe as in lactating, ketotic cows, when total blood ketone bodies have been reported to reach 30 mg/dl (6) and 45 mg/dl (4).

During fasting, BHBA and ACAC concentrations were approximately 2.5- and 5-fold greater than controls, but the increases were not significant. Baird et al. (3)
Table 3. Effects of 1,3-butanediol and phlorizin treatment and fasting on blood ketone body concentrations and urinary ketone body excretion in steers

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment</th>
<th>Control I</th>
<th>BDP (day 14)</th>
<th>BDP (day 28)</th>
<th>Control II</th>
<th>Fasted</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood β-hydroxybutyrate (mg/dl)</td>
<td></td>
<td>2.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.4</td>
</tr>
<tr>
<td>Blood acetoacetate (mg/dl)</td>
<td></td>
<td>.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>--</td>
<td>1.0&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>.2</td>
</tr>
<tr>
<td>Blood β-hydroxybutyrate: acetoacetate ratio</td>
<td></td>
<td>13</td>
<td>16</td>
<td>10</td>
<td>--</td>
<td>7</td>
<td>.9</td>
</tr>
<tr>
<td>Urinary β-hydroxybutyrate excretion (g/day)</td>
<td></td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>.5</td>
</tr>
<tr>
<td>Urinary acetoacetate excretion (g/day)</td>
<td></td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>.4</td>
</tr>
<tr>
<td>Urinary β-hydroxybutyrate: acetoacetate ratio</td>
<td></td>
<td>1.6</td>
<td>1.2</td>
<td>--</td>
<td>--</td>
<td></td>
<td>.1</td>
</tr>
</tbody>
</table>

<sup>a, b</sup>Means in rows with different superscript letters differ (P<.01).

<sup>c</sup>Not determined during the second control period.
reported that hepatic ketogenesis doubled when nonlactating cows were fasted for 6 days, causing moderate increases in blood ketone body concentrations. During fasting, concentrations of ketone bodies in blood increase due to increases in hepatic ketogenesis resulting from a greater influx of FFA into the liver (34). Data in Tables 2 and 3 indicate that ketone body concentrations in blood increased only slightly during marked increases in plasma FFA during fasting. Therefore, it seems that the rate of ketogenesis by the liver is not dependent solely on the rate of influx of FFA into the liver, suggesting that hepatic ketogenic regulatory factors exist. Bergman (6) reported that, in sheep, blood ketone bodies increased in direct proportion to increases in plasma FFA only when FFA were less than 1.5 mM. When plasma FFA exceeded 1.5 mM, however, blood ketone bodies increased exponentially. It is possible that the 1 mM FFA (Table 2) in fasted steers was not great enough to provide the stimulus for an exponential-type increase in blood ketone bodies, thereby explaining why fasted steers were not ketonemic.

Ketone body ratios in blood were not affected significantly by BDP or fasting, but a trend toward a decrease in ratios occurred during fasting (Table 3). Ratios of BHBA to ACAC in ketotic cows (1, 6) are much
lower than those reported here. Baird et al. (2) reported that ratios of BHBA to ACAC in blood decreased when lactating cows were fasted.

Steers did not excrete ketone bodies in urine in control and fasting periods, however, when BDP was given, ketonuria was evident (Table 3). The ratios of BHBA to ACAC in urine averaged 1.4 during BDP treatment. Because BHBA to ACAC ratios were much lower in urine than in blood, it seems that either extensive conversion of BHBA to ACAC occurred in the kidneys or that preferential excretion of ACAC over BHBA by the kidneys occurred. Prolonged BDP treatment (i.e., longer than 14 days) did not increase the severity of ketonemia or ketonuria.

In preliminary studies, we found that the severity of ketonemia in steers receiving BDP was related to the amount of BD fed. If more BD could be fed for a prolonged period (e.g., 1000 g/day for 7 days) in combination with P injections, more severe ketonemia could be produced. But caution must be taken when feeding BD, because if too much is fed, steers become anorexic and exhibit signs of extreme hyperactivity and nervousness (7).

**Kinetics of glucose metabolism**

Glucose irreversible loss (IL) and blood CO₂ transfer quotients (TQ) during periods 1, 2, and 4 are in Table 4.
Table 4. Effects of 1,3-butanediol and phlorizin treatment and fasting on kinetics of glucose metabolism in steers

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Variable</th>
<th>Control I (day 14)</th>
<th>BDP (day 28)</th>
<th>Fasted</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose irreversible loss (g/day)</td>
<td>371&lt;sup&gt;a&lt;/sup&gt;</td>
<td>481&lt;sup&gt;b&lt;/sup&gt;</td>
<td>600&lt;sup&gt;c&lt;/sup&gt;</td>
<td>182&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Blood CO₂ transfer quotient (%)</td>
<td>1.5&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.2&lt;sup&gt;f&lt;/sup&gt;</td>
<td>4.4&lt;sup&gt;g&lt;/sup&gt;</td>
<td>4.7&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a, b, c, d</sup> Means in rows with different superscript letters differ (P<.01).
<sup>e, f, g</sup> Means in rows with different superscript letters differ (P<.05).
Glucose kinetics were not determined during period 3 because control values from period 1 already had been determined. Compared with controls, glucose IL was 30% greater on day 14 and 62% greater on day 28 of BDP treatment and these differences are highly significant. Because the steers were fed every 2 h, they were in metabolic steady state, which is essential when conducting primed-continuous infusions to determine the kinetics of glucose metabolism. An increase in glucose irreversible loss in BDP-treated steers strongly suggests that net incorporation of substrates into glucose increased in response to ketonemia-glucosuria. In an earlier report (28), it was shown that glucosuria during BDP treatment was associated with an increase in glucose IL, indicating that a greater utilization of glucose caused net synthesis of glucose to increase. Previously, glucose IL was shown to increase by 40 to 65% in steers receiving short-term BDP treatment (28).

The mechanisms by which BDP mediates increases in glucose IL in steers is not known. It seems, however, that P-induced glucosuria may be a primary stimulus for increased gluconeogenesis, because BD, given alone, does not affect glucose IL (28). Kolodny et al. (22) observed rapid
increases in hepatic glucose output when dogs received P, and the increases could not be attributed to low glucose concentrations in blood perfusing the liver. The authors (22) speculated that the P-induced increases in hepatic glucose output were mediated by neural, neurohumoral, or humoral systems located in extrahepatic tissues.

On day 14 of BDP treatment, average urinary glucose excretion (Table 2) exceeded the average BDP-induced increase in glucose IL by 159 g (Table 4). On day 28 of BDP treatment, however, urinary glucose excretion was only 26 g in excess of the increase in glucose IL, suggesting that an improvement in glucose homeostasis occurred after prolonged BDP treatment.

As reported in the accompanying paper (27), in vitro gluconeogenic capacity of liver slices obtained by biopsy from the steers was greater when BDP was given than when the control treatment was given. Increases in hepatic gluconeogenic capacity may explain how glucose IL increased during BDP treatment. An increase in glucose IL strongly suggests that the net amount of substrates used for glucose synthesis increased. But equal amounts of feed were given in the control and BDP periods and BD is not a glucogenic precursor and has no known effect on rumen fermentation.
Therefore, the amount of dietary glucogenic precursors available for gluconeogenesis should have been identical during both periods. It is possible that utilization of endogenous precursors (i.e., amino acids, glycerol) for gluconeogenesis increased when BDP was given or that exogenous (dietary) and(or) endogenous precursors were selectively used for gluconeogenesis when BDP was given, thereby explaining how IL could increase when the amount of exogenous glucogenic precursors did not increase. Kronfeld et al. (25) reported that glucose IL was similar in ketotic and normal lactating cows, suggesting that ketonemia per se does not affect glucose IL.

Glucagon promotes hepatic glucose output by both gluconeogenesis and glycogenolysis (8). The increases in glucose IL during BDP treatment were not associated with increases in concentrations of plasma glucagon. Therefore, unless changes in glucagon concentrations in jugular vein blood are not representative of changes occurring in portal vein blood, it seems that increases in glucose IL were not mediated by increased glucagon secretion.

Glucose IL was reduced by 50% when steers were fasted, compared with control values (Table 4), suggesting that glucose was effectively conserved. It is likely that ketone bodies and FFA served as alternative energy sub-
strates during fasting. Low glucose IL during fasting also suggests that the supply of endogenous gluconeogenic precursors was not great enough to maintain normal rates of gluconeogenesis during prolonged starvation. Steele and Leng (38) fasted pregnant sheep for 4 days and reported a 51% decrease in glucose IL. Kronfeld and Raggi (24) fasted lactating cows for 3 to 4 days and reported a 50% decrease in glucose IL.

Blood CO₂ TQ (Table 4) was higher during BDP and fasting than during the control period. Control values are too low and may be in error, making comparisons with controls questionable. Veenhuizen (unpublished, ISU Master's Thesis, Ca. 1983) reported TQ values of 7% in steers receiving the same ration as used here.

Liver metabolite concentrations

Concentrations of triglyceride, glycogen, and ketone bodies in liver are in Table 5. Concentrations of TG in liver were slightly greater during BDP treatment than during the control periods, but the increases were not significant. Mills et al. (32) reported similar findings in steers treated with BDP for shorter periods. Therefore, even prolonged treatment with BDP does not cause significant development of fatty liver in steers. Ketotic
Table 5. Effects of 1,3-butanediol and phlorizin treatment and fasting on liver metabolite concentrations in steers

<table>
<thead>
<tr>
<th>Liver metabolite</th>
<th>Control I</th>
<th>BDP (day 14)</th>
<th>BDP (day 28)</th>
<th>Control II</th>
<th>Fasted</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglyceride (%)</td>
<td>.63</td>
<td>1.02</td>
<td>.82</td>
<td>.47</td>
<td>1.38</td>
<td>.15</td>
</tr>
<tr>
<td>Glycogen (%)</td>
<td>3.60^a</td>
<td>3.07^a</td>
<td>3.30^a</td>
<td>3.19^a</td>
<td>1.35^b</td>
<td>.26</td>
</tr>
<tr>
<td><strong>β-hydroxybutyrate</strong> (nmoles/g tissue)</td>
<td>398^a</td>
<td>2071^b</td>
<td>1565^b</td>
<td>616^a</td>
<td>622^a</td>
<td>175</td>
</tr>
<tr>
<td>Acetoacetate (nmoles/g tissue)</td>
<td>197^cd</td>
<td>292^de</td>
<td>366^e</td>
<td>232^de</td>
<td>64^c</td>
<td>32</td>
</tr>
<tr>
<td><strong>β-hydroxybutyrate to acetoacetate ratio</strong></td>
<td>2.0^c</td>
<td>7.1^cd</td>
<td>4.3^c</td>
<td>2.7^c</td>
<td>9.8^d</td>
<td>1.6</td>
</tr>
</tbody>
</table>

^a,b^ Means in rows with different superscript letters differ (P<.01).

^c,d,e^ Means in rows with different superscript letters differ (P<.05).
cows often have liver TG concentrations of 10% (6). The uptake of FFA by ruminant liver has been reported to be directly proportional to the rate of mobilization of FFA from adipose tissue (6). Because plasma FFA concentrations were not elevated markedly during BDP treatment, liver TG did not increase markedly either.

Concentrations of TG in liver increased by about two fold when steers were fasted, compared with control values, but the increases were not significant (Table 5). Because plasma FFA concentrations were increased significantly during fasting, it seems that liver TG should have increased also. It is possible, however, that the alternative pathways of FFA utilization in the liver (lipoprotein formation or oxidation) were sufficiently active to prevent formation of fatty liver. Reid et al. (34) fasted nonlactating cows for 6 days and found that TG content of liver was only 2.7%.

Glycogen content of liver was only slightly less during BDP treatment than during the first control period, however, values during the second control period were slightly less than those on day 28 of BDP treatment (Table 5). Previously, liver glycogen in steers was shown to not be affected by short-term BDP treatment (32). Therefore, it seems that even during prolonged BDP treatment,
glucosuria did not cause a carbohydrate loss or stress in steers great enough to cause serious metabolic consequences, as evidenced by normal glycogen concentrations in liver. Baird et al. (4) reported that liver glycogen in normal lactating cows was 4-fold greater than in ketotic cows, however, they could not attribute the lower glycogen levels directly to ketonemia because cows were also hypophagic during ketosis.

When steers were fasted, liver glycogen was 63% less than when steers received the control treatment (Table 5), obviously indicating that fasting induced carbohydrate stress to a much greater extent than did BDP treatment. Baird et al. (3) fasted nonlactating cows for 6 days and reported a 71% decrease in liver glycogen.

Concentrations of BHBA in liver (Table 5) were much greater during the BDP period than during the control or fasting periods and BHBA concentrations in blood and liver were highly correlated ($r = .99$). Butanediol is oxidized to BHBA in liver of rats (40) and steers (32), therefore, it seems that BHBA concentrations in blood depend on the extent to which BD is metabolized by liver. Concentrations of ACAC in liver (Table 5) did not increase as markedly as did BHBA when BDP was given, but a significant increase was noted on day 28 of BDP treatment.
Concentrations of ACAC in blood and liver were not highly correlated ($r = .56$).

Ratios of BHBA to ACAC in liver (Table 5) were greater, but not significantly so, when steers received BDP than when the control treatment was given, suggesting that BD is oxidized preferentially to BHBA rather than to ACAC. Blood ketone body concentrations (Table 3) during BDP treatment also suggest that BD is oxidized preferentially to BHBA. Mehlman et al. (29) reported that BD was, in some cases, oxidized preferentially to BHBA in liver of rats. When steers were fasted, BHBA to ACAC ratios increased significantly over controls, suggesting that fatty acids mobilized from adipose tissue were oxidized preferentially to BHBA. Baird et al. (2), however, reported that ratios of BHBA to ACAC in liver decreased when lactating cows were fasted.

