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Etiology of Fatty Liver of Dairy Cows

J. Young, E. Bogin, D. Beitz, A. McGillard

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Etiology of Fatty Liver of Dairy Cows

Investigator's Names

Investigator's Institutions

J. W. Young

Iowa State University

E. Bogin

Kimron Veterinary Institute

D. C. Beitz

Iowa State University

A. D. McGilliard

Iowa State University

Project's starting date: October 27, 1986

Type of Report: 1st Annual 2nd Annual Final x

Jerry W. Young
Signature

Principal Investigator

Michael E. Hasler
Signature Contract 5/Gents office,
7-23-90

Institution's Authorizing Official

Table of Contents

C.	Abstract	3
D.	Objectives of the original proposal	4
E.	Body	5
Section I:		
	Metabolites in Blood and Liver During Development and Early Treatment of Experimental Fatty Liver and Ketosis in Cows	6
Section II:		
	Fatty Liver and Ketosis are Not Induced by Either Feed Restriction or Dietary 1,3-Butanediol as Separate Treatments	17
Section III:		
	Subclinical Ketosis in Dairy Cows in Response to Feed Restriction and Dietary 1,3-Butanediol	32
Section IV:		
	Clinical Chemistry of Cows with Fatty Liver and Ketosis	51
F.	Description of Cooperation	61
G.	Evaluation of Research Achievements	61
H.	List of Publications	62

C. Abstract:

One experiment with high-producing cows showed that during a gradual experimental induction of fatty liver and ketosis, fatty liver was present for about 2 wk before clinical ketosis was diagnosed. Also, liver glycogen content was nearly zero for 2 wk before ketosis. Nonesterified fatty acids in blood increased when the induction protocol began but plateaued 2-3 wk before ketosis, whereas β -hydroxybutyrate increased continuously. One group of cows infused intraduodenally with 500 g of glucose/d did not develop fatty liver and ketosis. Another experiment showed clearly that feed restriction (i.e., energy restriction) as well as a source of ketone bodies (i.e., dietary 1,3-butanediol) are both necessary for the experimental induction of fatty liver and ketosis. A third experiment was designed to measure changes in metabolism, especially lipid metabolism, as fatty liver and ketosis develop. Total lipid, triglyceride and cholesterol increased in liver during induction, but the cows in this experiment only became subclinically ketotic in contrast to those in the two earlier experiments. Combined results from the three experiments give support to the idea that cows with a hepatic triglyceride-to-glycogen greater than about 2 are susceptible to fatty liver and eventual ketosis, whereas cows with a ratio below 2 are not susceptible. A fourth experiment showed that there is a strong relationship between fatty liver and the lipoprotein profile in blood. Determining the relative levels of LDL + VLDL in blood can be used to confirm the fatty liver situation. Further, cows with very low concentrations of HDL generally do not produce well and respond poorly to many treatments.

D. Objectives of the original proposal:

The specific objectives of the original proposal were to:

1. Evaluate the applicability of energy restriction plus 1,3-butanediol feeding as a model for fatty liver.
2. Characterize changes in lipid metabolism and gluconeogenesis during development of experimental fatty liver.
3. Determine criteria for detectability or diagnosis of fatty liver.
4. Characterize changes in lipid metabolism and gluconeogenesis during spontaneous fatty liver.

The basic objectives were not changed during the tenure of the project. The level of funding that was approved was not as great as that requested, however, which necessitated a decrease in the scope of measurements made under each objective. Under Objective 1 we deleted the starved group and worked with only a control and a FRBD group (20% feed restrictions plus dietary 1,3-butanediol). Detailed fatty acid composition of liver samples were not done, and blood plasma samples were not analyzed for lipoprotein concentrations or for enzymatic activities. For Objective 2, we did not take adipose tissue samples and evaluate fatty acid synthesis and its control. Further, total and esterified cholesterol, triglycerides, and very low density and very high density lipoprotein analyses were not done for plasma. Also plasma enzyme activities were not assayed. The major change for Objective 3 was that we did not use cows from 10 specific sire group, and we did not do liver biopsies on those cows. Under Objective 4 hepatocytes were not used because of the great difficulty in preparing them from puncture biopsy samples, and again we did not use 10 specific sire groups.

E. Body of the scientific report:

There are four sections to the main body of the final report. The format is that three manuscripts prepared for publication were shortened and included to give full details of the experiments. The abstract, introduction, some text, and all references were deleted, but tables and figures are included. These manuscripts form the first three sections of the report. The fourth section is a preliminary summary and evaluation of data that were obtained within the last few months of the project and are not yet prepared for publication. Numbering of tables and figures begins anew in each of the four sections.

SECTION I: Metabolites in Blood and Liver During Development and Early Treatment of Experimental Fatty Liver and Ketosis in Cows

The purpose of this experiment was to use our ketosis induction protocol to elucidate the developmental pattern of metabolic changes associated with ketosis. The experiment also was designed to test the effects of a continuous infusion of glucose into the duodenum during the time when ketosis was being induced. The experiment was designed to show specific metabolic changes at the interface between subclinical and clinical ketosis.

MATERIALS AND METHODS

Design of Experiment and Management of Cows

One Brown Swiss and 17 Holstein cows, averaging 595 kg BW at 4 d postpartum, were used in a completely randomized design. Six cows were assigned randomly to each of three treatments: control, a ketosis induction protocol (KIP), or a glucose-treated (GT) group. One Holstein cow did not respond to the ketosis induction protocol and was replaced to maintain equal numbers for the three treatments.

The experimental period for all cows began approximately 14 d before parturition and continued until 60 d postpartum for control and GT cows or two wk after ketosis detection for KIP cows. Cows were housed and fed hay ad libitum in a lot for dry, pregnant cows for 30 d before calving. After 4 d postpartum, all cows were housed in a tie-stall barn with other herd cows and fed for ad libitum intake a 60% forage, 40% concentrate diet. Fresh feed was given twice daily, between 900 and 1000 h and between 1600 and 1800 h. Long-stem alfalfa hay was fed before and after each pm milking and totaled approximately 6 kg/d. Orts were collected daily to determine exact feed intake. Control treatment consisted of ad libitum feeding throughout the experimental period.

KIP treatment consisted of cows gradually being induced into clinical ketosis by using our 20% feed restriction, 1,3-butanediol (FRBD) protocol, with the exception that cows were not overfed during the dry period. Specifically, at 2 wk postpartum, intake of silage plus grain mix was restricted to 80% of ad libitum intake of the previous 5 d and supplemented with the ketone body precursor, 1,3-butanediol (BD) at 4% of dry matter (DM) intake. At 18 d postpartum, BD supplementation was increased to 8% of DM intake. FRBD continued until ketosis was detected. Supplementation of BD was held at a constant percentage of DM intake after d 18 postpartum, which is in contrast to earlier work with FRBD. An average of 900 g BD/d was consumed, thereby resulting in 14.7% of the ME of the FRBD diet coming from BD.

Cows in the GT group were treated identically to those on KIP, with one exception. At day 23 of lactation, a small polyvinyl catheter was placed in the proximal duodenum about 20 cm caudal to the pylorus. Surgery was performed with cows standing upright and given Xylocaine along the right rib cage from the 9th to the 13th rib. An 18-cm incision was made at the 11th intercostal space in the region of the costochondral junction, blunt dissection was used to penetrate the peritoneal cavity, and the duodenum was externalized temporarily. An indwelling catheter was placed into the duodenum,

sutured to the duodenal surface by using an absorbent sponge for support, and externalized at the 12th intercostal space. The incision was closed with suture staples. Cows decreased feed intake for one feeding after surgery but returned to normal by the next morning. Milk production was not affected and inflammation at the incision site was minimal. At d 25 of lactation, tygon tubing was connected to the catheter and a peristaltic pump was used to infuse a dextrose solution (.35 g/ml) at $.96 \pm .03$ ml/min (i.e., 484 g/d) for 15 d. The infusion of glucose was intended to coincide with the time when subclinical and clinical ketosis would be detected for KIP. The FRBD protocol was continued for 7 d after the glucose infusion was terminated on d 39 postpartum. One infusion of glucose was terminated after 7 d because of hyperexcitability and nervousness of the cow. Two other cows showed extreme hyperexcitability during the last 3 d of glucose infusion.

Clinical ketosis was diagnosed by the Iowa State Veterinary Service at a mean of 44 d postpartum and within a range of 24 to 56 d postpartum for KIP cows. No GT cows became clinical ketotic. Diagnosis of ketosis was based upon the presence of high concentrations of ketone bodies in urine, altered behavior indicative of ketosis, decreased milk production, and further decreases in consumption of the FRBD diet. As soon as a cow was diagnosed as clinically ketotic, FRBD was terminated and she was returned to ad libitum intake. No further treatment was planned, but two cows required treatment with 500 ml of 50% dextrose intravenously when severe clinical ketosis was detected.