Increases in ketone body concentrations in liver during BDP treatment are similar to those reported for liver of ketotic cows (4). The absence of marked increases in ketone bodies in liver of the fasted steers does not agree with reports (3, 5) indicating that liver ketone bodies increased greatly when nonlactating cows were fasted. Concentrations of FFA in plasma of fasted cows (2, 6) are more than two fold greater than those
reported for these steers. Therefore, it seems that the greater influx of FFA into liver of fasting cows may account for the greater production of ketone bodies.
GENERAL DISCUSSION

Prolonged treatment with 1,3-butanediol and phlorizin (i.e., greater than 14 days) did not cause further changes in concentrations of metabolites in blood and liver, indicating that metabolic adaptations to ketonemia-glucosuria occurred rapidly. Ketotic cows exhibit decreases in plasma glucose and insulin and liver glycogen and increases in blood ketone bodies, plasma FFA, and liver triglyceride greater than those occurring in BDP-treated steers. These greater changes indicate that the metabolic stress imposed by lactation in cows fed ad libitum is greater than the stress imposed by BDP treatment in maintenance-fed steers.

Observations with ketotic cows sometimes are difficult to interpret because effects of ketonemia and lactation on metabolites in blood and liver often are confounded with effects of hypophagia, which usually accompanies ketosis. When steers received 1,3-butanediol and phlorizin, feed intake was not different from that during control periods, therefore, changes in blood and liver metabolites could be attributed solely to ketonemia-glucosuria.

When giving 1,3-butanediol and phlorizin to steers, assumptions are made that the only physiological
perturbations are ketonemia, ketonuria, and glucosuria. Butanediol does not seem to affect rumen fermentation (7) and, as discussed in (28), its successful use as an energy source in diets of animals is well-documented. Furthermore, BD is oxidized readily to BHBA by steer liver slices in vitro (32), indicating that BD is not toxic to animal tissues. Phlorizin prevents transport of glucose into cells of the proximal tubules in kidney and into cells of the intestinal brush border also (26). But it seems that phlorizin does not inhibit uptake of glucose by peripheral tissues (22). As discussed in the accompanying paper (27), however, phlorizin, under certain conditions, inhibits activities of some enzymes involved in carbohydrate metabolism, which indicates that the effects of phlorizin may not be limited to inhibition of glucose transport.

Due to lactation and hypophagia, ketotic cows probably experience much greater physiological stress than did steers treated with BDP. This greater stress probably explains why changes in blood and liver metabolites are much more marked in ketotic cows than in steers receiving BDP. Probably a more marked physiological stress could be imposed on steers if a sub-maintenance ration was fed during BDP treatment, but this would only invoke the
invalidity associated with feed-restricted ketosis models. Even though the BDP regime does not cause a ketosis in steers as severe as that seen in lactating cows, it can still be of great value in determining the effects of ketonemia-glucosuria on intermediary metabolism in the bovine.
REFERENCES CITED


SECTION III. IN VITRO HEPATIC GLUCONEOGENESIS AND KETOGENESIS AS AFFECTED BY PROLONGED KETONEMIA-GLUCOSURIA AND FASTING IN STEERS
ABSTRACT

Both 1,3-butanediol, which causes ketonemia, and phlorizin, which causes glucosuria, were given to four steers for 28 days to determine effects of prolonged ketonemia and glucosuria on hepatic gluconeogenesis and ketogenesis in vitro. Effects of prolonged fasting on these variables also were determined. In addition, effects of Ca**-free incubation media and in vitro additions of ketone bodies, fatty acids, defatted bovine serum albumin, NAD*, and niacin on hepatic gluconeogenesis were determined. Treatments were: control (1476 g corn, 3144 g alfalfa hay daily); control with butanediol plus phlorizin; and fasting for 9 days. Butanediol was mixed with the corn and phlorizin was injected twice daily. Liver, obtained by puncture biopsy, was incubated with carbon-14 labeled 10 mM propionate, lactate, alanine, glycerol, butyrate, and 1 mM palmitate. Glucose production [nmoles substrate converted to glucose/(100 mg liver x 2 h)] during control, butanediol plus phlorizin, and fasting averaged 467, 1441, and 2400 for propionate; 198, 760, and 2452 for lactate; 59, 151, and 444 for alanine; and 411, 1073, and 1155 for glycerol. The absence of Ca** in incubation media eclipsed the BDP-
and fasting-induced increases in hepatic gluconeogenesis. Additions of ketone bodies (5 mM), octanoate (1 mM), bovine serum albumin (3%), NAD⁺ (.45 mM), and niacin (.5 mM) did not markedly affect glucose production from propionate or lactate, however, some changes were noted. Stearate (1 mM), bound to albumin, greatly inhibited gluconeogenesis during control and butanediol plus phlorizin, but not during fasting. β-hydroxybutyrate production [nmoles/(100 mg liver x 2 h)] during control, butanediol plus phlorizin, and fasting averaged 414, 853, and 650 for butyrate and 12, 54, and 3 for palmitate. Hepatic gluconeogenesis was increased by both ketonemia-glucosuria and fasting, indicating that gluconeogenic capacity of ruminant liver is responsive to the physiological and nutritional status of the animal.
Lactation imposes a tremendous metabolic challenge on high-producing dairy cows, necessitating the synthesis of large quantities of glucose from noncarbohydrate precursors. Such cows often become ketotic during early lactation for reasons which still are not understood fully. Effects of ketosis on intermediary metabolism are not known completely and the lack of a consistent and predictable supply of spontaneously ketotic cows has impeded advances in ketosis research.

Effects of lactation ketosis on hepatic gluconeogenic enzyme capacities are not understood fully. Baird et al. (3) reported that concentrations of Krebs cycle and Embden-Meyerhof pathway intermediates in livers of cows were decreased by ketosis. Baird et al. (2) measured activities of gluconeogenic enzymes in livers of normal, lactating cows and of ketogenic, lactating cows and reported that, except for fructose diphosphatase, enzyme activities were greater in livers from ketogenic cows than in livers from normal cows, which suggests that hepatic gluconeogenic capacity would be increased during ketosis. Whether these in vitro observations are indicative of the true in vivo condition remains to be proven.
Interpretation of data regarding the effects of ketosis on metabolite concentrations and enzyme activities in tissues of cows is complicated by the uncertain differentiation between the true ketotic state (spontaneous ketosis) and the hypophagic state that later accompanies the disorder. Baird et al. (3) reported that healthy, lactating cows subjected to starvation became ketotic and exhibited changes in blood and liver metabolite concentrations similar to those observed in cows with spontaneous ketosis, which led those authors to question whether there were any differences in intermediary metabolism between cows suffering from starvation ketosis and those with spontaneous ketosis. Kronfeld (17), however, indicated major distinctions between spontaneous and starvation ketosis, which included higher plasma acetate and liver ketone body concentrations, greater mammary acetoacetate release, and greater glucose space and glucose transport rates in the former.

Earlier (8, 23, 28), and in an accompanying paper (22), we have reported attempts to create a ketosis model using steers fed 1,3-butanediol (BD) and injected with phlorizin (P). Butanediol is a ketogenic substrate and P is a compound that causes glucosuria. In (23), BD was fed for 14 days and P was injected during the last 7 days of
BD feeding. Steers were ketonemic when liver biopsies were performed and, compared with controls, in vitro hepatic gluconeogenic capacities increased. These observations were different from those in cows having an experimental ketosis, where in vitro hepatic gluconeogenic capacities were impaired (26).

Because our earlier work showed promise for causing a useful experimental ketosis, we wanted to determine the effects of extended treatment with BD and P on hepatic metabolism. The first objective was to determine the effects on in vitro hepatic gluconeogenic and ketogenic capacities caused by prolonged ketonemia and glucosuria in steers during treatment with BD and P for 28 days. Second, we determined effects of prolonged starvation on in vitro hepatic metabolism, thereby allowing comparisons between three physiological states: normal, ketonemia-glucosuria (experimental ketosis), and starvation.

Under some circumstances, long-chain fatty acids (LCFA) can increase gluconeogenesis from a variety of substrates in liver slices and in perfused rat liver (31). When LCFA are added to tissue preparations in a physiological manner, however, they do not stimulate in vitro hepatic gluconeogenesis in rats (30). The effects of LCFA and ketone bodies on gluconeogenesis in ruminant
liver in vitro are not known. Therefore, the third objective was to determine effects of LCFA and(or) ketone body additions on rates of in vitro hepatic gluconeogenesis in liver slices from steers receiving control or BDP treatments and from steers that were fasted.
MATERIALS AND METHODS

Experimental design

Care of the four steers, experimental design, and ration composition were described in the accompanying paper (22). Briefly, the trial consisted of four periods. In periods 1 and 3, the control ration was given; in period 2, control plus BD\(^1\) and P\(^2\) (BDP) was given for 28 days; and in period 4, steers were fasted for 9 days. The control treatment was repeated in period 3 to prevent possible carryover effects of BDP into the fasting period.

Liver biopsy

Liver biopsies were performed at the end of the first control period, at the middle (day 14) and end (day 28) of the BDP period, at the end of the second control period, and at the end of the fasting period. Tissue was obtained by the puncture biopsy technique (13), except for the fasting period, when liver was obtained via laparotomy just prior to sacrificing the steers. Puncture biopsy is advantageous because it allows repeated sampling from the

\(^1\)Butanediol was a gift from the Celanese Chemical Co., New York, NY.

\(^2\)Phlorizin was purchased from Fluka Chemical Corp., Hauppauge, NY.
same animal, but the disadvantages, especially in smaller animals, are difficulty in obtaining enough tissue for large-scale incubations and risk of death from excessive hemorrhage. Six to 8 g of liver normally were obtained, .5 g was rinsed immediately in cold .15 M NaCl and submerged in liquid nitrogen. The remaining liver tissue was kept in cold buffered (pH 7.4) .15 M NaCl. Frozen liver was stored in an airtight container at -80°C until assayed for metabolite concentrations. Fresh tissue was incubated as soon as possible, normally within 3 h.

**Incubation procedure**

Liver biopsy cores were sliced using a Stadie-Riggs tissue slicer. Seventy to 100 mg of slices were incubated with substrates (1 μCi of 14C-labeled substrate plus 30 μmoles of non-labeled substrate) in 25 ml Erlenmeyer flasks containing 3 ml of pH 7.4 Krebs-Ringer bicarbonate (KRB) buffer (9). When LCFA or ketone bodies were added, Ca++ was omitted from the buffer. Flasks were gassed with 95% O2 - 5% CO2, hanging wells containing folded filter paper were suspended for 14CO2 collection and flasks were stoppered. Incubations were for 2 h at 37°C in a metabolic shaker. Incubations with each substrate usually
were conducted in triplicate, however, on some occasions, tissue was limited and incubations then were only in duplicate.

Incubations were terminated by injecting .5 ml of 10% HClO₄ into the media. To trap ¹⁴CO₂, 100 µl of 25% NaOH was injected onto the filter paper in the hanging well. Acidified media was shaken for 1 h to evolve ¹⁴CO₂. Filter papers were removed and placed in vials. Radioactivity was determined by liquid scintillation counting.

Hepatic gluconeogenic capacities and substrate oxidation rates were measured by using Na-[2-¹⁴C]propionate, Na-L(+)[U-¹³C]lactate, L-[U-¹³C]alanine, and L-[U-¹⁴C]glycerol. All substrates were 10 mM. Effects of additions of ketone bodies (5 mM) and stearate conjugated to bovine serum albumin (1 mM stearate bound to defatted BSA, final BSA concentration 3%) on gluconeogenic capacities and substrate oxidation rates of liver slices using Na-[2-¹⁴C]propionate and Na-L(+)[U-¹³C]-lactate as substrates were determined on day 28 of BDP treatment, at the end of the second control period, and at the end of the fasting period. Effects of separate additions of octanoate (1 mM) and BSA (defatted, final
BSA concentration 3%) on gluconeogenic capacities and substrate oxidation rates of liver slices using Na-[2-\(^{14}\)C]-propionate as substrate were determined also. Effects of additions of NAD\(^+\) (β-, final concentration .45 mM) and niacin (final concentration .5 mM) on gluconeogenic capacities and substrate oxidation rates of liver slices using Na-[2-\(^{14}\)C]propionate as substrate were determined at the end of the fasting period.

Hepatic ketogenic capacities and substrate oxidation rates were measured by using the two substrates, Na-[1-\(^{13}\)C]butyrate (10 mM) and [1-\(^{13}\)C]palmitate-BSA complex (1 mM palmitate plus 1 μCi of [1-\(^{13}\)C] palmitate both bound to defatted BSA, final BSA concentration 3%). Ketone body production rates were corrected for endogenous liver ketone body content by measuring ketone bodies released from tissue incubated in media acidified with .5 ml of 10% HClO₄.

**Analytical procedures and presentation of data**

Following \(^{14}\)CO₂ collection, acidified media were spiked with 5500 DPM of [6-\(^3\)H]glucose. Flask contents were transferred to 50 ml tubes and centrifuged. The supernatant was neutralized with solutions of 5% and 10% KOH. Concentration of β-hydroxybutyrate (BHBA) in
neutralized medium was measured (37). Glucose in neutralized medium was isolated by using anion-cation exchange columns (27) and radioactivity and glucose concentration\(^3\) in the column eluate were both quantified. Recoveries of \(^{14}\text{C}\) radioactivity and glucose were corrected for recovery of the \([6-^{3}\text{H}]\text{glucose}\). Background radioactivity was measured in triplicate for each substrate by processing media that contained no tissue. Metabolic rates were based upon wet weights of tissue incubated because, as reported in the accompanying paper (22), changes in liver composition (i.e., glycogen and triglyceride content) were small.

Differences between treatment means for all variables were tested by using orthogonal comparisons (11, p. 237).

\(^3\text{Glucostat, Worthington Biochemicals, Freehold, NJ.}\)
RESULTS AND DISCUSSION

Kinetics of glucose metabolism in the steers were determined during this experiment and are reported, along with metabolite concentrations in plasma and liver, in (22).

Rates of gluconeogenesis and oxidation of gluconeogenic substrates

Effects of BDP treatment and of fasting on gluconeogenic rates and substrate oxidation rates in liver slices incubated in vitro are shown in Table 1. Alanine was not used as a substrate in the second control period (control II) because tissue was limited. Glycerol was used as a substrate in the first control period (control I); however, poor separation of $^{14}$C-glycerol from $^{14}$C-glucose by anion-cation exchange chromatography caused extremely high background counts. Subsequently, following incubation with liver, $^{14}$C-glycerol in the media was phosphorylated by using glycerol kinase and ATP, thereby allowing separation of the negatively charged $^{14}$C-glycerol-3-phosphate from $^{14}$C-glucose.

Incorporation of propionate, lactate, alanine, and glycerol into glucose by liver slices was greater during BDP treatment than during the control periods, but the
Table 1. Effects of 1,3-butandiol and phlorizin treatment and fasting on in vitro gluconeogenesis and substrate oxidation in liver slices from steers

<table>
<thead>
<tr>
<th>Gluconeogenic substrate</th>
<th>Treatment</th>
<th>Control I (day 14)</th>
<th>BDP (day 28)</th>
<th>Control II</th>
<th>Fasted</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>nmoles substrate converted/(100 mg liver x 2 h) ---</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionate:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>to glucose</td>
<td></td>
<td>466^d</td>
<td>1740^ef</td>
<td>1143^de</td>
<td>467^d</td>
<td>2400^f</td>
</tr>
<tr>
<td>to CO_2</td>
<td></td>
<td>83^de</td>
<td>191^f</td>
<td>161^ef</td>
<td>62^d</td>
<td>404^g</td>
</tr>
<tr>
<td>Lactate:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>to glucose</td>
<td></td>
<td>124^d</td>
<td>884^e</td>
<td>635^de</td>
<td>271^d</td>
<td>2452^f</td>
</tr>
<tr>
<td>to CO_2</td>
<td></td>
<td>288^de</td>
<td>476^e</td>
<td>317^de</td>
<td>213^d</td>
<td>789^f</td>
</tr>
<tr>
<td>Alanine:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>to glucose</td>
<td></td>
<td>59^d</td>
<td>203^d</td>
<td>99^d</td>
<td>---</td>
<td>444^e</td>
</tr>
<tr>
<td>to CO_2</td>
<td></td>
<td>108^d</td>
<td>116^d</td>
<td>60^d</td>
<td>---</td>
<td>252^e</td>
</tr>
<tr>
<td>Glycerol:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>to glucose</td>
<td></td>
<td>---</td>
<td>1312^d</td>
<td>83^de</td>
<td>411^e</td>
<td>1155^de</td>
</tr>
<tr>
<td>to CO_2</td>
<td></td>
<td>31^d</td>
<td>86^e</td>
<td>91^e</td>
<td>31^d</td>
<td>99^e</td>
</tr>
</tbody>
</table>

^aIncorporation into glucose expressed as nmoles substrate converted to glucose/(100 mg liver x 2 h).

^bOxidation to CO_2 expressed as nmoles substrate to CO_2/(100 mg liver x 2 h).

^cRefer to text for explanation of missing values.

^d,e,f,gMeans in rows with different superscript letter differ (P<.05).
increases were significant only for propionate, lactate, and glycerol on day 14 of BDP treatment. Mills et al. (28) reported that BD caused decreases and P caused increases in gluconeogenic capacity of steer liver slices. When BD and P were given in combination, however, the inhibitory effects of BD were negated by the stimulatory effects of P, resulting in greater rates of gluconeogenesis, compared with controls. Effects of P on hepatic gluconeogenic enzyme activities are not known fully, however, effects of BD have been studied extensively. Mehlman et al. (25) reported that pyruvate carboxylase (PC) and phosphoenolpyruvate carboxykinase (PEPCK) activities increased in liver and kidney of rats fed BD. If these enzymes were affected similarly in steers fed BD, it could explain the increases in incorporation of propionate, lactate, and alanine into glucose, but not of glycerol, because this substrate bypasses the PC and PEPCK reactions by entering the gluconeogenic pathway as a triose phosphate.

Data regarding effects on hepatic metabolism of spontaneous ketosis in cows are limited. Mills (26) reported that gluconeogenic capacity of liver slices was decreased when lactating cows became ketotic. Hibbitt and
Baird (12) found that liver slices from ketotic cows had respiration rates that were 35% lower than liver slices from normal cows.

Gluconeogenic capacity of liver slices increased markedly with all substrates when steers were fasted (Table 1) and, with the exception of glycerol, incorporation of substrates into glucose was greater during fasting than during BDP treatment. Mills et al. (28) reported increases of in vitro gluconeogenic capacities and oxidation rates in liver slices from feed-restricted steers. But other reports (14, 19) involving ruminants have indicated that in vitro hepatic gluconeogenic capacity decreased in response to fasting. Effects of fasting on activities of gluconeogenic enzymes have been studied extensively. Baird et al. (3) reported that the activities of pyruvate kinase and PEPCK in livers of starved cows were much less than in livers of fed cows. Young et al. (38), however, reported little change in activity of PEPCK when cattle were fasted. Another report (4) noted that fasting caused a 26% decrease in propionyl-CoA carboxylase activity in livers of cows. Ballard et al. (5) found increased PC activity in fasted cows, compared with fed, nonlactating cows. In work with nonruminants (29, 31, 32), in vitro hepatic gluconeogenic capacity increased in response to fasting.
As reported in (22), increases of in vivo glucose irreversible loss (IL) were observed in these steers during BDP treatment. The BDP-induced increases of in vitro hepatic gluconeogenic capacities may explain the BDP-induced increases in glucose IL, suggesting that in vitro observations were representative of in vivo conditions. We also reported (22) that glucose IL decreased when steers were fasted. The fasting-induced increases of in vitro hepatic gluconeogenic capacities are not consistent with the fasting-induced decreases in glucose IL in vivo. But one would not expect to find a concomitant decrease for in vitro gluconeogenic capacity and for in vivo glucose IL during fasting because gluconeogenic substrates were limited in vivo, but not in vitro. Lindsay (20) reported that hepatic gluconeogenic rate is related more to nutrient supply than to changes in efficiency of nutrient utilization.

Oxidation of propionate, lactate, and glycerol to CO₂ by liver (Table 1) was greater during BDP treatment than during the control periods. Oxidation of alanine, however, increased only slightly on day 14 of BDP treatment and then decreased below its control value on day 28 of BDP treatment.

Oxidation of propionate, lactate, and alanine to CO₂ by
liver from fasted steers was markedly greater than when steers received either control or BDP treatments. But with glycerol, oxidation rates during fasting were similar to rates during BDP treatment, however, values during fasting were greater than control values. Increases in substrate oxidation rates during BDP and fasting paralleled the increases in gluconeogenic rates, however, the magnitude of the increases were usually greater for gluconeogenesis than for oxidation. The concomitant increases in gluconeogenic and oxidation capacities in liver slices during BDP treatment and fasting suggest that gluconeogenic substrates were not utilized preferentially either for glucose synthesis or for oxidation. Therefore, it seems that the BDP- and fasting-induced increases in hepatic metabolism were of a general nature rather than specific.

Gluconeogenic capacities and substrate oxidation rates were greater on day 14 of BDP than on day 28 of BDP, except when glycerol was the substrate, when oxidation rates were nearly equal. Although these decreases were not statistically significant, they suggest that an adaptation in utilization of substrates by liver may have begun to occur during prolonged BDP treatment, with metabolic capacities shifting back toward control values.
Effects of Ca**, ketone bodies, and fatty acids on gluconeogenesis

Effects of Ca**-free buffer and additions of ketone bodies, fatty acids, defatted BSA, NAD*, and niacin on in vitro hepatic gluconeogenic capacity are in Table 2. When stearate was added to incubation media, Ca** was omitted from the buffer solution to prevent formation of insoluble Ca**-stearate salts. Because Ca**-free buffer was used when stearate was added, Ca** also was omitted when octanoate, ketone bodies, and BSA were added, so that valid comparisons could be made. When the buffer did not contain Ca**, incorporation of propionate into glucose was not increased by BDP treatment or fasting and incorporation of lactate into glucose was not affected by BDP treatment, and was increased only slightly by fasting. But when the buffer contained Ca** (2.5 mM), incorporation of both propionate and lactate into glucose during BDP treatment and fasting was much greater than incorporation during the control period. We are not aware that anyone previously has reported a major differential effect of Ca** upon gluconeogenesis between liver slices from two different physiological conditions. This intriguing observation should be investigated further until an explanation is found.
Table 2. Effects of Ca\textsuperscript{++}-free buffer and additions of ketone bodies, fatty acids, BSA, NAD\textsuperscript{+}, and niacin on in vitro gluconeogenic capacity of liver slices from steers

<table>
<thead>
<tr>
<th>Flask contents</th>
<th>Substrate</th>
<th>Propionate</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control II</td>
<td>BDP (day 28)</td>
</tr>
<tr>
<td>KRB\textsuperscript{a} Without Ca\textsuperscript{++}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With Ca\textsuperscript{++}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRB without Ca\textsuperscript{++} plus:</td>
<td>Ketone bodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>β-hydroxybutyrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acetoacetate</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stearate-BSA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ketone bodies plus Stearate-BSA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Propionate</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(100 mg liver x 2 h)</td>
</tr>
</tbody>
</table>

KRB\textsuperscript{a}: Without Ca\textsuperscript{++} with Ca\textsuperscript{++}:

<table>
<thead>
<tr>
<th></th>
<th>Propionate</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control II</td>
<td>BDP</td>
</tr>
<tr>
<td>530\textsuperscript{cd}</td>
<td>486\textsuperscript{ef}</td>
<td>646\textsuperscript{d}</td>
</tr>
<tr>
<td>467\textsuperscript{cdx}</td>
<td>1143\textsuperscript{cx}</td>
<td>2400\textsuperscript{cy}</td>
</tr>
</tbody>
</table>

KRB without Ca\textsuperscript{++} plus:

<table>
<thead>
<tr>
<th></th>
<th>Propionate</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control II</td>
<td>BDP</td>
</tr>
<tr>
<td>403\textsuperscript{cdx}</td>
<td>637\textsuperscript{dxy}</td>
<td>910\textsuperscript{dy}</td>
</tr>
<tr>
<td>617\textsuperscript{cdx}</td>
<td>640\textsuperscript{dex}</td>
<td>1340\textsuperscript{dy}</td>
</tr>
<tr>
<td>750\textsuperscript{cx}</td>
<td>273\textsuperscript{fgy}</td>
<td>578\textsuperscript{dxy}</td>
</tr>
<tr>
<td>35\textsuperscript{ex}</td>
<td>18\textsuperscript{ex}</td>
<td>967\textsuperscript{dy}</td>
</tr>
<tr>
<td>31\textsuperscript{ex}</td>
<td>25\textsuperscript{ex}</td>
<td>1118\textsuperscript{dy}</td>
</tr>
<tr>
<td>10\textsuperscript{dx}</td>
<td>4\textsuperscript{ex}</td>
<td>183\textsuperscript{dy}</td>
</tr>
<tr>
<td></td>
<td>BSA</td>
<td>Octanoate</td>
</tr>
<tr>
<td>----------</td>
<td>------</td>
<td>-----------</td>
</tr>
<tr>
<td></td>
<td>372^dx</td>
<td>205^fgx</td>
</tr>
<tr>
<td></td>
<td>599^cd</td>
<td>817^d</td>
</tr>
</tbody>
</table>

Krebs-Ringer bicarbonate buffer.

β-hydroxybutyrate and acetoacetate, each 5 mM.

Means in columns with different superscript letters differ (P<.05).

For each substrate, means in rows with different superscript letters differ (P<.05).
Although effects of Ca^{++} on in vitro gluconeogenic capacity of liver from ruminants have not been reported previously, effects of other ions have been reported. Leng and Annison (19) indicated that incorporation of propionate into glucose by sheep liver slices was favored by increased Na^+ and decreased K^+ content in incubation media. Kimmich and Rasmussen (15) reported that 100 mM Ca^{++} additions to incubation media inhibited PC activity in isolated mitochondria of rat liver, indicating that Ca^{++} may play an important role in regulating the initial reactions of gluconeogenesis. Although Ca^{++} has been shown to inhibit magnesium-activated enzymes (7), our data indicate that capacities for gluconeogenesis and substrate oxidation in liver slices were enhanced by Ca^{++} during BDP treatment and fasting, possibly suggesting an increase in the activities of enzymes associated with the gluconeogenic and tricarboxylic acid cycle pathways.

Additions of BHBA plus ACAC to Ca^{++}-free buffer (Table 2) did not significantly affect incorporation of propionate and lactate into glucose. Addition of BHBA (5 mM) to Ca^{++}-free buffer increased incorporation of propionate into glucose in all treatment periods, but despite increases of up to two fold, effects were not significant.
Addition of ACAC (5 mM) caused gluconeogenesis from propionate to increase slightly during the control period, however, values tended to decrease when ACAC was added to liver obtained from BDP-treated and fasted steers. With the possible exception of BHBA addition to liver from fasted steers, it seems that ketone bodies, either separately or in combination, had no effect on hepatic gluconeogenesis from propionate. Krebs et al. (16) reported that ACAC promoted glucose synthesis in rat kidney cortex slices by serving as a fuel for respiration, thereby sparing gluconeogenic precursors from oxidation, and by activating pyruvate carboxylase via increased acetyl CoA formation from ACAC.

Addition of 1 mM stearate bound to BSA (Table 2) essentially stopped (93% reduction) incorporation of propionate and lactate into glucose during control and BDP periods. But in liver slices prepared from fasted steers, stearate-BSA did not inhibit incorporation of propionate and lactate into glucose. Thus, the liver slices from fasted steers again show a differential response. No previous data regarding the effects of LCFA on in vitro gluconeogenesis in liver from ruminants are available, however, data presented here indicate that
stearate-BSA is a potent inhibitor of gluconeogenesis from propionate and lactate, except in liver from fasted steers.