Sampling and Analytical Procedures

Milk production was recorded daily and cows were weighed on alternate days. An explanation of terminology and abbreviations, timing of sampling of blood and liver, and feeding protocols is outlined in Table 1.

TABLE 1. Summary of sampling blood and liver and feeding schedule for ketosis-induced (KIP) and glucose-treated (GT) cows¹.

Stage of experiment	Feeding protocol	Sampling time	Average day of sampling ²
Prepartum (PP)	Ad lib hay	Estimated 2 wk prepartum	-11 ± 1.5
Early postpartum (EPP)	Ad lib feeding of herd forage and grain mix plus 6 kg alfalfa hay	Between 4 and 7 d postpartum	5.5 ± .4
Pre-induction (PI)	Same as for EPP	One day before feed restriction and 1,3-butanediol feeding began	13 ± .3
Early induction (EI)	Restriction of feed intake to 80% of ad libitum; supplementation with 1,3-butanediol at 4% for 4 d and at 8% of DM intake thereafter (FRBD)	About 1 wk after beginning FRBD	21 ± .3
Mid-induction (MI)	Same as for EI	About 2 wk after beginning FRBD	28 ± .3
Late-induction (LI)	Same as for EI	About 3 wk after beginning FRBD	35 ± .4
Ketotic (K)	Same as for EI	Within 48 h before or after clinical ketosis was diagnosed	44 ± 5.8
Recovery (R)	Ad libitum feed intake. Same as for EPP	About 2 wk after diagnosis of clinical ketosis	60 ± 3.7

¹Sampling times were similar for control cows, however, ad libitum feeding was continued throughout the period for the controls.

²Parturition is the reference point. Means of sampling day ± SE for EPP, PI, EI, MI, and LI are from all 17 cows. Means of sampling day ± SE for K and R are for the five KIP cows and control and treated cows were sampled at similar times. Sampling days for one KIP cow were delayed because of an injury (29), and her sampling days are not included in means of sampling day.

A blood sample was taken approximately 2 wk prepartum from the jugular vein. After parturition, weekly blood samples from the jugular vein were collected into heparinized syringes containing 150 μ l of a 4% NaF solution, which acts as a glucose preservative. Samples were taken just before the morning feeding, and plasma was prepared and stored at -20°C . Plasma was assayed for concentrations of glucose, non-esterified fatty acids (NEFA), and acetate. Protein-free filtrates of plasma were prepared and assayed for β -hydroxybutyrate (BHBA).

Three to 4 g of liver were taken by puncture biopsy immediately after blood samplings. Approximately 1 g was frozen immediately at -20°C for later determination of glycogen and triglyceride content. The remaining liver was used within 1 h for in vitro incubations.

Frozen liver samples later were thawed to remove them from sample vials, dropped into liquid nitrogen, and pulverized by grinding with a mortar and pestle. One-half of each sample then was weighed and homogenized in five volumes by weight of 10% perchloric acid. The resulting suspension was used for determination of glycogen.

The remaining half of each sample was placed in 10 ml of a 2:1 chloroform:methanol solution. Samples were allowed to shake for at least 2 h, 4 ml H_2O was added, and samples were vortexed vigorously and centrifuged. After centrifugation the methanol-water layer and liver tissue were removed and the remaining chloroform layer was evaporated under nitrogen. The lipid extract then was brought into solution in 95% ethanol and assayed for triglycerides.

Statistical Analysis

Data were analyzed by using general linear models to compare stage differences within treatments and treatment differences at each stage of the experiment. A split-plot design (143 df) was used with treatments (2 df) as the main effect and with cows within treatment as error A (15 df), and with stage (7 df) and treatment by stage interaction (14 df) as subplots. One KIP cow only reached the subclinical ketosis stage, and data for the ketosis and recovery stages were not obtained. Also, three control cows were not sampled at 35 d postpartum. Least square means were calculated.

RESULTS

Milk Production, Dry Matter Intake, and Body Weight Loss

Averages for milk production, dry matter intake, and body weight loss are shown in Figure 1 for each treatment at each stage of the experiment. Milk production (Figure 1A) increased in all cows between d 5 and d 13 postpartum; differences were significant within KIP and GT ($P < .05$), but not within controls. Production of milk for both control and GT cows remained relatively constant for the remainder of the experiment. KIP cows

Figure 1. Milk production (A), dry matter intake (B), and body weight changes (C) for control cows, cows given the ketosis induction protocol (KIP), and cows given the glucose treatment (GT) daily. The * for significance at $P < .05$ refers to differences for a particular day for KIP or GT vs. control. Statistical differences relating to changes with time within a particular treatment are given when discussed in the text. Standard errors calculated individually for each day and averaged for control, KIP, and GT groups are: A (milk production) .84, 1.39, and 1.05; B (dry matter intake) 1.6, 1.6, and 1.5; C (body weight change) 21.7, 7.4, and 10.6.

gradually decreased in production during treatment but did not show a significant decrease ($P > .2$) when compared to controls until there was a major decrease at ketosis. This result further suggests that the FRBD protocol given to cows soon after parturition can establish conditions that are similar to on-farm ketosis. Values for milk production in KIP cows at d 35 were significantly less than those at d 13 ($P < .05$), but not from those at d 21 or d 28. These changes represent an average loss of only .3 kg of milk daily, emphasizing the subtle but significant losses in milk production that can occur during subclinical ketosis.

Data for dry matter intake data (Figure 1B) for KIP and GT cows reflect the feed restriction from d 14 through the ketotic stage at d 44. GT cows consistently ate all of the feed offered during the induction, but KIP cows showed a trend to decrease intake further at ketosis ($P < .15$), with three of the six KIP cows becoming anorexic.

Body weight changes (Figure 1C) indicate that endogenous nutrient stores were being used for energy in all three groups of cows, and the rate of body weight loss was similar up through d 28. From then through d 35 and d 44, however, KIP and GT cows lost weight rapidly. Even though GT cows continued to lose weight, milk production was maintained (Figure 1A), and they showed no outward signs of ketosis. The two-week recovery period, marked by a return to normal feed intake (Figure 1B), was sufficient to restore body weight to control values for both KIP and GT cows (Figure 1C). The body weight losses seen in KIP cows were more variable but not as severe as those seen earlier with cows overfed in the dry period.

Metabolites in Blood

Plasma glucose concentrations (Figure 2A) generally fluctuated more for control and KIP treatments than for the GT cows. In general, glucose decreased after calving in control cows, but was significantly decreased only at d 28 and d 35 ($P < .05$), possibly when cows were in the most negative energy balance. KIP cows had lower glucose concentrations compared to controls at d 13, but this also corresponded with higher milk production for KIP at d 13 (Figure 1A). Glucose concentration decreased significantly ($P < .05$) from d 13 and remained low at d 28 and d 44. Glucose concentration of KIP cows did not parallel the dramatic drop in milk production seen at ketosis. The increase at d 35 is unexplainable, but two cows had unusually high glucose values. After recovery, plasma glucose concentrations of KIP cows returned to values observed at d 5 and 13 but were significantly lower than controls ($P < .01$).

Figure 2. Concentrations in blood plasma of glucose (A), nonesterified fatty acids (B), β -hydroxybutyrate (C), and acetate (D) for control cows, cows given the ketosis induction protocol (KIP), and cows given the glucose treatment (GT). The * for significance at $P < .05$ refers to differences for a particular day for KIP or GT vs. control. Statistical differences relating to changes with time within a particular treatment are given when discussed in the text. Standard errors calculated individually for each day and averaged for control, KIP and GT groups are: A (glucose) 2.1, 3.6, and 3.7; B (NEFA) .10, .17, and .11; C (BHBA) 5.7, 4.1, and 2.2; D (acetate) .10, .49, .15.

Glucose concentrations in GT cows for d 5 and 13 were unexplainably higher than for KIP cows but were not significantly higher than controls. Whether this condition affected the treatment of GT cows is unknown. Glucose infusions, begun at d 25 postpartum, resulted in increases in plasma glucose over controls at d 35 ($P < .05$), and the elevated glucose concentrations persisted until d 44 even though glucose infusions were terminated at day 39. Two GT cows developed severe nervousness and hyperexcitability during the last wk of glucose infusion. These two cows later were shown to have glucose concentrations greater than 90 mg/dl.