Addition of ketone bodies and stearate-BSA in combination (Table 2) caused marked reductions in incorporation of propionate and lactate into glucose during control and BDP periods. But in liver slices obtained from fasted steers, ketone bodies plus stearate-BSA did not inhibit gluconeogenesis from propionate or lactate, although a nonsignificant decrease in incorporation of lactate into glucose was seen. Additions of ketone bodies plus stearate-BSA caused effects similar to those seen when stearate-BSA was added alone. Therefore, it seems that under the conditions reported here, ketone bodies per se do not significantly affect incorporation of propionate or lactate into glucose by liver slices prepared from control, BDP-treated, or fasted steers. The curious response of liver slices from fasted steers is again evident when ketone bodies plus stearate-BSA is present.

Because LCFA are insoluble in aqueous solutions (plasma, KRB buffer) at pH 7.4, they must be bound to protein. To determine if the BSA component of the stearate-BSA complex affected gluconeogenic rates, liver
slices were incubated in Ca\textsuperscript{2+}-free KRB buffer containing 3\% defatted BSA. Incorporation of propionate into glucose decreased by 30\% and 58\% (Table 2) when BSA was added to media containing liver slices from control and BDP-treated steers, however, the decreases were not significant. These data indicate that, although BSA may cause a slight decrease in gluconeogenesis from propionate, most of the inhibition caused by the stearate-BSA complex can be attributed to stearate. Schimmel and Knobil (32) found that defatted BSA did not affect gluconeogenesis from pyruvate in rat liver slices. Incorporation of propionate into glucose increased by 88\% (Table 2) when BSA was added to liver slices prepared from fasted steers, which was similar to the increases noted when stearate-BSA and ketone bodies plus stearate-BSA were added. Although these increases were not significant, it seems that BSA additions may stimulate in vitro gluconeogenesis from propionate in liver slices from fasted steers.

Octanoate was added (1 mM) to incubation media to determine the effects of a medium-chain, water soluble fatty acid on gluconeogenesis from propionate (Table 2). Octanoate caused a small increase in incorporation of propionate into glucose by liver slices prepared during the control treatment. But during BDP treatment and
fasting, octanoate caused more marked increases in gluconeogenesis from propionate, however, the increases were significant only during BDP treatment. Clark et al. (6) reported that butyrate, a short-chain fatty acid, increased gluconeogenic capacity of isolated lamb liver cells. Leng and Annison (19) reported similar findings from liver slices from starved sheep, but with fed sheep, butyrate did not affect hepatic gluconeogenesis.

Effects of niacin and NAD$^+$ on gluconeogenesis

Niacin is effective in the treatment of bovine lactation ketosis (10) and has been shown to have an anti-lipolytic effect in cattle (35). The effects of supplemental niacin on carbohydrate metabolism in ruminants are not known fully. Because niacin promotes an increase in blood glucose (34), workers have speculated, however, that niacin potentiates gluconeogenesis in the ruminant. It is possible that the therapeutic actions of niacin may be mediated through NAD$^+$ and NADP$^+$, important coenzymes involved with carbohydrate and lipid metabolism, of which niacin is a component.

Sufficient liver was obtained from the biopsy at the end of the fasting period to allow additions of NAD$^+$ and niacin to liver slice incubations to determine if either
compound affected gluconeogenesis from propionate. Addition of NAD+ (0.45 mM) to KRB containing Ca++ caused incorporation of propionate into glucose to increase by 22% and addition of niacin (0.50 mM) caused values to increase by 28%, when compared with controls (KRB with Ca++), but the increases were not statistically significant (Table 2). Concentration of NAD+ in liver of normal and fasted cows is approximately 1.1 and 0.56 mM (3), therefore, our NAD+ additions were not in excess of physiological concentrations. NAD+ in liver was not measured in this experiment. It is possible that NAD+ was decreased in liver of fasted steers, thereby limiting availability of NAD+ for gluconeogenesis, which would explain why NAD+ additions caused higher rates of gluconeogenesis from propionate. The slight niacin-induced increase in gluconeogenesis indicates that either the vitamin itself may have been stimulatory or that liver slices utilized the excess niacin to synthesize more NAD+. In either case, provided the niacin-induced increase was real, it seems that these observations in vitro support the observations that niacin promotes increases in blood glucose concentrations in vivo.

Factors affecting substrate oxidation

Data on effects of Ca++-free buffer and additions
of ketone bodies, fatty acids, defatted BSA, NAD+, and niacin on substrate oxidation rates in vitro are summarized in Table 3. Omission of Ca++ from KRB buffer and additions of ketone bodies, either in combination or alone, did not cause any consistent changes in oxidation of propionate or lactate. Addition of stearate-BSA, alone and in combination with ketone bodies, caused oxidation rates to decrease. When propionate was the substrate, BSA caused decreased oxidation rates, however, octanoate, NAD+ and niacin additions did not cause any marked changes. Overall, effects of in vitro additions on substrate oxidation were, in most cases, similar to effects observed on gluconeogenic capacity of liver slices (Table 2).

Concentrations of glucose in incubation media (see appendix) and rates of substrate oxidation were correlated with gluconeogenic rates (Table 4). Positive correlations were found between media glucose and gluconeogenic rates, with a possible exception occurring when lactate was the substrate, when probability only approached .05. Positive correlations indicate that increases in gluconeogenic rates were associated with increases in media glucose concentration. Sources of glucose in incubation media include free glucose in liver, glucose released from liver
Table 3. Effects of Ca\textsuperscript{++}-free buffer and additions of ketone bodies, fatty acids, BSA, NAD\textsuperscript{+}, and niacin on in vitro substrate oxidation by liver slices from steers

<table>
<thead>
<tr>
<th>Flask contents</th>
<th>Substrate</th>
<th>Control (day 28)</th>
<th>BDP</th>
<th>Fasted</th>
<th>Control (day 28)</th>
<th>BDP</th>
<th>Fasted</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRB\textsuperscript{a}: Without Ca\textsuperscript{++}</td>
<td>Propionate</td>
<td>164\textsuperscript{ex}</td>
<td>116\textsuperscript{cx}</td>
<td>418\textsuperscript{cdy}</td>
<td>382\textsuperscript{d}</td>
<td>245\textsuperscript{ed}</td>
<td>322\textsuperscript{d}</td>
</tr>
<tr>
<td>With Ca\textsuperscript{++}</td>
<td></td>
<td>62\textsuperscript{cdx}</td>
<td>161\textsuperscript{cx}</td>
<td>404\textsuperscript{cdy}</td>
<td>213\textsuperscript{cx}</td>
<td>317\textsuperscript{cx}</td>
<td>789\textsuperscript{cy}</td>
</tr>
<tr>
<td>KRB without Ca\textsuperscript{++} plus: Ketone bodies\textsuperscript{b}</td>
<td></td>
<td>173\textsuperscript{ex}</td>
<td>257\textsuperscript{dx}</td>
<td>531\textsuperscript{cey}</td>
<td>472\textsuperscript{dxy}</td>
<td>222\textsuperscript{dx}</td>
<td>591\textsuperscript{cy}</td>
</tr>
<tr>
<td>(\beta)-hydroxybutyrate</td>
<td></td>
<td>203\textsuperscript{ex}</td>
<td>175\textsuperscript{cx}</td>
<td>650\textsuperscript{ey}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetoacetate</td>
<td></td>
<td>345\textsuperscript{fxy}</td>
<td>120\textsuperscript{cx}</td>
<td>411\textsuperscript{cdy}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stearate-BSA</td>
<td></td>
<td>22\textsuperscript{cx}</td>
<td>15\textsuperscript{ex}</td>
<td>241\textsuperscript{dy}</td>
<td>27\textsuperscript{ex}</td>
<td>22\textsuperscript{ex}</td>
<td>348\textsuperscript{dy}</td>
</tr>
<tr>
<td>Ketone bodies plus Stearate-BSA</td>
<td></td>
<td>27\textsuperscript{cx}</td>
<td>27\textsuperscript{ex}</td>
<td>381\textsuperscript{cdy}</td>
<td>43\textsuperscript{ex}</td>
<td>33\textsuperscript{ex}</td>
<td>197\textsuperscript{dy}</td>
</tr>
</tbody>
</table>

Note: The table presents the mmoles substrate oxidized to CO\textsubscript{2}/(100 mg liver x 2 hr).
<table>
<thead>
<tr>
<th></th>
<th>BSA</th>
<th>Octanoate</th>
<th>KRB with Ca²⁺ plus:</th>
<th>NAD⁺</th>
<th>Niacin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>38&lt;sup&gt;cx&lt;/sup&gt;</td>
<td>48&lt;sup&gt;ex&lt;/sup&gt;</td>
<td>255&lt;sup&gt;dy&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>131&lt;sup&gt;dex&lt;/sup&gt;</td>
<td>150&lt;sup&gt;cx&lt;/sup&gt;</td>
<td>432&lt;sup&gt;cdy&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Krebs-Ringer bicarbonate buffer.

<sup>b</sup>β-hydroxybutyrate and acetoacetate, each 5 mM.

<sup>c,d,e,f</sup>Means in columns with different superscript letters differ (P<.05).

<sup>x,y</sup>For each substrate, means in rows with different superscript letters differ (P<.05).
Table 4. Correlation coefficients for media glucose vs. gluconeogenic rate and substrate oxidation rate vs. gluconeogenic rate in liver slice incubations

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Media glucose vs. gluconeogenic rate</th>
<th>P&gt;R</th>
<th>Substrate oxidation rate vs. gluconeogenic rate</th>
<th>P&gt;R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionate</td>
<td>.48</td>
<td>.0001</td>
<td>.89</td>
<td>.0001</td>
</tr>
<tr>
<td>Lactate</td>
<td>.23</td>
<td>.092</td>
<td>.84</td>
<td>.0001</td>
</tr>
<tr>
<td>Alanine</td>
<td>.39</td>
<td>.008</td>
<td>.92</td>
<td>.0001</td>
</tr>
<tr>
<td>Glycerol</td>
<td>.91</td>
<td>.0001</td>
<td>.74</td>
<td>.0001</td>
</tr>
</tbody>
</table>
glycogen by phosphorolytic and hydrolytic cleavage, and gluconeogenesis by liver slices.

Glucose effectively suppresses gluconeogenesis in vivo (1). Seto et al. (33) reported that glucose formation from propionate was reduced by 10 mM glucose in vitro. In the present experiment, glucose in media did not exceed 2 mM; therefore, gluconeogenesis should not have been inhibited by high concentrations of glucose. Positive correlations between gluconeogenic rates and media glucose concentrations substantiate the lack of glucose inhibition.

Substrate oxidation rates were correlated positively (Table 4) with gluconeogenic rates (data from Table 1), indicating that gluconeogenic substrates were not preferentially utilized for either glucose synthesis or oxidation during BDP- or fasting-induced increases in hepatic metabolic rates. This lack of preference suggests that effects of BDP treatment and fasting on capacities of hepatic enzymes were not specific, i.e., enzymes of both the gluconeogenic pathway and the tricarboxylic acid cycle were affected.

Ketogenesis and oxidation of ketogenic substrates

Ketogenic capacities and substrate oxidation rates,
as affected by BDP treatment and fasting, are summarized in Table 5. Production of BHBA during the first control period (control I) is not reported because BHBA concentration in media were not determined. In these samples, Universal Indicator\(^4\) was used to show neutral pH during titration with KOH. It was discovered later that the indicator interfered with enzymatic determination of BHBA. Subsequently, indicator sticks\(^5\) were used to show neutrality.

Net BHBA production and substrate oxidation rates were greater from butyrate than from palmitate, regardless of treatment. Greater rates of BHBA production from butyrate may be caused by selective entry into mitochondria. Butyrate freely enters mitochondria, but palmitate requires the carnitine acyltransferase (CAT) system for entry. The CAT system plays an important role in regulation of hepatic fatty acid oxidation and hence of ketogenesis (36). Net BHBA production from


\(^5\)Color pHast indicator sticks (pH 0-14), MCB Reagents, Gibbstown, NJ.
Table 5. Effect of 1,3-butanediol and phlorizin treatment and fasting on in vitro ketogenesis and substrate oxidation in liver slices from steers

<table>
<thead>
<tr>
<th>Ketogenic substrate</th>
<th>Treatment</th>
<th>Control I (day 14)</th>
<th>BDP (day 28)</th>
<th>Control II</th>
<th>Fasted</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butyrate*</td>
<td>BHBA produced</td>
<td>--</td>
<td>1051^c</td>
<td>655^cd</td>
<td>414^d</td>
<td>650^d</td>
</tr>
<tr>
<td></td>
<td>Substrate oxidized</td>
<td>266^c</td>
<td>137^d</td>
<td>143^d</td>
<td>154^d</td>
<td>292^c</td>
</tr>
<tr>
<td>Palmitate:</td>
<td>BHBA produced</td>
<td>--</td>
<td>71^c</td>
<td>37^f</td>
<td>12^de</td>
<td>3^e</td>
</tr>
<tr>
<td></td>
<td>Substrate oxidized</td>
<td>3</td>
<td>2.9</td>
<td>3.3</td>
<td>2.4</td>
<td>3.3</td>
</tr>
</tbody>
</table>

*a*Refer to text for explanation of missing values.

^b*nmoles/(100 mg liver x 2 h).*

^c,d,e*Means in rows with different superscript letters differ (P<.05).*
butyrate in liver slices was significantly greater on day 14 of BDP treatment than during the control period, however, values on day 28 of BDP and during fasting were only 60% greater than controls. Production of BHBA from palmitate was significantly greater on day 14 of BDP treatment than during the control period. By day 28 of BDP, however, values decreased, but were still 3-fold greater than controls. In liver slices prepared from fasted steers, BHBA production from palmitate was lower than controls, but the difference was not significant.