Plasma NEFA concentrations give an indication of the interaction between the rate of lipolysis from adipose tissue and uptake of NEFA by liver. While NEFA concentrations for control and GT cows (Figure 2B) were relatively constant during the first 40 d postpartum, NEFA for KIP cows increased significantly at the beginning of ketosis induction (d 21) and remained elevated through ketosis at d 44, reflecting the need to mobilize body reserves after a decrease of caloric intake. Values for NEFA for KIP cows at d 21 and d 28 were significantly different ($P < .05$) from d 13. Values at d 13 had increased postpartum, but combined with low plasma glucose concentration, this suggests that lipid is being mobilized to maintain high milk production (Figure 1A). Values for GT cows reflect the trend of normalcy already shown for milk production and plasma glucose concentrations. NEFA values increased nonsignificantly at the beginning of FRBD, but decreased after the initiation of glucose infusions and remained low.

Concentrations of BHBA in plasma (Figure 2C) generally paralleled NEFA values for all treatments, with the exception that BHBA for KIP cows continued to increase from d 21 to d 35 rather than to decrease. For KIP cows, BHBA concentrations at d 13, 21, 28, and 35 were progressively significantly different from each other ($P < .05$), and at d 45 were different from d 60 and d 21. The results suggest that even though NEFA concentration increases and peaks early, BHBA concentration in plasma increases continually, peaking at about 1 wk before signs of clinical ketosis are detected. BHBA seems to exert a regulatory effect in plasma by inhibiting lipolysis of adipose tissue, and such regulation could explain the gradual decrease in NEFA concentration in the latter stages of ketosis.

Plasma acetate concentrations (Figure 2D) increased for the KIP group at d 28 and remained high until ketosis was detected. This seems to indicate production of acetate by the liver in response to increased ketogenesis. Acetate production in the liver is an alternative to ketogenesis and can provide an end product for incomplete metabolism of acetyl-CoA. The delay in acetate response compared with BHBA supports the idea that plasma acetate concentrations are increased during spontaneous ketosis but decreased during fasting ketosis. Plasma acetate concentrations of GT and control cows were constant throughout the experiment and were not different from each other.

Liver Composition

Effects of KIP and GT on liver composition are shown in Figure 3. For control cows, glycogen in liver (Figure 3A) decreased slightly after parturition and remained relatively stable thereafter. Glycogen content of liver decreased from d 5 to d 13 for KIP cows, but for unknown reasons it did not decrease for GT cows; in fact it increased. For KIP cows, a decreased glycogen content of liver probably reflects the severity of negative energy balance. Therefore, assuming GT cows are in negative energy balance, the cause of increased glycogen at d 13 before any dietary modifications were made is unclear. Glycogen content of the KIP cows decreased significantly at d 28, reached nearly zero by d 35, and then increased slightly by d 44. The timing of changes in glycogen for KIP cows was essentially opposite of changes in liver triglycerides (Figure 3B).

Figure 3. Concentrations of glycogen (A) and triglycerides (B) in liver of control cows, cows given the ketosis induction protocol (KIP), and cows given the glucose treatment (GT). The * for significance at $P < .05$ refers to differences for a particular day for KIP or GT vs. control. Statistical differences relating to changes with time within a particular treatment are given when discussed in the text. Standard errors calculated individually for each day and averaged for control, KIP, and GT groups are: A (glycogen) .47, .86, and .46; B (TG) .56, 1.00, and .33.

Triglycerides (Figure 3B) increased significantly for KIP cows at d 28 and remained steady through d 44, averaging nearly 10% of liver wet weight. Unlike other metabolites, liver triglycerides did not return to control values after a 2-wk recovery period, indicating a continuing inability to transport fat out of the liver. Liver triglycerides of GT cows were lower at d 5 and 13 than control or KIP cows and remained low throughout the experiment.

DISCUSSION

Inducing clinical ketosis in KIP cows by using the FRBD protocol supports earlier results. Studies with fasted cows and with spontaneously-ketotic cows have shown similar blood and liver metabolite profiles as seen at ketosis for our KIP cows. There seems to be several types of ketosis, each with a different underlying cause. The FRBD protocol established two specific experimental conditions. The first is involuntary feed restriction. Assuming that 1,3-butanediol was oxidized completely to CO_2 , the FRBD protocol resulted in a 9% decrease in energy intake, but a 20% decrease in protein intake. The second is mild hyperketonemia. Because lipids are mobilized for energy and mild fat accretion has been shown to occur in liver of some high-producing cows in early lactation, mild hyperketonemia seems to be within physiological normalcy for a cow in early lactation. Also, another study in our laboratory has shown that neither feed restriction nor 1,3-butanediol supplementation given separately is sufficient to induce ketosis. Therefore, interaction between negative energy balance and increased ketones in blood seems to be important in development of ketosis.

Previous work with FRBD suggested that changes in blood and liver metabolites occur before ketosis and lead to the symptoms of clinical ketosis. From Figures 2 and 3, we now can summarize for KIP cows that: a) plasma NEFA concentrations peaked 3 wk before ketosis and remained high, b) increases in BHBA in plasma began 3 wk before and peaked 1 wk before ketosis, c) liver glycogen content decreased 2 wk before and was nearly zero 1 wk before ketosis, and d) liver triglyceride content increased dramatically 2 wk before ketosis and remained high. These changes did not occur in GT cows given about 500 g glucose/d in the duodenum. Unfortunately, cows that were assigned randomly before calving to the KIP treatment tended to have higher milk production, lower plasma glucose, higher liver triglyceride content, and higher plasma NEFA concentration immediately after calving than control or GT cows. The differences for metabolites were not statistically significant, and it is assumed that these pretreatment tendencies do not affect the conclusions drawn from the results.

The changes in plasma NEFA and glucose concentrations after beginning KIP (Figure 2) suggest fat mobilization in response to an energy deficiency. The subsequent continual increase in BHBA concentration seems to indicate an increased ketogenesis by the liver to metabolize the free fatty acids, which also is implied by a gradual decrease in plasma NEFA concentration after d 21 (Figure 2B). Significant increases of plasma acetate (Figure 2D) and liver triglyceride (Figure 3B), however, do not occur until after d 28. Liver

triglyceride synthesis, however, was not increased during ketosis. These data suggest that the liver is utilizing alternate pathways to metabolize acetyl-CoA and that lipid transport is impaired, indicating the beginning of the disease state.

The onset of clinical ketosis at an average of d 44 was noted most by the drastic decrease in milk production (Figure 1A). Concentrations of metabolites in blood and liver did not change at ketosis from values 1 and 2 wk earlier. Thus, the data reveal no obvious trigger mechanism that marks the transition from subclinical to clinical ketosis. Understanding effects of ketosis development on liver function may provide valuable insights into the onset of clinical ketosis.

Two major differences can be noted between the present versus earlier results where a similar FRBD protocol was used, but the cows were overfed in the dry period. First, prepartum liver glycogen content of the present cows was about half that of the earlier cows. This most likely reflects differences between normal feeding versus overfeeding in the dry period. Second, both control and KIP cows increased liver triglycerides slightly in early postpartum in the present study. In the previous study, a 6-fold increase in liver triglycerides after calving was reported, which led to the conclusion that both a rapid decrease in glycogen and an increase in triglyceride content of liver are prerequisites for a cow to be susceptible to ketosis from the FRBD protocol.

Explanations for the beneficial effects of glucose infusion into the duodenum on preventing fatty liver and ketosis in GT cows center on two main effects: 1) providing glucose carbon for lactose synthesis in the mammary gland, and 2) increasing carbohydrate sufficiency or stimulating regulatory mechanisms to increase hepatic gluconeogenesis. The persistency of milk production in GT cows (Figure 1A) suggests that the duodenal glucose acted to reverse the effects of FRBD. At d 44, differences in milk production between the KIP and GT groups were 9.0 kg/d. Assuming that milk is 5% lactose, 450 g of glucose would be required to synthesize the extra milk if all other precursors were available. If 65% of the infused glucose was absorbed, a maximum of 340 g glucose/d would be available to the mammary gland. Further, it is generally estimated for low-producing cows that about 70% of the glucose available is used for milk lactose by the mammary gland, and infusions of glucose into the abomasum of healthy cows in negative energy balance did not increase milk production. Thus, it seems that milk production of GT cows was maintained by more than just glucose carbon supplied from the intestine.

Because both 1,3-butanediol and about 500 g of glucose/d were added, ME intake of GT cows was 95% of control cows. GT cows showed increases in both BHBA and NEFA after beginning FRBD and before glucose infusion, but the magnitude of response was different than for KIP cows (Figure 2C, 2D). These confounding data prevent specific conclusions from being drawn about the mechanism of action of duodenal glucose. However, four major differences in metabolism between KIP and GT cows were observed. First, glucose concentrations in plasma were much greater in GT cows. Second, BHBA

concentrations in plasma did not rise as in KIP cows, suggesting a decrease in hepatic ketogenesis or an increase in extrahepatic utilization of ketone bodies. Third, glycogen content of liver from GT cows remained at levels observed for control cows, whereas glycogen in liver of KIP cows was nearly depleted. Fourth, there was no increased accretion of triglyceride in liver of GT cows. Body weight loss (Figure 1C), however, did change similarly for KIP and GT cows, presumably indicating a loss of gut fill from feed restriction.

The net result of GT treatment in early-lactation cows was to establish a metabolic profile of cows with slightly elevated plasma BHBA concentration and no fatty liver. Clinical ketosis symptoms subsequently were not observed. Further work in elucidating the effects of duodenal glucose in cows already suffering from fatty liver would provide more insight on whether duodenal glucose reverses or prevents the severe effects of ketosis.

In summary, the use of FRBD for inducing ketosis resulted in an underfeeding, hyperketonemic model for ketosis and provided cows that developed subclinical ketosis and progressed into clinical ketosis. Increases in plasma NEFA and BHBA occurred shortly after FRBD began. Increases in liver triglyceride, decreases in liver glycogen, and increases in plasma acetate were seen 2 wk before clinical ketosis developed. No plasma or liver metabolites changed significantly at ketosis when compared with 1 and 2 wk before ketosis. Thus, fatty liver seemed to occur as a result of negative energy balance and hyperketonemia, but what actually triggers the onset of clinical ketosis is unclear. Infusion of about 500 g glucose/d into the duodenum of cows given the FRBD protocol resulted in a normal metabolic profile at the time when KIP cows were ketotic.

SECTION II: FATTY LIVER AND KETOSIS ARE NOT INDUCED BY EITHER FEED RESTRICTION OR DIETARY 1,3-BUTANEDIOL AS SEPARATE TREATMENTS

The objective of this experiment was to answer four questions. First, would either feed restriction or dietary butanediol separately, starting at 14 d postpartum, induce fatty liver or ketosis in dairy cows? Second, could fatty liver and ketosis be induced by combining feed restriction and butanediol at d 43 of lactation? Third, would feed restriction or butanediol separately cause a decrease of *in vitro* hepatic gluconeogenesis? Fourth, does butanediol directly affect metabolism of propionate by liver slices from normal or ketonemic cows?

MATERIALS AND METHODS

Cows, Treatments, and Management

Sixteen multiparous Holstein cows were assigned randomly at calving to three groups: control, FR (feed restriction), or BD (fed ad libitum plus butanediol). There were five, six, and five cows per group, respectively. Production of milk in the previous lactation (305-d mature equivalent) was 9465 kg (+676 kg over herdmates).

From parturition through d 13 postpartum, all cows were fed a silage and grain mixture for ad libitum intake, which continued throughout for control cows (Figure 1). The pretreatment stage was d 7 to 13 postpartum, or wk 0. For FR cows, intake was restricted at d 14 to 80% of the average ad libitum intake of the previous 5 d. Cows in group BD continued to be fed for ad libitum intake at d 14, but butanediol was included at 5.5% of DM. Cows in both groups FR and BD continued their respective separate treatments from wk 1 through wk 4 after beginning treatments. On d 43, butanediol was added to the ration at 7% of DM for cows in group FR, and feed for group BD was restricted to 80% of ad libitum intake with the amount of butanediol remaining constant. Both groups then were maintained on the combined treatment for wk 5 and 6.

Amounts of butanediol fed and degree of feed restriction were similar to those of a previous experiment in which they were given as a combination. Butanediol was introduced by feeding half of the final amount for 3 d and then beginning the full amount. Average daily intakes of butanediol were 885 g for group BD during wk 1 to 4 and 783 and 1000 g for groups FR and BD, respectively, during wk 5 to 6.

Cows had access as a group to a limited amount of alfalfa hay before each milking and individually after the p.m. milking. Estimated consumption of hay was 4 kg of DM daily. Cows were housed in tie stalls and fed silage and grain mixture twice daily, with orts weighed once daily. Cows were milked twice daily and were weighed every second day. Development of ketonemia was monitored by use of urine ketone test strips (Keto-Diastix^R, Ames Division, Miles Laboratories, Inc., Elkhart, IN).

Sampling Procedures

Puncture biopsies of about 5 g of liver were obtained under local anesthesia from all cows at four times: on d 13, 28, 42, and 56. One portion of liver was frozen and stored at -20° C. The remaining portion was placed immediately into an ice-cold solution of phosphate-buffered (.015 M) .9% NaCl (pH 7.4) and used within 1.5 h for in vitro metabolic studies.

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

Group:

Legend:

Ad libitum intake: Restricted intake:

Dietary 1,3-butanediol: -----

Figure 1. Schematic representation of dietary treatments

Incubation Procedures

Incubations were essentially as for earlier experiments. Briefly, liver tissue was sliced, blotted, weighed, and placed into 25-ml flasks that contained 3 ml of pH 7.4 Krebs-Henseleit bicarbonate buffer, .03% bovine serum albumin, 10 mM Na-propionate, 1 μ Ci Na-[2- 14 C]-propionate (ICN Biomedicals, Inc., Costa Mesa, CA), and 2 mM Ca^{++} . Gluconeogenic rates are maximized by physiological concentrations of Ca^{++} . To determine effects of butanediol on in vitro metabolism of propionate, flasks also contained either 10 mM 1,3-butanediol (Aldrich Chemical Co., Inc., Milwaukee, WI) or no addition. Flasks were gassed with a 95:5 mixture of O_2 : CO_2 and incubated in triplicate for 2 h at 37° C. Incubations were terminated by injecting .5 ml of 1.5 N H_2SO_4 . Radiolabelled CO_2 and glucose were measured.

Analytical Methods

Frozen liver was powdered under liquid N_2 , and a portion used for determination of glycogen and free glucose. Lipid was extracted from remaining powdered liver by shaking overnight in 2:1 (v:v) chloroform-methanol. The chloroform layer was filtered through glass wool and evaporated under moving air to determine total lipid gravimetrically. The dried lipid extract was dissolved in 3:2 (v:v) hexane-isopropanol, and concentration of triglyceride was determined by using a modification of a commercial kit (Sigma triglyceride kit no. 338, Sigma Chemical Co., St. Louis, MO). Sample plus reagent were incubated for 45 min at 37° C to evaporate the hexane and allow the reaction to go to completion.

Concentrations in plasma of β -hydroxybutyrate (BHBA), glucose, and acetate were determined. Concentration of nonesterified fatty acids (NEFA) was determined by using a modification of a commercial kit (NEFA-C kit, WAKO Chemical Co. USA, Dallas, TX) with the following modifications: Color reagents A and B and their respective diluents were combined with 16 and 33 ml of water, respectively. To 25 μ l of sample or standard were added .4 ml reagent A and .8 ml reagent B. The reaction was linear through 2000 μ equiv/l.

Concentrations of immunoreactive insulin and glucagon in plasma were measured by validated radioimmunoassays. Plasma for glucagon contained 2500 IU of aprotinin (Trasylol^R, Mobay Chemical Corp., FBA Pharmaceuticals, New York, NY) per ml. All samples for each hormone were assayed concurrently, with intraassay coefficients of variation of 8% and 12% for glucagon and insulin, respectively.

Contents of fat and protein in milk samples taken weekly at four consecutive milkings were determined (Milk-O-Scan 203, Foss Food Technology, Eden Prairie, MN), and FCM and SCM were calculated. The mixed ration was sampled monthly, and DM was determined by drying at 60° C for 2 d in a forced-air oven. Samples then were composited for the experiment, and contents of crude protein (Kjeldahl) and ADF were determined.

Statistical Analyses

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

To evaluate effects of time (weeks), data involving repeated measurements also were subjected to analysis of variance for a split-plot design by using GLM. Treatment groups were the main plot and were tested by using the error term of cows within groups. Times of sampling (weeks) were subplots and were tested against residual error, as was the treatment by time interaction. Conservative df were used to evaluate all F-tests containing time as a factor. Use of conservative df, which are obtained by dividing df for both terms of the F-test by df for time, compensates for lack of randomization of the subplots when using a split-plot design to analyze repeated-measures data. Conditional comparisons between treatments at each sampling time were evaluated by using Bonferroni t-tests.

Analysis of variance for in vitro propionate metabolism contained a third factor of type of addition (butanediol or none) to incubation media. Because interactions of incubation additions with treatment or time were not significant, mean rates of propionate metabolism by type of addition are presented.

RESULTS AND DISCUSSION

General Health of Cows

All five control cows were healthy throughout the experiment. Five cows assigned to FR showed no visual health problems, but the sixth cow developed ketosis only 4 d after initiation of feed restriction. A blood sample was obtained, and then she was given 500 g dextrose intravenously, 250 g dextrose subcutaneously, and 20 mg dexamethasone (Azium^R, Schering Veterinary, Kenilworth, NJ) intramuscularly. Recovery was rapid, and she was retained on experiment. Data for liver function and metabolites in plasma of this cow at time of ketosis are not included in statistical analyses but will be discussed separately.