With both butyrate and palmitate, the measured ketogenic capacity of liver slices was greatest on day 14 of BDP treatment and an adaptation in utilization of ketogenic substrate by liver had occurred during prolonged BDP treatment, causing ketogenic capacity on day 28 of BDP to approach control levels. A similar adaptation was noted for utilization of glucogenic substrates for gluconeogenesis (Table 1). Mills et al. (28) reported that in vitro hepatic ketogenic capacity was not affected by short-term BDP treatment in steers. Ketogenic capacity of liver slices was depressed markedly when lactating cows became ketotic (26) and ketogenic capacity of rat liver homogenate was not affected by starvation and subsequent ketonemia (24).
Oxidation of butyrate to CO₂ by liver slices was highly variable during the control periods, thereby making comparisons with BDP and fasting periods difficult. But it is apparent that oxidation of butyrate was greater during fasting than during BDP treatment. Oxidation of palmitate to CO₂ by liver slices was minimal and not affected by BDP treatment or fasting. The increased capacity for beta oxidation when butyrate, rather than palmitate, was the substrate suggests that regulation occurred prior to beta oxidation, probably at the CAT system.
GENERAL DISCUSSION

During the control and BDP periods, feed intake was constant, therefore, effects of ketonemia-glucosuria on hepatic metabolism are not confounded with variations in feed intake. During the early stages of spontaneous ketosis, cows exhibit normal behavior, however, as the disease progresses, they soon become hypophagic. Therefore, in ketotic cows or in a starvation ketosis, effects of ketonemia on hepatic metabolism are confounded with effects of reduced feed intake.

The physiological status of these steers (i.e., ketonemia-glucosuria and fasting) markedly affected hepatic glucose synthesis from propionate, lactate, alanine, and glycerol (Table 1). BDP treatment effectively caused an increase in the gluconeogenic capacity of liver slices, and because BDP caused ketonemia (22), it seems that ketonemia in vivo does not inhibit, and may even stimulate, in vitro gluconeogenesis. Whether ketonemia affects in vivo hepatic gluconeogenesis is not known, but reports (17, 18) indicate that glucose irreversible loss is not affected during the early stages of spontaneous ketosis when cows exhibit normal feeding behavior. The BDP-induced increases of substrate incorporation into glucose
cannot be attributed solely to increases in PC and PEPCK activities because incorporation of glycerol into glucose increased also during BDP treatment. BDP also increased oxidation of glucogenic substrates by liver slices (Table 1), suggesting that ketonemia-glucosuria not only affected gluconeogenic enzymes, but also affected enzymes involved in the tricarboxylic acid cycle.

It seems that the maximal effects of BDP treatment on in vitro hepatic gluconeogenic and ketogenic capacities occur within 14 days and that continuation of BDP treatment does not promote greater changes. Data presented in (22) indicate that changes in blood and liver metabolite concentrations were maximal by day 14 of BDP treatment.

Incorporation of substrates into glucose and oxidation of gluconeogenic substrates to CO₂ were increased markedly by fasting (Table 1), suggesting that decreased supply of dietary gluconeogenic substrates in vivo either directly or indirectly caused hepatic enzyme activities to increase. As with BDP treatment, it seems that fasting not only increased capacities of gluconeogenic enzymes, but also increased capacities of tricarboxylic acid cycle enzymes, as evidenced by greater rates of substrate oxidation. Therefore, the fasting-induced
increases in hepatic metabolic rates appear to be general.

The absence of Ca\textsuperscript{++} in incubation media eclipsed the BDP- and fasting-induced increases in hepatic glucoseogenesis (Table 2), thereby suggesting that Ca\textsuperscript{++} may either directly or indirectly affect hepatic glucoseogenesis in ruminants. It is possible that Ca\textsuperscript{++} may be required for increases in activities of gluconeogenic enzymes to occur, or that Ca\textsuperscript{++} may allow greater entry of substrates into hepatocytes for glucoseogenesis. Further studies may determine whether the Ca\textsuperscript{++} effects are an artifact of in vitro tissue incubations or are of any physiological significance.

Stearate-BSA effectively inhibited incorporation of propionate and lactate into glucose (Table 2) and oxidation of these substrates to CO\textsubscript{2} (Table 3) by liver slices prepared from control and BDP-treated steers. As with BDP treatment and fasting, it seems that the inhibitory effects of stearate-BSA were general, rather than enzyme-specific. The effects of stearate-BSA additions in vitro may be of physiological significance, because FFA concentrations in plasma of ketotic cows often exceed 1 mM and may be at least partly responsible for the decrease seen in glucose irreversible loss during the advanced
stages of spontaneous ketosis. But it is possible also that effects of protein-bound LCFA on gluconeogenesis and substrate oxidation in vitro can be attributed entirely to the unphysiological conditions associated with in vitro tissue incubations. The lack of stearate-induced inhibition of gluconeogenesis and substrate oxidation in liver slices prepared from fasted steers is in contrast to observations made during the control and BDP periods. It is possible that the mechanisms responsible for the fasting-induced increases in gluconeogenesis negated the inhibitory effects of stearate-BSA.

As discussed in (22), other than causing ketonemia-ketonuria, it seems that 1,3-butanediol has no adverse effects on metabolism in cows. But the physiological effects of phlorizin may not be limited to its effects on transport of glucose across cell membranes. Phlorizin has been shown to inhibit activity of glucose-6-phosphatase in rabbit intestine (21). Zanobini et al. (39) reported that P did not inhibit activities of purified, commercially prepared enzymes involved in glucose metabolism in vitro; however, P did inhibit the enzymes in rat kidney in vivo. Results from our laboratory, however, do not indicate that treatment in vivo with P causes inhibition of gluconeogenic enzymes. When liver
slices prepared from P-treated steers were incubated with gluconeogenic substrates, rates of incorporation of substrates into glucose tended to increase (28). Data presented in Table 1 indicate that when P is given in combination with BD in vivo, rates of incorporation of substrate into glucose increased. Previous reports (23, 28) and data presented here suggest that 1,3-butanediol and phlorizin, given at amounts reported herein, cause no physiological perturbations other than ketonemia, ketonuria, and glucosuria.
REFERENCES CITED


GENERAL SUMMARY

The primary objective of this dissertation project was to evaluate the efficacy of 1,3-butanediol and phlorizin for causing an induced ketosis in steers. Physiological effects of BDP treatment in steers were compared with reported effects of ketosis in lactating cows to determine if the physiological stress created by BDP treatment is as severe as that imposed by lactation.

In the first trial (Section I), when BD was fed for 14 days and P was injected during the last 7 days of BD feeding, glucosuria and ketonuria were evident, concentrations of glucose in plasma decreased, and concentrations of ketone bodies and FFA increased. The changes, however, were not as great as those occurring in lactating, ketotic cows. Treatment with BDP did not cause significant changes in concentrations of insulin, glucagon, or growth hormone in plasma (4) or in concentrations of glycogen and triglyceride in liver (6), which also indicated that the physiological stress in steers treated with BDP was not as great as that in lactating cows.

When steers received BDP, glucose IL, glucose pool size, and glucose space were greater than when steers received the control treatment. The increase in glucose
IL suggests that hepatic gluconeogenesis increased in response to urinary glucose excretion and that, under normal physiological conditions, gluconeogenic capacity of liver is not utilized fully in steers. The amount of glucose excreted in urine was similar to the increase in glucose IL during BDP treatment, indicating that steers maintained glucose homeostasis. Mills et al. (6) reported that in vitro gluconeogenic capacity of liver slices prepared from steers receiving BDP was greater than that in steers during the control period, which may explain how glucose IL increased in vivo.

Because physiological perturbations in steers receiving the 14-day BD, 7-day P treatment were not as great as those in cows during lactation ketosis, the length of treatment with both BD and P was increased to 28 days (Sections II and III) in an attempt to cause greater changes in blood and liver metabolite concentrations. In addition, effects of a 9-day fast (Sections II and III) on blood and liver metabolite concentrations and on glucose IL also were determined to see if the physiological stress of BDP treatment was comparable to that of fasting.

Treatment with BDP for 28 days did not cause changes in blood or liver metabolite concentrations or glucose IL greater than those caused by short-term BDP treatment,
suggesting that physiological adaptations to ketonemia-glucosuria in steers occurred rather rapidly (i.e., within 14 days) and that, despite glucosuria, glucose homeostasis was maintained. The physiological stress imposed by fasting was greater than that imposed by extended BDP treatment, as evidenced by lower concentrations of liver glycogen and higher concentrations of plasma FFA during fasting. Even after fasting, however, plasma glucose concentrations were still 85% of control values, but glucose IL was only 50% of control values. The slight decrease in plasma glucose during the marked decrease in glucose IL suggests that homeostatic mechanisms spared glucose from oxidation in order to maintain plasma glucose concentrations at physiological concentrations. It is likely that ketone bodies, FFA, and amino acids served as alternate energy sources during fasting.

In addition to determining in vivo effects of BDP treatment and fasting, effects on in vitro hepatic gluconeogenesis and ketogenesis also were determined. BDP treatment and fasting both caused greater rates of incorporation of propionate, lactate, alanine, and glycerol into glucose, and the increases for fasting were greater than those for BDP treatment. Mills et al. (6) reported
previously that, compared with control steers, in vitro hepatic gluconeogenic capacity was greater when steers received BDP or were fasted. The mechanisms by which hepatic gluconeogenesis increased in response to BDP and fasting are not known. It is possible that capacities of gluconeogenic enzymes were increased by BDP and fasting, however, Young et al. (7) reported that PEPCK activity in liver of cattle was not affected by fasting. Other reports have indicated that in vitro hepatic gluconeogenic capacity (1) and activities of PEPCK (2) and propionyl-CoA carboxylase (3) decreased in response to fasting in cattle. Therefore, it seems that effects of fasting on gluconeogenesis in ruminants are not known fully.

Rates of oxidation of butyrate and palmitate to BHBA were increased by BDP treatment, but fasting did not cause any changes. Butyrate was oxidized at a much greater rate than palmitate, suggesting that entry of substrates into mitochondria is selective. Mills (5) reported that neither BDP treatment nor fasting had an effect on rates of hepatic ketogenesis.

In ketotic cows, concentrations of ketone bodies and FFA in blood are elevated greatly above normal. The effects of ketone bodies and FFA on hepatic gluconeogenesis in ruminants are not known; therefore, both were added to in
vitro liver slice incubations to determine if hepatic gluconeogenic capacity was affected. In addition, effects of omission of Ca\(^{++}\) from incubation media on hepatic gluconeogenesis were determined also.

In vitro additions of ACAC (5 mM) and BHBA (5 mM) did not significantly affect gluconeogenic capacity of liver slices prepared from steers receiving the control, BDP, or fasting treatments. Therefore, it is possible that ketonemia (ketone bodies) during ketosis does not directly affect hepatic gluconeogenesis in vivo. In vitro additions of stearate (1 mM) bound to bovine serum albumin greatly reduced gluconeogenic capacity of liver when steers received the control or BDP treatments, but values tended to increase for liver slices from fasted steers. Whether LCFA affects gluconeogenesis in vivo in ruminants is not known and it is possible that the effects of LCFA reported in this study are artifacts of the in vitro system. The differential effect of LCFA on gluconeogenesis during fasting cannot be explained by results of this study.

When Ca\(^{++}\) was omitted from incubation media, gluconeogenesis from propionate and lactate was not affected when liver slices were from control steers. But during BDP treatment and fasting, omission of Ca\(^{++}\) caused large decreases in incorporation of substrates into glucose by
liver slices. Effects of Ca\(^{++}\) on in vitro hepatic gluconeogenic capacity in ruminants have not been reported previously, however, results of this study suggest that, under some conditions, Ca\(^{++}\) may play an important role in regulating gluconeogenesis.

Studies of the effects of lactation on intermediary metabolism have provided researchers with a basic understanding of the etiology of ketosis, however, the effects of ketonemia, hypoglycemia, and elevated plasma FFA during ketosis on intermediary metabolism are not known fully and further study is needed. Continued improvements in management, feeding, and breeding of dairy cattle will result in additional increases in milk production and, because high-producing cows are more susceptible to ketosis, the incidence of the disease likely will increase in the future.

Research in bovine lactation ketosis is difficult because of the lack of a predictable supply of ketotic cows. Just as the development of the alloxan-diabetic rat has allowed great advances in understanding human diabetes, development of a valid experimental ketosis model should allow for major advances in understanding lactation ketosis. Further attempts, therefore, should be made to develop a suitable ketosis model. Although the current BDP model of
ketosis in steers does not fully simulate the physiological conditions in the ketotic cow, it will still be of value in the study of intermediary metabolism in ruminants that are affected by ketonemia and glucosuria.

Results of this study created more questions than were answered, but this is a common characteristic of basic research. Further attempts should be made to improve the BDP ketosis model. Improvement possibly could be accomplished by using overconditioned steers abruptly changed to maintenance or submaintenance rations at the initiation of BDP treatment. Overconditioned cows are more susceptible to lactation ketosis; therefore, overconditioned steers may be more susceptible to experimental ketosis. Other suggestions for future research include:

1. Determine why omission of Ca\(^{++}\) from incubation media negated the BDP- and fasting-induced increases in hepatic gluconeogenesis. This may best be accomplished by using isolated perfused rat liver and by using isolated hepatocytes from ruminants. Furthermore, would in vitro additions of EDTA to perfusion and incubation media, with and without Ca\(^{++}\), cause more marked inhibition of hepatic gluconeogenesis?
2. Determine why liver slices obtained from steers during fasting showed differential responses to in vitro additions of stearate-BSA and defatted BSA. Is liver of fasted steers histologically similar to liver of fed steers?

Niacin supplementation seems to be beneficial in the prevention and treatment of lactation ketosis, supposedly by inhibiting lipolysis and by "enhancing carbohydrate metabolism". Therefore, investigations into the effects of supplemental niacin on kinetics of glucose metabolism in vivo and on hepatic gluconeogenic capacity in vitro may conclusively determine whether niacin increases hepatic gluconeogenesis. If so, then niacin should routinely be added to rations of dairy cows.
REFERENCES CITED


ACKNOWLEDGEMENTS

Appreciation is extended to Dr. J. W. Young for his guidance during my graduate program at Iowa State University and for his editorial comments during preparation of this dissertation. Dr. Young also is recognized for his invaluable assistance in preparation of manuscripts for publication.