Four cows in group BD remained healthy, but during wk 3, one cow went off-feed, which increased concentrations of NEFA and BHBA and decreased glucose in plasma. The cow recovered quickly and was retained on experiment, but data for that week were not used in statistical analyses.

Milk Production and Composition

Production of milk (Table 1) by FR and BD cows was less than that of controls

TABLE 1. Milk Production and Composition From Control Cows and Cows Feed-Restricted (FR) or Fed 1,3-Butanediol (BD) During the Separate-Treatment Period and Then Given Both Treatments During the Combination-Treatment Period.

	Separate-treatment period (wk 1-4) ¹					Combination-treatment period (wk 5-6) ²				
	Treatment group					Treatment group				
Item ³	Control	FR	BD	SE	Contrasts ⁴	Control	FR	BD	SE	Contrasts ⁴
Milk, kg/d	36.5	33.8	33.3	.6	A, .003	35.8	32.1	30.6	1.5	A, .03
Protein, %	2.9	3.0	3.2	.09	A, .12	2.8	3.2	3.2	.12	A, .02
Fat, %	3.3	3.6	3.6	.19		3.1	3.9	3.8	.21	A, .02
SCM, kg/d	33.4	32.0	32.1	.8		32.6	31.6	30.0	.8	A, .10 B, .19
FCM, kg/d	33.5	32.3	31.9	.8		32.8	31.8	29.9	.7	A, .05 B, .10

¹Weeks 1 through 4 of experiment. Means were corrected for covariate of week 0 (pretreatment).

²Weeks 5 and 6 of experiment. Means were corrected for covariate of week 0 (pretreatment).

Separate analyses of covariance were conducted for the individual-treatment and combination-treatment periods.

³Means of 4, 5, and 4 cows for control, FR, and BD, respectively, except milk production, which is mean of 5, 6, and 5 cows.

⁴Probabilities of greater F for orthogonal contrasts: A = control vs. FR plus BD; B = FR vs. BD.

during both wk 1 to 4 and wk 5 to 6 ($P < .003$ and $.03$, respectively). Production of milk was similar for cows in FR and BD, even though BD cows were fed ad libitum during wk 1 to 4. Yields of fat and protein were similar among groups, whereas percentages of fat and protein in milk from cows in FR and BD groups tended to increase (Table 1).

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

Intake and Body Weight Change

Intake of DM from the silage-grain mixture (Table 2) was not significantly different for cows in control and BD groups during wk 1 to 4 when both groups were fed for ad libitum intake, but was less for FR as designed. Intake averaged 84% of ad libitum for FR, which was slightly more than the desired 80% because of two cows that had very low pretreatment intakes and were restricted only to about 90% of pretreatment intake. Intake of ME by cows in group FR was estimated to be 83% of requirements at wk 0 (24). During wk 5 to 6, restricted intakes were similar for cows in FR and BD, with intakes for both groups less than that of controls.

Intake of hay was only estimated, and caution must be exercised, therefore, in interpretation of intake data. It seems, however, that cows in group BD consumed less DM as a percentage of body weight than did control cows (Table 2), which in part was attributable to cows in group BD being heavier at the start of the experiment (Table 2). The estimated decrease of DM intake by BD cows was sufficient to account for the decrease in milk production discussed already. Cows in group FR tended ($P < .10$) to have greater loss of weight than control cows during the first week of feed restriction (data not shown).

Metabolites and Hormones in Plasma

Average concentrations of glucose, NEFA, and acetate in plasma (Table 3) during wk 1 to 4 were not different among groups. Concentration of BHBA tended ($P < .12$) to be higher for cows in groups FR and BD than in control cows. During wk 5 to 6, glucose tended to be lower ($P < .12$) and BHBA higher ($P < .06$) in plasma of cows in groups FR and BD than in control cows.

Figure 2 shows weekly concentrations of NEFA, acetate, and BHBA. Concentrations of NEFA (Figure 2a) and acetate (Figure 2b) were greater at wk 1 in FR cows than in control cows ($P < .05$). Concentration of BHBA (Figure 2c) tended to be greater for FR cows compared with controls at wk 1 ($P < .10$) and wk 5 ($P < .10$), and greater for BD cows compared with controls at wk 1 ($P < .10$) and wk 6 ($P < .10$).

TABLE 2. Average daily intakes and body weight of control cows and cows feed-restricted (FR) or fed 1,3-butanediol (BD) during weeks 1 through 4 and then given both treatments during weeks 5 and 6¹.

Item and time	Treatment			SE
	Control	FR	BD	
Intake, silage-grain mixture, kg DM/d				
Week 0	16.7	14.6	15.8	2.4
Weeks 1 to 4	19.6 ^a	12.3 ^b	19.2 ^a	1.1
Weeks 5 and 6	18.5 ^a	12.5 ^b	15.1 ^b	1.1
Estimated total DM intake ² , % of body weight				
Week 0	3.6	3.4	3.2	.2
Weeks 1 to 4	4.2 ^a	3.1 ^c	3.8 ^b	.1
Weeks 5 and 6	4.0 ^a	3.1 ^b	3.2 ^b	.2
Body weight, kg				
Week 0	573 ^{a,b}	546 ^b	625 ^a	25
Week 4	567 ^b	529 ^b	614 ^a	16
Week 6	564 ^{a,b}	530 ^b	608 ^a	22
Body weight loss, kg/d				
Weeks 1 to 4	.22	.62	.36	.47
Weeks 5 and 6	.30	.12	.74	.89

¹Values are means resulting from separate analyses of variance conducted for each period. Means in a row with a common superscript are not different ($P > .05$).

²Actual intake of dry matter (DM) from forage-grain mixture plus estimated consumption of 4 kg DM from alfalfa hay.

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

Constituent	<u>Separate-treatment period (wk 1-4)¹</u>				<u>Combination-treatment period (wk 5-6)²</u>			
	<u>Treatment group</u>		SE	Contrasts ³	<u>Treatment group</u>		SE	Contrasts ³
	Control	FR	BD		Control	FR	BD	
Glucose, mg/dl	60.2	60.2	58.1	1.9	63.6	58.7	61.4	1.6 A,12
NEFA ⁴ , uequiv/l	389	494	347	85	409	305	411	108
Acetate, umol/l	891	941	818	104	846	792	700	63
BHBA ⁵ , mg/dl	4.7	6.5	8.8	1.4	4.4	7.4	6.6	1.0 A,06
Insulin, pg/ml	311	286	352	21	343	347	331	30
Glucagon, pg/ml	152	154	149	8	151	159	151	8
Insulin:glucagon, molar	1.3	1.2	1.4	.1	1.3	1.4	1.3	.2

¹Weeks 1 through 4 of experiment. Means were corrected for covariate of week 0 (pretreatment).

²Weeks 5 and 6 of experiment. Means were corrected for covariate of week 0 (pretreatment). Separate analyses of covariance were conducted for the individual-treatment and combination-treatment periods.

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

⁴Nonesterified fatty acids.

⁵ β -Hydroxybutyrate.

Figure 2. Concentrations of metabolites in plasma from control cows and cows feed-restricted (FR) or fed butanediol (BD) during weeks 1 through 4 and then given the combination during weeks 5 and 6. At any week, treatment means with the same letter are not different ($P > .1$). Week 0 is d 7 to 13 postpartum.

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

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

C. B-Hydroxybutyrate (SED = 2.0). Treatment, $P = .31$; week, $P = .31$; treatment x week, $P = .25$.

Insulin in plasma (Table 3) was greater ($P < .05$) for group BD than group FR during wk 1 to 4 but was not different among groups during wk 5 to 6. It is uncertain whether the difference during wk 1 to 4 is a result of decreased insulin in group FR or of increased insulin in group BD, but insulin tended ($P < .2$) to be greater in group BD than in controls.

Concentration of glucagon was similar among groups during both periods, resulting in a slightly higher, although nonsignificant, ratio of insulin to glucagon for cows in group BD during wk 1 to 4 (Table 3). Changes in hormones and metabolites in plasma in FR cows were similar to those caused by feed restriction in earlier studies, and indicate adaptive responses to the decrease in energy intake.

Liver Composition

When averaged over all groups, concentration of glycogen in liver increased significantly ($P < .05$) from wk 0 to wk 6 (data not shown). Average concentration of glycogen (Table 4) was less ($P < .05$) in cows in group FR compared with those in group BD during wk 1 to 4, but was similar among groups and essentially normal by wk 6. Concentration of free glucose in liver was not affected by treatment (Table 4).

Concentration of total lipid in liver decreased ($P < .004$) from wk 0 to wk 6 when averaged over all treatments (data not shown). Average total lipid in liver (Table 4) from cows in group BD tended to be greater ($P < .12$) during wk 1 to 4 and was significantly greater ($P < .005$) at wk 6 than for cows in group FR. This small increase in total lipid was not reflected in significant increases in triglyceride (Table 4); other lipid constituents were not determined.

For the cow that became ketotic after 4 d of feed restriction, contents of total lipid and triglyceride in liver were decreased slightly 2 d after ketosis compared with wk 0 (total lipid: 4.1 vs. 5.5% of wet weight; triglyceride: 1.1 vs. 2.2% of wet weight, respectively).

TABLE 4. Composition of liver and metabolism of propionate by liver slices from control cows and cows feed-restricted (FR) or fed 1,3-butanediol (BD) during the separate-treatment period and then given both treatments during the combination-treatment period.

Constituent	Separate-treatment period (wk 1-4) ¹				Combination-treatment period (wk 5-6) ²					
	Treatment group		SE	Contrasts ³	Treatment group		SE	Contrasts ³		
	Control	FR			Control	FR				
	--(% of liver wet weight)--				--(% of liver wet weight)--					
Glycogen	3.6	2.8	4.0	.3	B,.05	3.7	3.5	3.8	.5	
Glucose	.5	.4	.5	.04		.5	.4	.5	.04	
Total lipid	3.7	3.6	4.1	.2	B,.12	3.6	3.3	4.2	.2	B,.005
Triglyceride	.8	.7	.9	.1		.7	.5	.7	.1	
Product from propionate	$(\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{g}^{-1}\text{ liver wet wt.})$				$(\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{g}^{-1}\text{ liver wet wt.})$					
Glucose	10.2	9.8	9.1	1.2		8.7	9.6	6.9	1.1	B,.11
C0 ₂	.6	.6	.6	.07		.5	.5	.4	.08	
Glucose/C0 ₂	18.1	17.5	16.1	---	18.9	18.0	17.4	---		

¹Weeks 1 through 4 of experiment. Means were corrected for covariate of week 0 (pretreatment).

²Weeks 5 and 6 of experiment. Means were corrected for covariate of week 0 (pretreatment). Separate analyses of covariance were conducted for the individual-treatment and combination-treatment periods.

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

In Vitro Metabolism of Propionate

Rates of conversion of propionate to glucose and CO₂ were linear through 3 h of incubation (data not shown). Conversion of propionate to glucose and CO₂ by liver slices decreased ($P < .02$ and $.01$) from wk 0 to wk 6 but was not significantly different among treatment groups (data not shown).

When adjusted for pretreatment rates (Table 4), conversion of propionate to glucose was not different among groups during wk 1 to 4 but at wk 6 tended ($P < .11$) to be lower for cows in group BD compared with those in group FR. Conversion of propionate to CO₂ and the ratio of glucose to CO₂ produced were not different among groups.

Rates of conversion of propionate to glucose by liver slices from the one cow that became ketotic in group FR were decreased by 38% compared with pretreatment rates observed 1 wk earlier (5.2 vs. 8.3 $\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{g}^{-1}$). It is not known whether gluconeogenic rates were decreased as an effect of ketosis or by treatment with dexamethasone 2 d before the liver biopsy was obtained. It was observed previously that activity of phosphoenolpyruvate carboxykinase was decreased in liver of ketotic cows 48 h after treatment with dexamethasone. Decreased gluconeogenic capacity in the ketotic cow was observed despite concentration of glucose in plasma of 99.0 mg/dl (compared with 51.6 mg/dl when ketosis was diagnosed), and content of liver glycogen of 6% of wet weight (compared with 2.5% 1 wk before ketosis).

When added to incubation media, butanediol decreased oxidation of propionate to CO₂ (Table 5), which increased the ratio of glucose to CO₂ produced. Oxidation of butanediol to BHBA could spare oxidation of propionate by increasing the supply of reducing equivalents.

TABLE 5. Effect of butanediol in incubation media on conversion of propionate to glucose or carbon dioxide by liver slices in vitro¹

Product	Addition to media		SE
	None	10 mM 1,3-Butanediol	
-----($\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{g}^{-1}$ liver wet weight)-----			
Glucose	10.4	10.3	.25
CO ₂	.7	.4 ^a	.02
Ratio glucose to CO ₂	14.6	25.2	---

^aSignificant effect of addition of 1,3-butanediol, $P < .05$.

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

GENERAL DISCUSSION

The primary significance of these results is to indicate that neither moderate feed restriction nor dietary butanediol separately caused the gradual development of fatty liver and lactation ketosis. Combining feed restriction and butanediol at d 43 postpartum also did not cause changes leading to fatty liver or ketosis, in contrast to beginning the combination at d 14 as in earlier experiments. Our findings indicate that both an energy deficit and an exogenous ketone source are necessary, beginning early in lactation, to induce fatty liver and ketosis.

Failure of our combined protocol to induce ketosis after d 43 postpartum agrees with the greatest incidence of spontaneous ketosis occurring during the first 3 to 4 wk postpartum. Reasons for the observed loss of susceptibility to fatty liver and ketosis sometime between d 14 to 42 are unclear, but likely include the lessening of negative energy balance as intake increases and milk production decreases after peak. Changes of glucose and NEFA in plasma and glycogen and lipid in liver from wk 0 to wk 4 indicate a lessening of energy deficit and occurred regardless of treatment. Future research should attempt to delineate biochemical causes of the decrease in susceptibility to induction of fatty liver and ketosis.

Neither feed restriction nor dietary butanediol separately, or the two combined, markedly decreased *in vitro* hepatic gluconeogenesis from propionate, in contrast to studies with ketosis induced by a combination of feed restriction and butanediol. Addition of butanediol to incubation media also did not decrease gluconeogenesis. These findings point to the decrease in gluconeogenic capacity as a specific metabolic aberration related to onset of clinical ketosis rather than as a result of either feed restriction or metabolism of butanediol.

Cows given butanediol separately maintained normal concentrations of metabolites in plasma and liver, with the exception of slightly increased BHBA in plasma. With our diet fed *ad libitum*, butanediol in amounts similar to those in earlier studies did not cause fatty liver, which seems to exclude butanediol "toxicity" as a cause of fatty liver.

The early case of ketosis in one cow in the feed-restriction group is the first to occur during feed restriction in three experiments with a total of 16 cows, which is an incidence (6.25%) not greatly different from that reported for spontaneous ketosis. Ketosis in this cow occurred after feed restriction, but its development does not seem completely typical of either "spontaneous" ketosis or our induced ketosis for three reasons. First, up to 50% feed restriction alone did not cause clinical ketosis in 10 cows in two previous studies. Second, the rapid onset of ketosis after initiation of feed restriction contrasts with the gradual development of ketosis induced by feed restriction plus butanediol. Finally, the cow did not have the significantly increased

lipid in liver that is observed both in spontaneous and induced ketosis. Although the nature and significance of this one case are unclear, it is still valid to conclude that feed restriction alone is not a reliable technique for producing ketosis experimentally.

Results of this experiment provide further insights on development of fatty liver and ketosis during early lactation. Cows subjected to feed restriction were mobilizing greater amounts of lipid during wk 1, as indicated by increases in NEFA, acetate, and BHBA in plasma and increased loss of body weight. Changes in concentrations of metabolites were qualitatively similar to those reported for cows that eventually developed fatty liver and ketosis. By wk 2, however, concentrations of metabolites in feed-restricted cows were returning to values similar to those in control cows. It seems, therefore, that our cows were able to adjust metabolically to a moderate degree of feed restriction, even though they already were in negative energy balance.

We suggest that, when individual effects of feed restriction and butanediol are combined in cows early in lactation and in substantial negative energy balance, cows are unable to make the metabolic adjustments observed in our cows that were feed-restricted only. Furthermore, this detrimental synergism between feed restriction and butanediol leads first to development of fatty liver and, eventually, to clinical ketosis. Reasons for the inability to adjust metabolically to the stressors of feed restriction and butanediol are unknown, but a possible developmental sequence can be outlined.

The events probably start with a decreased availability of gluconeogenic precursors caused by feed restriction and the resultant increase in mobilization of NEFA from adipose tissue. It has been suggested that ketone bodies may directly regulate their own production in ruminant liver via an inhibition of carnitine palmitoyltransferase I, and BHBA was shown to decrease oxidation of palmitate by bovine liver slices. Conversion of butanediol to BHBA, therefore, could decrease oxidation of NEFA. Alternatively, oxidation of butanediol to BHBA, which occurs in hepatic cytosol and mitochondria, could decrease oxidation of NEFA by increasing the supply of reducing equivalents. The cytosol of liver from ketotic cows was more reduced chemically, suggesting a similarity between induced and spontaneous ketosis. Regardless of the mechanism, metabolism of butanediol to BHBA in feed-restricted cows may provide increased ketogenesis while decreasing hepatic oxidation of NEFA during their increased mobilization from adipose tissue.