Recognition and thanks are given to Dr. D. C. Beitz, Dr. A. D. McGilliard, Dr. J. R. Russell, Dr. A. H. Trenkle, and Dr. W. F. Wedin for serving as members of my graduate committee. Thanks are also given to Dr. D. R. Buxton for serving as a substitute member of my graduate committee.

Although too numerous to name individually, I must recognize the laboratory and farm help provided by work-study and part-time employees, without which, results of this research would not yet be known.

Thanks are given to my wife, Patty, for her support and understanding during my graduate program.

And last, but not least, appreciation is extended to the staff of Beyer Hall laundry room, for faithfully providing me with clean towels during my graduate program.
Table A1. Effects of in vivo treatments and in vitro additions on amount of glucose in incubation media

<table>
<thead>
<tr>
<th>Flask contents</th>
<th>Propionate</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BDP Control</td>
<td>(day 28)</td>
</tr>
<tr>
<td></td>
<td>BDP Control</td>
<td>(day 28)</td>
</tr>
<tr>
<td>KRB: without Ca++</td>
<td>341&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>400&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>with Ca++</td>
<td>210&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>543&lt;sup&gt;ac&lt;/sup&gt;y</td>
</tr>
<tr>
<td>KRB without Ca++ plus: Ketone bodies</td>
<td>400&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>424&lt;sup&gt;abc&lt;/sup&gt;x</td>
</tr>
<tr>
<td>ACAC</td>
<td>394&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>294&lt;sup&gt;b&lt;/sup&gt;y</td>
</tr>
<tr>
<td>BHBA</td>
<td>336&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>570&lt;sup&gt;c&lt;/sup&gt;y</td>
</tr>
<tr>
<td>Stearate-BSA</td>
<td>423&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>400&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ketone bodies plus stearate-BSA</td>
<td>242&lt;sup&gt;abcx&lt;/sup&gt;</td>
<td>469&lt;sup&gt;ac&lt;/sup&gt;y</td>
</tr>
<tr>
<td>BSA</td>
<td>457&lt;sup&gt;b&lt;/sup&gt;</td>
<td>420&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Octanoate</td>
<td>330&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>572&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>NAD&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Niacin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup> Means in columns with different superscript letters differ (P<.05).

<sup>x,y</sup> For each substrate, means in rows with different superscript letters differ (P<.05).
Table A2. Coefficients of correlation between amount of glucose in incubation media and hepatic gluconeogenic rate

<table>
<thead>
<tr>
<th>Substrate and in vitro addition&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Correlation</th>
<th>P&gt;R</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRO</td>
<td>.48</td>
<td>.0001</td>
</tr>
<tr>
<td>LAC</td>
<td>.23</td>
<td>.092</td>
</tr>
<tr>
<td>ALA</td>
<td>.39</td>
<td>.008</td>
</tr>
<tr>
<td>GLY</td>
<td>.91</td>
<td></td>
</tr>
<tr>
<td>PRO - no Ca&lt;sup&gt;++&lt;/sup&gt;</td>
<td>.63</td>
<td>.0001</td>
</tr>
<tr>
<td>PRO + KB</td>
<td>.77</td>
<td>.0001</td>
</tr>
<tr>
<td>PRO + ACAC</td>
<td>.72</td>
<td>.0001</td>
</tr>
<tr>
<td>PRO + BHBA</td>
<td>.68</td>
<td>.0001</td>
</tr>
<tr>
<td>PRO + stearate</td>
<td>.22</td>
<td>.195</td>
</tr>
<tr>
<td>PRO + KB + stearate</td>
<td>.40</td>
<td>.015</td>
</tr>
<tr>
<td>PRO + BSA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>-.02</td>
<td>.915</td>
</tr>
<tr>
<td>PRO + NAD&lt;sup&gt;+&lt;/sup&gt;</td>
<td>.75</td>
<td>.008</td>
</tr>
<tr>
<td>PRO + niacin</td>
<td>.77</td>
<td>.004</td>
</tr>
<tr>
<td>LAC - no Ca&lt;sup&gt;++&lt;/sup&gt;</td>
<td>.19</td>
<td>.428</td>
</tr>
<tr>
<td>LAC + KB</td>
<td>.47</td>
<td>.005</td>
</tr>
<tr>
<td>LAC + stearate</td>
<td>.67</td>
<td>.0001</td>
</tr>
<tr>
<td>LAC + KB + stearate</td>
<td>-.26</td>
<td>.189</td>
</tr>
</tbody>
</table>

<sup>a</sup>PRO = propionate, LAC = lactate, ALA = alanine, GLY = glycerol, KB = ACAC (5 mM) + BHBA (5 mM), BSA = defatted bovine serum albumin (3% final concentration).
Table A3. Coefficients of correlation between in vitro hepatic substrate oxidation rate and gluconeogenic rate

<table>
<thead>
<tr>
<th>Substrate and in vitro addition&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Correlation</th>
<th>P&gt;R</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRO</td>
<td>.89</td>
<td>.0001</td>
</tr>
<tr>
<td>LAC</td>
<td>.84</td>
<td>.0001</td>
</tr>
<tr>
<td>ALA</td>
<td>.92</td>
<td>.0001</td>
</tr>
<tr>
<td>GLY</td>
<td>.74</td>
<td>.0001</td>
</tr>
<tr>
<td>PRO - No Ca&lt;sup&gt;++&lt;/sup&gt;</td>
<td>.75</td>
<td>.0001</td>
</tr>
<tr>
<td>PRO + KB</td>
<td>.75</td>
<td>.0001</td>
</tr>
<tr>
<td>PRO + ACAC</td>
<td>.72</td>
<td>.0001</td>
</tr>
<tr>
<td>PRO + BHBA</td>
<td>.89</td>
<td>.0001</td>
</tr>
<tr>
<td>PRO + stearate</td>
<td>.90</td>
<td>.0001</td>
</tr>
<tr>
<td>PRO + KB + stearate</td>
<td>.96</td>
<td>.0001</td>
</tr>
<tr>
<td>PRO + BSA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>.82</td>
<td>.0001</td>
</tr>
<tr>
<td>PRO + NAD&lt;sup&gt;a&lt;/sup&gt;</td>
<td>.85</td>
<td>.002</td>
</tr>
<tr>
<td>PRO + niacin&lt;sup&gt;a&lt;/sup&gt;</td>
<td>.99</td>
<td>.0001</td>
</tr>
<tr>
<td>LAC - no Ca&lt;sup&gt;+&lt;/sup&gt;</td>
<td>.93</td>
<td>.02</td>
</tr>
<tr>
<td>LAC + KB</td>
<td>.95</td>
<td>.0001</td>
</tr>
<tr>
<td>LAC + stearate</td>
<td>.96</td>
<td>.0001</td>
</tr>
<tr>
<td>LAC + KB + stearate</td>
<td>.97</td>
<td>.0001</td>
</tr>
</tbody>
</table>

<sup>a</sup>Refer to Table A2 for explanation of abbreviations.
ASSAY OF LOW CONCENTRATIONS OF LONG-CHAIN FATTY ACIDS IN PLASMA BY FORMING $^{63}\text{Ni}$ SALTS
The section presented herein, "Assay Of Low Concentrations Of Long-Chain Fatty Acids in Plasma By Forming $^{63}$Ni Salts", summarizes my attempts to improve the original procedure published by Ho (14) titled "Radiochemical Assay Of Long-Chain Fatty Acids Using $^{63}$Ni As Tracer". After many months of testing, it was decided that the modified radiochemical assay of FFA was reliable, and the procedure was used to determine FFA concentrations in plasma in Section I of this dissertation.

Originally, my intention was to submit the modified radiochemical assay for publication. But after further use of the procedure, it was noted that precision of the method began to decrease. Additionally, in measuring plasma FFA concentrations, determinations using the $^{63}$Ni assay were compared with determinations using the enzymatic method of Shimizu et al. (27) titled "Enzymatic micro-determination of serum free fatty acids". Correlation between the two procedures was low ($r = .5$) and, because the precision of the enzymatic method was much greater than that of the $^{63}$Ni assay, it was decided that the modified $^{63}$Ni assay was not worthy of publication. The preliminary manuscript, however, was included in this dissertation to permanently document my attempts to improve the assay.
INTRODUCTION

Ayer (1) reported that Cu, Ni and Co formed chloroform-soluble salts with long chain free fatty acids (FFA). Colorimetric methods (8, 11, 16, 17, 18, 19, 20, 24, 26) for measuring FFA are based on the reaction of diethylthiocarbamate or diphenylcarbazide with Co or Cu bound to FFA in organic solution. Major problems involving colorimetric methods include low sensitivity and poor repeatability, due partially to contamination of the final extract with traces of Co or Cu from the aqueous reagent. In 1969, Ho and Meng (15) reported a new method for the quantitative measurement of FFA involving a radiochemical assay of $^{60}$Co-FFA complexes, giving a direct measurement of the molar quantity of FFA in plasma or incubation media. The recovery of $^{60}$Co-FFA complex in the labeling solution was 100% for FFA with 14 or more carbon atoms. The FFA:Co molar ratio in the labeling solution was 2.05 indicating that one mole of Co bound approximately two moles of FFA. Lactic acid did not interfere with the assay but Ho and Meng (15) did not determine if this was the case for other plasma metabolites, such as β-hydroxybutyric acid or lecithin.

Ho (14) subsequently modified the $^{60}$Co radiochemical
assay by using $^{63}$Ni as the tracer. $^{63}$Ni is a weak beta emitter, is easily counted by liquid scintillation, and is safer to use than $^{60}$Co, a high-energy gamma emitter. Ho (14) used Dole's extraction mixture (7) to extract FFA from plasma. Lactic acid and tripalmitin did not interfere with the assay. However, lecithin and cephalin formed a chloroform-heptane soluble complex with Ni, thereby necessitating the separation of phospholipids from FFA in the extraction mixture. The FFA:Ni molar ratio in the organic phase following the labeling of stearic acid standards was 2.04.

Marinetti et al. (21) reported that phospholipids were first separated on a silicic acid column by D. M. Rathmann, who described the procedure in a thesis submitted to the University of Rochester in 1944. Since McCarthy and Duthie (22) reported that silicic acid binds phospholipids, it has routinely been used to separate FFA from phospholipid in organic extract of plasma or tissue samples (16, 20). Ho (14) modified the Dole and Meinertz (7) FFA extraction procedure by adding silicic acid to the organic extract of plasma to bind phospholipids. However, reports (3, 11, 14, 26) indicated that silicic acid not only binds phospholipids, but also binds FFA. Ho (14)
using \(^{14}\)C-palmitic acid, reported that with 200 mg silicic acid per assay, more than 60\% of the \(^{14}\)C radioactivity was not recovered from an unspecified volume of chloroform-heptane. When Ho used 5 mg silicic acid per 500 \(\mu l\) chloroform-heptane, FFA binding to silicic acid was non-significant. However, for plasma FFA determination, Ho used 5 mg silicic acid in 160 \(\mu l\) chloroform-heptane, a level which exceeded the silicic acid concentration used in the FFA binding test by more than 3 fold.

Itaya and Ui (18) used phosphate buffer to prevent coextraction of phospholipid and FFA from serum. They reported that lipid phosphorous extracted by chloroform in the presence of phosphate buffer was less than 1.3\% of the lipid phosphorous extracted by methanol-chloroform. Other reports (11, 12, 24) indicated that phosphate buffer effectively prevented coextraction of phospholipids and FFA from plasma or serum. With colorimetric FFA procedures (4, 9, 19, 23, 28), coextraction of phospholipids and FFA from plasma is reportedly prevented by the aqueous Co or Cu reagent. Noma et al. (25) reported that only 1.7\% of chloroform-methanol extractable phospholipid was recovered in the organic phase when serum FFA was extracted in the presence of an aqueous Cu reagent.
DeBrabander and Verbeke (6) modified the radiochemical assay of Ho (14) by replacing the centrifugation step, following FFA labeling using an aqueous solution of $^{63}$Ni, with absorption of water using anhydrous sodium sulfate. Tracer in aqueous solution not bound to FFA was thereby removed from the organic phase. This modification caused an increased repeatability and a higher recovery of the FFA-Ni complex. However, this modification was tested only on solutions of stearic acid in heptane and not on extracts of plasma.

In this paper, we report on the reliability of the proposed extraction procedure and the $^{63}$Ni labeling procedure. We determined the extent of FFA binding to silicic acid and evaluated the efficacy of phosphate buffer to remove phospholipid from organic extracts of plasma. The effect of phosphate buffer pH on efficiency of extraction of FFA from plasma or bovine serum albumin (BSA) solutions was determined. We also determined if Ni formed a chloroform-heptane soluble complex with lactic, pyruvic, acetic and β-hydroxybutyric acids and with lecithin or cephalin. Finally, we observed the effects of Ni specific radioactivity (SRA) in the $^{63}$Ni working solution on the molar ratios of FFA and Ni existing as FFA:Ni complex by labeling standard solutions of stearic
acid with $^{63}\text{Ni}$ to determine if the FFA:Ni molar ratio reported by Ho (14) was constant.
MATERIALS AND METHODS

Solutions

EXTRACTION SOLUTION: 32 ml of chloroform was mixed with 65 ml of n-heptane and 3 ml of methanol.

PHOSPHATE BUFFER: This was prepared as described by Falholt et al. (11). The pH was adjusted to 6.4 using .5N NaOH solution.

LABELING SOLUTION: 55 ml of chloroform was mixed with 45 ml of n-heptane.