If hepatic uptake of NEFA remained unchanged, decreased oxidation of NEFA should increase their esterification. Continued mobilization of NEFA from adipose tissue could, therefore, result in accumulation of triglyceride because of the limited capacity of ruminant liver to export triglyceride in lipoproteins. We suggested earlier that lactation ketosis is preceded by development of fatty liver. Ketogenesis *in vitro* increased only after the rate of esterification began to decrease. Loss of hepatic

glycogen during continued feed restriction and development of fatty liver may deplete citric acid cycle intermediates, which in turn may increase ketogenesis from NEFA. Increased hepatic lipid also may contribute in some manner to the eventual impairment of gluconeogenesis that occurs at clinical ketosis.

Continued production of ketone bodies from butanediol and NEFA, coupled with depletion of hepatic and systemic carbohydrate, eventually may result in overproduction of ketone bodies relative to the ability of peripheral tissues of the cow to metabolize them. The end result of this scenario is clinical ketosis. Our results provide only indirect support for these suggestions, in that liver composition and function were not altered by feed restriction or butanediol separately, and ketosis did not develop.

In summary this experiment supports the hypothesis that, normally, neither feed restriction nor dietary butanediol as separate treatments will induce fatty liver and/or ketosis in high-producing dairy cows. Subjecting cows to a combination of feed restriction and butanediol starting at d 43 postpartum rather than at d 14 postpartum also did not induce either fatty liver or ketosis. Neither feed restriction or butanediol as separate treatments, nor the later combined treatment, caused the inhibition of *in vitro* gluconeogenesis observed in earlier studies with induced ketosis. Butanediol added to incubation media also did not decrease *in vitro* gluconeogenesis by liver slices. Future experiments with the feed restriction plus butanediol model should help to define the nature of the interactions among mobilization of NEFA, carbohydrate insufficiency, and increased ketogenesis in dairy cows during early lactation.

SECTION III: SUBCLINICAL KETOSIS IN DAIRY COWS IN RESPONSE TO FEED RESTRICTION AND DIETARY 1,3-BUTANEDIOL

The objective of this experiment was to characterize progressive changes in milk composition, energy balance, concentrations of plasma insulin and glucagon, concentrations of serum lipoproteins, hepatic lipid composition, and *in vitro* hepatic oxidation of palmitate that occur in feed-restricted cows given dietary 1,3-butanediol.

MATERIALS AND METHODS

Treatments, Design of Experiment, and Management of Cows

Thirteen multiparous Holstein cows were assigned alternately at calving to two treatment groups, designated "control" or "FRBD" (i.e., "feed-restriction and 1,3-butanediol"). Milk production in the previous lactation (305-d mature equivalent) averaged 8,763 kg (+358 kg over herdmates). All cows were fed a mixed silage and grain diet for *ad libitum* intake from parturition through d 13 postpartum, and control cows continued to be fed *ad libitum* through d 56 postpartum (Figure 1).

Group:

Legend:

Ad libitum intake, no butanediol:

Restricted intake, plus butanediol:

Figure 1. Experimental protocol.

Intake for FRBD cows was restricted at d 14 to 80% of the average intake from d 9 to 13; 1,3-butanediol (butanediol) was included at 7% of ration DM. Average daily intake of butanediol was 890 ± 175 g. Cows in group FRBD continued the protocol through d 42 postpartum, at which time butanediol was discontinued and cows were returned to ad libitum intake. The period from d 9 through 13 (wk 0) is referred to as the pretreatment period, d 14 through 42 (wk 1 to 4) as the treatment period, and d 43 through d 56 (wk 5 to 6) as the recovery period (Figure 1).

Cows were housed in a tie-stall barn, milked twice daily, and weighed three times weekly. The mixed ration and 1.6 kg of alfalfa hay were fed individually to each cow twice daily, with orts removed and weighed once daily. Samples of feeds and milk were obtained and analyzed. Butanediol was introduced by feeding one-half of the final daily amount for 3 d and starting the full amount on d 4. Development of ketonemia was monitored by use of urine ketone test strips (Keto-Diastix^R, Ames Division, Miles Laboratories, Inc., Elkhart, IN).

Sampling Procedures

Puncture biopsies of 5 to 6 g of liver were obtained under local anesthesia before the a.m. feeding on each of six days for each cow: d 13, 21, 28, 35, 42, and 56. A 1 g piece of liver was frozen and stored at -20° C until used for determination of liver composition. The remaining liver was placed into ice-cold phosphate-buffered saline and used within 1.5 h for in vitro metabolic studies.

Weekly samples of jugular venous blood were obtained from each cow on the day before liver biopsy. Serum obtained from 12 ml of blood by using commercial serum separator tubes (Corvac^R, Monoject Scientific, Sherwood Medical, St. Louis, MO) was frozen at -20° C. The remaining 20 ml was divided between two heparinized tubes. One tube contained 150 μ l 4% NaF to inhibit glycolysis. Two ml of whole blood from the tube without NaF were deproteinized immediately by adding 2 ml of ice-cold 10% (w/v) perchloric acid while vortexing the tube. After centrifugation, the supernatant was frozen at -20° C until analyzed. Plasma was harvested by centrifugation from the remaining 18 ml of blood, aliquoted, and stored at -20° C.

Incubation Procedures

For, incubations, liver slices were blotted, weighed, and placed into 25-ml flasks that contained 3 ml of calcium-free Krebs-Henseleit bicarbonate buffer at pH 7.4. Sodium palmitate (2 mM) and .5 μ Ci of [$1-^{14}$ C]-palmitic acid were complexed with fatty acid-free bovine serum albumin (BSA; item No. 7030, Sigma Chemical Co., St. Louis, MO). Final concentration of BSA was 4% (w/v), giving a molar ratio of palmitate to BSA of 4:1.

Flasks were gassed for 15 sec with 95:5 O₂:CO₂ and incubated in triplicate for

2 h at 37° C with constant shaking. Incubations were terminated by injecting 3 ml of 10% (w/v) perchloric acid. Radioactive CO₂ was collected and quantitated. Carryover of radioactive substrate through analytical procedures was measured in zero-time flasks acidified immediately after addition of the liver slice.

Analytical Procedures

Flasks were allowed to cool after collection of CO₂. Contents were transferred to polyethylene tubes, centrifuged at 2000 x g for 10 min, and then 2 ml of supernatant were transferred to glass tubes and neutralized to pH 7 with 2 M K₂CO₃. After centrifugation at 2500 x g for 10 min, .5 ml of the second supernatant was transferred to a scintillation vial, 10 ml of scintillation cocktail was added, and ¹⁴C in total acid-soluble metabolites (ASM) was determined. The ASM fraction consists mainly of ketone bodies, acetate, and short-chain esters of carnitine, which are products of incomplete oxidation of long-chain fatty acids, but it also includes dicarboxylic acids and amino acids that become labeled in the citric acid cycle. Thus, ASM provides a more complete estimate of total fatty acid oxidation than only measuring CO₂ production or ketogenesis.

Frozen liver was powdered in liquid N₂, and concentrations of glycogen and glucose were determined. Lipid was extracted from powdered liver with 2:1 (v:v) chloroform-methanol, and concentration of total lipid was determined gravimetrically after drying under moving air, which was determined to be equivalent to drying under N₂ for subsequent analyses. The lipid extract was redissolved in 3:2 (v:v) hexane-isopropanol, and concentrations of triglyceride (9) and total cholesterol (Sigma total cholesterol kit no. 352, Sigma Chemical Co., St. Louis, MO) were determined. A portion of the extract was dried and redissolved in chloroform, and concentration of total phospholipid was determined from a standard curve prepared from a combination (7:3 w:w) of bovine phosphatidylcholine and phosphatidylethanolamine (both prepared from bovine liver, obtained from Sigma Chemical Co., St. Louis, MO).

Concentration of BHBA was determined in perchloric acid filtrates, which first were neutralized with 2 M K₂CO₃. Plasma was analyzed for concentrations of glucose, acetate, and nonesterified fatty acids (NEFA). Concentrations of insulin and glucagon in plasma were determined by validated radioimmunoassays. Plasma for glucagon was stored with 2500 KIU of aprotinin (Trasylol^R, Mobay Chemical Corp., FBA Pharmaceuticals, New York, NY) per ml to inhibit proteolysis. All samples for each hormone were assayed concurrently, with intraassay coefficients of variation of 11 and 5% for insulin and glucagon, respectively.