STOCK SOLUTIONS: (1) Saturated aqueous solution of sodium sulfate. (2) Saturated aqueous solution of potassium sulfate. (3) Nicklous nitrate stock solution: 0.2 mmole of nicklous nitrate and 0.8 ml of glacial acetic acid were dissolved in the sodium sulfate stock solution to a volume of 100 ml. (4) $^{63}$Ni primary solution: 2.0 mCi of $^{63}$Ni nitrate was diluted with nicklous nitrate stock solution to a volume of 10 ml (2μCi/10μl).

$^{63}$Ni WORKING SOLUTION: The working solution should be used within 24 hours after preparation. One ml of nicklous nitrate stock solution, 0.85 ml of saturated potassium sulfate solution, 150 μl
of triethanolamine, and 10 µl of $^{63}$Ni primary solution were mixed. 100 µl of this solution contains 100 nmoles Ni$^{2+}$ and 0.0995 µCi $^{63}$Ni (SRA=2209 DPM/nmole Ni). This solution was prepared according to Ho (14).

**STEARIC ACID STANDARD SOLUTIONS:** A 1 mM solution of stearic acid in heptane was prepared, and aliquots of this solution were diluted with heptane to give standard solutions ranging from .06 to .75 mM stearic acid.

**Procedure**

The FFA radiochemical assay consists of three separate stages: I. extraction of FFA from the sample, II. labeling the FFA extract with Ni, III. labeling the stearic acid standards with Ni. The extraction tubes were acid-washed before use. Plasma samples and stearic acid standards were assayed in triplicate.

I. Extraction of FFA from plasma or incubation media.

To a silanized screw-top culture tube (extraction tube) are added 50 µl of plasma or incubation media, and 4 ml of extraction solution. Tubes are vortexed rapidly for 2 minutes, allowed to stand for 10 minutes, and
vortexed slowly for 1 minute. If vortexed too vigorously, stubborn emulsions may form. Tubes are centrifuged at 4°C at 2500 RPM for 10 minutes to give complete separation of the aqueous and organic phases. An aliquot (2 ml suggested) of the upper organic phase is transferred to a silanized 13 x 100 mm disposable culture tube (labeling tube), and is evaporated to dryness under a stream of air. The extraction procedure also is performed using 50 μl aliquots of water (water blanks).

II. Labeling FFA from extracted sample with Ni.

To each labeling tube containing the FFA extract are added 1 ml labeling solution, and 100 μl $^{63}$Ni working solution. The tube is stoppered immediately and vortexed rapidly for 20 seconds, taking care not to allow contents to contact the rubber stopper. Approximately 1 g of anhydrous sodium sulfate is added, the tube is stoppered, vortexed for approximately 5 seconds, and allowed to stand until the solution clears, indicating that all $^{63}$Ni working solution has been absorbed by the sodium sulfate. A 250 μl aliquot of the labeling solution is transferred to a scintillation vial, and evaporated to dryness under a stream of air to avoid chemical quenching. Nine ml of EP-Readysolv scintillation fluid are added, and vials are counted in a liquid scintillation counter.
III. Labeling stearic acid standards with Ni

To silanized 13 x 100 mm disposable culture tubes are added 200 µl of stearic acid standard solutions. It was found that 3.0 to 200.0 nmoles FFA per assay provided a sufficient range for the standard curve. The standard solution aliquot is evaporated to dryness under a stream of air. Tube contents are labeled with Ni according to the procedure in stage II. It is essential that sample extracts and standards are labeled using the same $^{63}$Ni working solution.

A linear regression of counts per minute in the labeling solution versus nmoles FFA per assay is obtained from the standard solutions. Water blank counts are subtracted from sample counts and these values are factored according to the fraction of FFA extract dried for labeling, and nmoles FFA per assay are calculated from the standard curve. FFA per assay is factored to give FFA per liter of plasma (e.g., 15 nmoles FFA per assay per 50 µl plasma = 300 nmoles FFA/ml plasma = 0.3 mM FFA in plasma). Standard solutions of stearic acid are not subjected to the sample extraction procedure because the FFA are not protein bound, and the extraction procedure, in this case, would simply be a dilution procedure. A zero stearic acid
standard should not be used because subtraction of water blank counts from sample counts corrects for background radioactivity associated with the labeling procedure.
RESULTS AND DISCUSSION

To determine if a linear response could be obtained using the proposed procedure, cow plasma, with volumes ranging from 10 to 600 μl per assay, was extracted and labeled with Ni. The relationship between volume of plasma per assay and amount of Ni recovered in the labeling solution was linear (Table A4). Plasma FFA concentrations calculated from these data were essentially the same regardless of the volume of plasma extracted, except for the assays using 10 μl of plasma (Table A5). Results indicate that the assay, using a $^{63}$Ni working solution with a SRA of 1120 CPM per nmole Ni, was not sensitive enough to allow accurate determination of less than 2.5 nmoles of FFA per assay. However, assay sensitivity can be increased if Ni SRA in the working solution is increased (14).

Table A4. Amount of Ni recovered in labeling solution as affected by volume of plasma per assay

<table>
<thead>
<tr>
<th>Ni recovered (nmoles)</th>
<th>Volume of plasma (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>.3</td>
<td>10</td>
</tr>
<tr>
<td>1.0</td>
<td>25</td>
</tr>
<tr>
<td>1.7</td>
<td>50</td>
</tr>
<tr>
<td>3.0</td>
<td>100</td>
</tr>
<tr>
<td>6.6</td>
<td>200</td>
</tr>
<tr>
<td>11.6</td>
<td>400</td>
</tr>
<tr>
<td>16.5</td>
<td>600</td>
</tr>
</tbody>
</table>
Table A5. Effect of sample volume on FFA determinations

<table>
<thead>
<tr>
<th>Plasma volume (µl)</th>
<th>FFA per assay (nmoles)</th>
<th>Plasma FFA concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>.70</td>
<td>.060</td>
</tr>
<tr>
<td>25</td>
<td>2.5</td>
<td>.101</td>
</tr>
<tr>
<td>50</td>
<td>5.3</td>
<td>.106</td>
</tr>
<tr>
<td>100</td>
<td>9.5</td>
<td>.095</td>
</tr>
<tr>
<td>200</td>
<td>22.5</td>
<td>.113</td>
</tr>
<tr>
<td>400</td>
<td>39.8</td>
<td>.100</td>
</tr>
<tr>
<td>600</td>
<td>57.1</td>
<td>.095</td>
</tr>
</tbody>
</table>

*Calculated from standard curve of stearic acid (0 to 100 nmoles per assay).

To determine if silicic acid binds FFA, solutions of [U-14C] stearate conjugated to BSA (10) were extracted using the mixture of Dole and Meinertz (7). Two ml of the heptane layer was transferred to tubes containing 0, 50, 100 or 200 mg of silicic acid and then two ml of chloroform were added. Tubes were vortexed, centrifuged, and an aliquot of the silicic acid-treated organic solution was counted. The recovery of 14C-stearate from the organic solution decreased as the amount of silicic acid increased (Table A6).

Table A6. Amount of 14C-stearate recovered from labeling solution as affected by silicic acid

<table>
<thead>
<tr>
<th>14C-stearate recovered (%)</th>
<th>Silicic acid per assay (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>0</td>
</tr>
<tr>
<td>87</td>
<td>50</td>
</tr>
<tr>
<td>82</td>
<td>100</td>
</tr>
<tr>
<td>71</td>
<td>200</td>
</tr>
</tbody>
</table>
The efficiency and linearity of extraction using the proposed extraction solution, Dole's extraction solution (7), and Dole's extraction solution with two levels of silicic acid (Ho's extraction procedure) was tested using \(^{14}\)C-stearate conjugated to BSA. Results are in Table A7. Using the proposed extraction solution, recovery of \(^{14}\)C-stearate was linear over the range of volumes extracted and recovery was at least 93% of initial radioactivity. However, recovery of \(^{14}\)C-stearate decreased as sample volume increased. Dole's extraction solution produced lower recoveries of \(^{14}\)C-stearate than the proposed extraction solution. When silicic acid, at a concentration of 12.5 mg per ml chloroform-heptane, was used with Dole's extraction solution, recovery of \(^{14}\)C-stearate decreased, except for the 25 \(\mu\)l aliquot. Recovery of \(^{14}\)C-stearate seemed to decrease as sample volume increased. When 37.5 mg of silicic acid per ml of chloroform-heptane was used following extraction with Dole's solution, recovery of \(^{14}\)C-stearate for all sample volumes decreased further. Ho (14) recommended using 5 mg of silicic acid per 160 \(\mu\)l of chloroform-heptane (31 mg per ml) to prevent coextraction of FFA and phospholipid from plasma. The data presented herein indicate that the proposed extraction
Table A7. Efficiency of extraction of $^{14}$C-stearate from BSA in aqueous solution as affected by extraction solution and silicic acid

<table>
<thead>
<tr>
<th>Extraction method</th>
<th>Volume $^{14}$C-Stearate-BSA solution per assay (μl)</th>
<th>25</th>
<th>50</th>
<th>75</th>
<th>100</th>
<th>125</th>
<th>150</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proposed solution</td>
<td>102 99 97 95 97 93</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dole's solution</td>
<td>94 94 93 92 93 91</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dole's solution plus:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 mg silicic acid</td>
<td>97 88 87 85 87 82</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150 mg silicic acid</td>
<td>90 88 83 80 82 81</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

solution gave complete or near complete extraction of $^{14}$C-stearate from BSA solutions. Recovery of $^{14}$C-stearate using Dole's extraction solution was lower than recoveries using the proposed extraction solution, but recovery was still acceptable. However, $^{14}$C-stearate recovery from chloroform-heptane containing silicic acid was markedly reduced, indicating that silicic acid should not be used to bind phospholipid when quantitative extraction of FFA is necessary.

The effects of pH of phosphate buffer on recovery of $^{14}$C-stearate from BSA solution and the relative recovery of Ni in organic extracts of plasma are in Table A8. The recovery of $^{14}$C-stearate from the extraction solution was
Table A8. Efficiency of extraction as affected by pH of phosphate buffer

<table>
<thead>
<tr>
<th>Phosphate buffer pH</th>
<th>Counts recovered (%)</th>
<th>Relative recovery of Ni in labeling solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>96</td>
<td>91</td>
</tr>
<tr>
<td>6.4</td>
<td>97</td>
<td>100</td>
</tr>
<tr>
<td>5.7</td>
<td>106</td>
<td>96</td>
</tr>
<tr>
<td>5.5</td>
<td>103</td>
<td>89</td>
</tr>
<tr>
<td>2.8</td>
<td>105</td>
<td>90</td>
</tr>
<tr>
<td>2.0</td>
<td>102</td>
<td>91</td>
</tr>
</tbody>
</table>

\(^a\)100 \mu l of \(^{14}C\)-stearic acid-BSA complex was extracted with 4 ml CHM, using 3 ml phosphate buffer (initial counts = 3990 ± 229 CPM).

\(^b\)50 \mu l of steer plasma was extracted with 4 ml CHM, using 3 ml phosphate buffer. Values are relative to pH 6.4 phosphate buffer.

not markedly affected by pH of the phosphate buffer but recovery seemed to be lower when pH 7.0 and 6.4 buffers were used. Error in pipetting probably accounts for recoveries greater than 100%. Itaya and Ui (18) reported maximal extraction of FFA from BSA solution when the pH of the phosphate buffer was between 6 and 7. The amount of Ni recovered in organic extracts of plasma is an indication of the amount of FFA extracted from a sample. Ni recovery was maximum when pH 6.4 phosphate buffer was used (Table A8). At lower and higher pH, Ni recovery was slightly reduced, indicating lower FFA extraction
from plasma. Itaya and Ui (18) reported similar but more marked reductions in efficiency of FFA extraction from plasma when phosphate buffer pH was lower or greater than 6.2.

To determine if FFA was quantitatively recovered following extraction of the sample, pipetting the organic extract, and drying the labeling solution, plasma plus known amounts of stearic acid was extracted and labeled. Values for recovery of added stearic acid from the extraction solution are in Table A9. Results indicate that recovery of stearic acid following the extraction and labeling procedures was quantitative.

To determine if lactic, pyruvic, \( \beta \)-hydroxybutyric or acetic acids and if lecithin or cephalin formed a chloroform-heptane soluble complex with Ni, 100 \( \mu \)l of \( ^{63}\)Ni working solution were added to standard solutions of each compound. After vortexing, sodium sulfate was added to tubes to remove the aqueous Ni phase. Aliquots of labeling solution were counted. Results are in Table A10. Using water as a zero or background reference, physiological or excess amounts of \( \beta \)-hydroxybutyric, acetic and pyruvic acids did not form a chloroform-heptane soluble complex with Ni. Physiological amounts of lactic acid did not bind Ni, but excess lactic acid bound slightly
Table A9. Recovery of stearic acid additions from steer plasma

<table>
<thead>
<tr>
<th>Assay contents</th>
<th>FFA per assay (nmoles)</th>
<th>Stearic acid recovered (nmoles)</th>
<th>Recovery of added stearic acid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 µl plasma</td>
<td>15.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µl plasma plus:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>49.6 nmoles stearic acid</td>
<td>67.9</td>
<td>52.3</td>
<td>105.4</td>
</tr>
<tr>
<td>99.2 nmoles stearic acid</td>
<td>115.6</td>
<td>100</td>
<td>100.8</td>
</tr>
<tr>
<td>198.4 nmoles stearic acid</td>
<td>218.9</td>
<td>203.3</td>
<td>102.5</td>
</tr>
</tbody>
</table>

aStearic acid in heptane was added to extraction tubes, evaporated, 50 µl of plasma from a phlorizin-treated steer was added, and the contents were extracted and labeled.

greater amounts of Ni. Levels of lactic acid this high are not likely to be encountered, even following strenuous exercise in humans or during periods of lactic acidosis in ruminants. Lecithin did not bind significant amounts of Ni when present in subnormal, physiological, or excess amounts. Chlouverakis and Hojnicki (3) reported that lecithin did not form a chloroform-heptane soluble complex with $^{60}$Co. Cephalin, at all levels tested, formed a chloroform-heptane soluble complex with Ni.
Table A10. Formation of chloroform-heptane soluble complexes with $^{63}$Ni by stearic acid and other metabolites

<table>
<thead>
<tr>
<th>Assay contents $^a$</th>
<th>Equivalent concentration in plasma $^b$ (mM)</th>
<th>Ni recovered in labeling solution (mnoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stearic acid (100 nmoles)</td>
<td>2.0</td>
<td>23.4</td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td>.8</td>
</tr>
<tr>
<td>BHBA: 20 nmoles</td>
<td>.4</td>
<td>.8</td>
</tr>
<tr>
<td>300 nmoles</td>
<td>6.0</td>
<td>.8</td>
</tr>
<tr>
<td>Acetate: 30 nmoles</td>
<td>.6</td>
<td>.8</td>
</tr>
<tr>
<td>150 nmoles</td>
<td>3.0</td>
<td>.8</td>
</tr>
<tr>
<td>Pyruvate: 10 nmoles</td>
<td>.2</td>
<td>.7</td>
</tr>
<tr>
<td>200 nmoles</td>
<td>4.0</td>
<td>.7</td>
</tr>
<tr>
<td>Lactate: 100 nmoles</td>
<td>2.0</td>
<td>.8</td>
</tr>
<tr>
<td>2000 nmoles</td>
<td>40.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Lecithin: 25 nmoles</td>
<td>.5</td>
<td>.8</td>
</tr>
<tr>
<td>50 nmoles</td>
<td>1.0</td>
<td>1.3</td>
</tr>
<tr>
<td>100 nmoles</td>
<td>2.0</td>
<td>1.3</td>
</tr>
<tr>
<td>200 nmoles</td>
<td>4.0</td>
<td>.8</td>
</tr>
<tr>
<td>Cephalin: 25 nmoles</td>
<td>.5</td>
<td>8.9</td>
</tr>
<tr>
<td>50 nmoles</td>
<td>1.0</td>
<td>12.5</td>
</tr>
<tr>
<td>100 nmoles</td>
<td>2.0</td>
<td>13.9</td>
</tr>
<tr>
<td>200 nmoles</td>
<td>4.0</td>
<td>13.5</td>
</tr>
</tbody>
</table>

$^a$Assay contents were not extracted or washed with phosphate buffer prior to labeling.

$^b$If 50 µl of plasma is used per assay, amounts per assay range from normal physiological levels to levels far in excess of physiological.
Total plasma phospholipid concentrations average 2.2 mM in humans (29), 1.25 mM in cows (2), and 0.7 mM in sheep (13). In ruminants and non-ruminants, 80% of the total lipid phosphorus in plasma is in lecithin (plus lyssolecithin). Cephalin accounts for 2% of the total lipid phosphorous in non-ruminants, 2.4% in sheep, and is not detectable in plasma of cows (5). Amounts of cephalin used in this experiment greatly exceeded physiological amounts in plasma. The results of the Ni binding experiment indicate that lactic, β-hydroxybutyric, acetic, and pyruvic acids do not interfere with the assay. We observed that, following addition of 100 µl of $^{63}$Ni working solution, lecithin and cephalin solutions remained cloudy long after addition of sodium sulfate, indicating that the solubility of the aqueous $^{63}$Ni working solution in the labeling solution was enhanced by these phospholipids. If the labeling solution is not allowed enough time to clear following addition of sodium sulfate, erroneously high $^{63}$Ni counts will be obtained due to solubilized Ni, rather than phospholipid-bound Ni, in the labeling solution. It was observed that the aqueous $^{63}$Ni working solution could not be separated from labeling solution containing lecithin or cephalin by centrifugation.
The effects of lactic, β-hydroxybutyric, acetic, and pyruvic acids and lecithin on the amount of Ni recovered in the labeling solution in the presence of stearic acid following dilution with the extraction solution and washing with phosphate buffer are in Table A11. The effect of cephalin on Ni recovery was not examined because it usually is absent, or is very low in concentration in plasma. Using the amount of Ni bound by 100 nmoles of stearic acid as a reference, results indicate that, with one exception, the amount of Ni recovered in the organic phase did not increase by more than 3.9% in response to the addition of these plasma metabolites. When 200 nmoles lecithin was added to each assay, Ni recovery was 12% greater than when no lecithin was added. However, this quantity of lecithin is greater than quantities found in 50 μl of plasma.

To determine if lecithin affected recovery of Ni in organic extracts of plasma, lecithin was added to plasma, extracted, washed with phosphate buffer, and labeled with $^{63}$Ni. Results are in Table A12. Using the amount of Ni recovered in the labeling solution of plasma extracts as a reference, 25 nmoles of lecithin per assay unexplainably caused a decrease in Ni recovery, but 50,
Table A11. Effect of metabolites on the amount of Ni recovered in the labeling solution in the presence of stearic acid

<table>
<thead>
<tr>
<th>Assay contents</th>
<th>Ni recovered in labeling solution (nmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stearic acid (100 nmoles)</td>
<td>23.5</td>
</tr>
<tr>
<td>Stearic acid (100 nmoles) plus:</td>
<td></td>
</tr>
<tr>
<td>Lactate - 100 nmoles</td>
<td>23.4</td>
</tr>
<tr>
<td>2000 nmoles</td>
<td>24.3</td>
</tr>
<tr>
<td>BHBA - 20 nmoles</td>
<td>23.7</td>
</tr>
<tr>
<td>300 nmoles</td>
<td>23.2</td>
</tr>
<tr>
<td>Acetate - 30 nmoles</td>
<td>23.1</td>
</tr>
<tr>
<td>150 nmoles</td>
<td>23.1</td>
</tr>
<tr>
<td>Pyruvate - 10 nmoles</td>
<td>23.3</td>
</tr>
<tr>
<td>200 nmoles</td>
<td>23.6</td>
</tr>
<tr>
<td>Lecithin - 25 nmoles</td>
<td>23.2</td>
</tr>
<tr>
<td>50 nmoles</td>
<td>24.4</td>
</tr>
<tr>
<td>100 nmoles</td>
<td>23.5</td>
</tr>
<tr>
<td>200 nmoles</td>
<td>26.2</td>
</tr>
<tr>
<td>Water</td>
<td>.8</td>
</tr>
<tr>
<td>Chloroform</td>
<td>.8</td>
</tr>
</tbody>
</table>

*Samples were extracted with 4 ml of extraction solution and then washed with 3 ml of phosphate buffer.
Table A12. Effect of lecithin on amount of Ni recovered in labeled solutions of steer plasma extracts

<table>
<thead>
<tr>
<th>Assay contents</th>
<th>Ni recovered in labeling solution (nmoles)</th>
<th>Calculated concentration of FFA in plasma$^b$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma (50 µl)</td>
<td>5.4</td>
<td>.32</td>
</tr>
<tr>
<td>Plasma (50 µl) plus:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lecithin - 25 nmoles</td>
<td>5.0</td>
<td>.29</td>
</tr>
<tr>
<td>50 nmoles</td>
<td>5.9</td>
<td>.36</td>
</tr>
<tr>
<td>100 nmoles</td>
<td>5.6</td>
<td>.33</td>
</tr>
<tr>
<td>200 nmoles</td>
<td>5.7</td>
<td>.35</td>
</tr>
<tr>
<td>Water</td>
<td>.8</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Samples were extracted with 4 ml of extraction solution and then washed with 3 ml of phosphate buffer.

$^b$Values calculated by using net DPM in labeling solution.
100, and 200 nmoles of lecithin caused an increase in Ni recovery. Because lecithin did not form a complex with Ni (Table A10), it seems that the observed increase in Ni recovery is the result of a phospholipid-induced increase in the solubility of aqueous $^{63}$Ni working solution in the labeling solution, rather than the result of the formation of a chloroform-heptane soluble lecithin-Ni complex. Using radioactivity in the labeling solution and a standard curve to calculate plasma FFA concentrations, addition of 25 nmoles lecithin to plasma caused a 9.4% decrease in calculated FFA concentrations, but 50, 100, and 200 nmoles of lecithin caused a 12.5, 3.1, and 9.4% increase in values. Differences in calculated FFA concentrations seem to be random and may be partly accounted for by error associated with the assay. Furthermore, because the plasma aliquot already contained naturally occurring lecithin, any lecithin addition increased concentrations of phospholipid above normal physiological levels. As occurred with the lecithin and cephalin assays reported in Table A10, it was observed that additional phospholipid in plasma extracts caused the labeling solution to remain cloudy long after addition of sodium sulfate. This apparent phospholipid-induced increase in
solubility of aqueous $^{63}$Ni working solution in the labeling solution may have caused Ho (14) to conclude that phospholipids formed a chloroform-heptane soluble complex with Ni.

Ho (14) using 100 nmoles nicklous nitrate and 0.0995 μCi $^{63}$Ni (SRA = 2200 DPM/nmole Ni) to label 1 to 40 nmoles stearic acid, reported the FFA:Ni molar ratio in the labeling solution, following centrifugation to remove the aqueous Ni working solution, was 2.04, indicating that one mole of Ni bound approximately two moles of FFA. DeBrabander and Verbeke (6) using 1000 nmoles nicklous nitrate and 0.0995 μCi $^{63}$Ni (SRA = 220 DPM/nmole Ni) to label 40 to 400 nmoles stearic acid reported the FFA:Ni molar ratio in the labeling solution following centrifugation was 2.47 and the ratio following absorption of aqueous $^{63}$Ni working solution by sodium sulfate was 1.92. Ho (14) reported that because the FFA:Ni molar ratio in the labeling solution consistently approached 2.0, the FFA content of a sample could be calculated directly from the amount of Ni recovered in the labeling solution. The amount of Ni in the labeling solution existing as a FFA-Ni complex is easily calculated if the SRA of the $^{63}$Ni working solution is known. The effects of different Ni
specific radioactivities in the $^{63}\text{Ni}$ working solution on the FFA:Ni molar ratio in the labeling solution, following absorption of the aqueous phase by sodium sulfate were investigated to determine if the amount of Ni recovered in the labeling solution was an accurate indicator of the amount of FFA per assay. Zero, 6, 12, 25, 60, 99, and 198 nmoles of stearic acid were labeled with Ni using $^{63}\text{Ni}$ working solutions of varying SRA. Results are in Table A13. The SRA of the $^{63}\text{Ni}$ working solution markedly affected the FFA:Ni molar ratios in the labeling solution. As Ni SRA increased, the FFA:Ni molar ratio increased. Theoretically, a given quantity of FFA should bind a constant amount of Ni. But, as reported herein, when Ni SRA increased, the amount of Ni bound by each amount of stearic acid decreased. The rate of increase in Ni SRA was greater than the rate of decrease in the amount of Ni bound by a given quantity of FFA, resulting in a disproportionate shift in the amount of radioactive and non-radioactive Ni bound by FFA. In the present study, as opposed to the study of Ho (14), the FFA:Ni molar ratio in the labeling solution approached 2.0 only when Ni SRA was 215 DPM/nmole. However, our observations are in agreement with the observations of DeBrabander and
Verbeke (6). These results indicate that calculation of FFA quantity directly from the amount of Ni recovered in the labeling solution could lead to serious errors and that a standard curve should be used for FFA determinations.

Table A13. Molar ratio of FFA to Ni in labeling solution as affected by specific radioactivity of $^{63}$Ni working solution

<table>
<thead>
<tr>
<th>FFA per assay (nmole)</th>
<th>Specific radioactivity of $^{63}$Ni (DPM/nmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>81</td>
</tr>
<tr>
<td>0</td>
<td>8.5</td>
</tr>
<tr>
<td>6.2</td>
<td>12.1</td>
</tr>
<tr>
<td>12.4</td>
<td>18.7</td>
</tr>
<tr>
<td>24.8</td>
<td>22.9</td>
</tr>
<tr>
<td>49.6</td>
<td>37.0</td>
</tr>
<tr>
<td>99.2</td>
<td>66.8</td>
</tr>
<tr>
<td>198.4</td>
<td>128.8</td>
</tr>
</tbody>
</table>

| FFA:Ni ratio           | 1.66             | 1.94              | 2.31              | 2.72              | 3.68               | 6.02               |

In vivo studies of intermediary metabolism frequently require intravenous injections of isotopically labeled compounds. In our laboratory, for instance, $[^{14}C]$- and $[6^{-3}H]$glucose is given intravenously to cattle to measure kinetics of glucose metabolism. Since the proposed FFA procedure is a radiochemical assay, we wanted to determine
if any radioactivity originating from $^{14}$C- and $^3$H-labeled glucose in plasma was retained by the organic phase following the extraction procedure. The radioactivity in a plasma sample taken from a steer which had received an intravenous injection of $^{14}$C- and $^3$H-glucose was 117 DPM per 50 µl. Equal volumes of plasma were extracted, according to the proposed procedure, and the radioactivity in the organic phase following a phosphate buffer wash was 33 DPM. Background radiation was 30 DPM. These results indicate that 100% of the radioactivity originating from $^{14}$C- and $^3$H-labeled compounds in plasma was retained by the aqueous phase of the extraction mixture and did not contaminate the organic phase.

Within-run precision of the assay was determined using plasma from phlorizin-treated steers and a non-lactating cow. Results are in Table A14. Because the coefficient of variation was less than 10% (average C.V. = 6.5%) precision was acceptable, indicating that the assay was reliable.
<table>
<thead>
<tr>
<th>Number of observations</th>
<th>Mean FFA concentration in plasma (mM)</th>
<th>SD</th>
<th>SEM</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>.293</td>
<td>.026</td>
<td>.010</td>
<td>8.87</td>
</tr>
<tr>
<td>10</td>
<td>.326</td>
<td>.020</td>
<td>.006</td>
<td>6.13</td>
</tr>
<tr>
<td>5</td>
<td>.129</td>
<td>.014</td>
<td>.006</td>
<td>4.65</td>
</tr>
</tbody>
</table>
REFERENCES CITED


18 Itaya, K., and M. Ui. 1965. Colorimetric determination of free fatty acids in biological fluids. J. Lipid Res. 6:16.