To estimate concentration of triglyceride-rich lipoproteins, serum was thawed and subjected to dextran sulfate (molecular weight 5000, Sigma Chemical Co., St. Louis, MO) precipitation, with amounts of serum and reagents halved. Concentrations of

cholesterol (Sigma total cholesterol kit no. 352, Sigma Chemical Co., St. Louis, MO) and triglyceride (Sigma triglyceride kit no. 339, Sigma Chemical Co., St. Louis, MO) were determined in the precipitate, supernatant, and total serum. Preliminary experiments showed that freezing serum did not change distribution of either triglyceride or cholesterol among the fractions analyzed.

Presentation of Data

Metabolic rates and composition of liver are expressed as a function of wet weight of liver. This is justified because changes in metabolic rates observed in liver slices from ketotic cows with fatty liver were similar when expressed either on the basis of wet weight or per unit of DNA. Other workers showed that protein content of liver changed only slightly in comparison with changes in lipid over the range of hepatic lipid concentrations observed in our experiment.

Statistical Analyses

One cow assigned to FRBD quickly developed a severe clinical ketosis only 9 d after initiating FRBD, which is somewhat sooner than expected from earlier studies. Because the response to FRBD by this cow differed markedly from that of the other six cows, her data were excluded from statistical analysis but are discussed later for comparison. Statistical analyses, therefore, were conducted by using six cows per treatment.

All repeated measurement data were subjected to analysis of variance for a split-plot design by using the General Linear Models (GLM) package of SAS. Dietary treatment (control vs. FRBD) was the main plot, and time (week of sampling) was the subplot. Conservative df were used to evaluate F-tests containing time as a factor. Conditional comparisons of treatments at each week were made by using t-tests, with approximate degrees of freedom calculated by Satherwaite's procedure. Standard errors were calculated by dividing the appropriate error term by the harmonic mean of number of observations per treatment group. Because some cows were not sampled every week, least squares means are presented throughout. Tests of significance with $P \leq .05$ were considered statistically significant.

RESULTS AND DISCUSSION

To our initial surprise, only one of the seven cows assigned to FRBD developed fatty liver and clinical ketosis, which is a much lower incidence of induction than expected. The other six cows, however, did develop ketonemia and signs of subclinical ketosis and will be emphasized. Careful analysis and interpretation of the data provides important information on why some cows are susceptible to clinical ketosis whereas others are not. It is from this perspective that we present and discuss the results.

Milk Production and Composition

Average production of milk by FRBD cows was less than that for controls ($P < .03$). Production began to decrease during wk 1 and was less than controls by wk 2 (Figure 2a). Pretreatment milk production by FRBD cows was less than that of cows earlier induced into ketosis by FRBD. Production of milk fat was not different between groups, but production of protein and total solids was less for FRBD (data not shown), which resulted in yield of SCM being less ($P < .02$) for FRBD cows (28.1 vs 33.4 kg/d). The tendency of FRBD cows to maintain fat yield was attributable to a greater ($P < .05$) content of fat (Figure 2b). Contents of protein (3.10 vs 3.15%) and total solids (12.13 vs 12.66%) were not different ($P > .1$) between control and FRBD groups.

Intakes, Change in Body Weight, and Energy Balance

Average daily intake of DM during FRBD treatment was 69% of that of controls (15.3 vs. 22.2 kg). Consumption of DM by FRBD cows averaged about 2.8% of BW during treatment (Figure 3a), whereas control cows increased intake of DM from 3.4 to nearly 4.6% of BW. By the second week of recovery, intakes for FRBD cows were similar to controls. During the treatment period, average intakes of net energy for FRBD and controls were 80% and 93% of initial net energy requirements calculated at wk 0 (24).

Cows on FRBD lost BW at an average of 1.8 kg/d, reaching a cumulative loss of 52 kg by wk 4 (Figure 3b). Control cows, however, began regaining BW by wk 3, coinciding with attainment of energy balance (Figure 3c), and had returned to pretreatment BW by wk 5. Weight loss by our FRBD cows was not as extreme as the 3.1 kg/d observed earlier for overconditioned cows induced into ketosis by FRBD, but was similar to the weight loss of 2.0 kg/d for cows induced into ketosis by FRBD in another experiment. Calculated net energy balance (Figure 3c) was less for FRBD cows than control cows from wk 1 to 5, and was most negative during wk 1 (-6.6 Mcal/d). Crude protein content of the total diet (14.5% of DM) was somewhat low for cows in early lactation, and may have decreased the extent of negative energy balance for both groups by limiting milk production.

Metabolites and Hormones in Blood

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

Figure 2. Average daily production of milk and fat content in control cows or cows subjected to feed restriction plus dietary butanediol (FRBD) during weeks 1 through 4 (d 14 to 42 postpartum). Asterisks denote significant differences ($P < .05$) between treatments at that week.

A. Daily milk production (standard error of the difference (SED) between treatment means at any week = 2.3). Probabilities of greater F for effects in model: treatment, $P = .03$; week, $P = .17$; treatment x week, $P = .03$. B. Milk fat content (SED = .34). Treatment, $P = .22$; week, $P = .13$; treatment x week, $P = .20$.

Figure 3. Average daily intake of dry matter (DM) as a percentage of body weight (BW), cumulative body weight change (week 0 as reference), and energy balance of control cows or cows subjected to feed restriction plus dietary butanediol (FRBD) during weeks 1 through 4 (d 14 to 42 postpartum). Asterisks denote significant differences ($P < .05$) between treatments at that week.

A. DM intake, % of BW (standard error of the difference (SED) between treatment means at any week = .3). Probabilities of greater F for effects in model: treatment, $P = .02$; week, $P = .66$; treatment x week, $P = .09$.

B. Cumulative weight change (SED = 13.7). Treatment, $P = .02$; week, $P = .04$; treatment x week, $P = .08$.

C. Energy balance (SED = 2.3). Treatment, $P = .02$; week, $P = .006$; treatment x week, $P = .04$.

Figure 4. Concentrations of metabolites in plasma from control cows or cows subjected to feed restriction plus dietary butanediol (FRBD) during weeks 1 through 4 (d 14 to 42 postpartum). Asterisks denote significant differences ($P < .05$) between treatments at that week.

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

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

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

D. B-Hydroxybutyrate (SED = 2.3). Treatment, $P = .07$; week, $P = .08$; treatment x week, $P = .05$.

Concentrations of total cholesterol and triglyceride in serum were not significantly different between groups (Figures 5a and b), although triglyceride tended to be higher in FRBD cows early in the treatment period and then again during recovery. Circulating triglycerides usually are decreased in cows with spontaneous ketosis. Dextran sulfate-precipitable (DSP)-cholesterol and DSP-triglyceride (Figures 5c and d) were greater ($P < .05$) in FRBD cows during wk 1 but were similar to controls during the remainder of the experiment. The mechanism(s) responsible for and the physiological significance of the short-lived increases in DSP-lipids cannot be determined from available data. Detailed study of lipoprotein metabolism in cows subjected to FRBD is needed.

Insulin in plasma was similar between groups during FRBD treatment (Figure 6a), but was increased ($P < .03$) in FRBD cows compared with controls during the recovery period. For FRBD cows to maintain a concentration of insulin similar to that of controls during FRBD treatment was unexpected because of the greater negative energy balance of FRBD cows (Figure 3c). Earlier, cows subjected to FRBD had decreased concentrations of insulin (30). Concentration of glucagon in plasma was similar between groups (Figure 6b).

Composition of Liver

Concentration of glycogen in liver (Figure 7a) was less ($P < .05$) in FRBD cows during pretreatment but increased steadily thereafter, whereas liver glycogen in control cows changed much less. The increasing glycogen during FRBD contrasts with previous results where it decreased and became almost depleted at least 1 wk before clinical ketosis.

Concentrations of total lipid (data not shown) and triglyceride (Figure 7b) were greater in control cows during pretreatment and decreased steadily thereafter. Liver triglyceride increased during FRBD and then decreased to control values during recovery, but the increases were much less than seen earlier. Concentrations of total cholesterol and phospholipid were not different between groups during either treatment or recovery periods (Figure 7c and d), but total cholesterol was greater ($P < .05$) in controls during pretreatment.

In Vitro Hepatic Oxidation of Palmitate

Rates of oxidation of palmitate to CO_2 were linear for 3 h, whereas rates to ASM were somewhat greater during the first 30 min; rates then remained linear but at a slightly lower rate for the next 2.5 h (data not shown).

Oxidation of palmitate to CO_2 was greater ($P < .05$) in liver slices from FRBD cows at wk 1 (Figure 8a). This early increase in CO_2 production in FRBD cows could indicate increased oxidation of palmitate to meet energy demands of the liver when feed restriction decreased supply of other fuels. Oxidation of palmitate to ASM by

Figure 5. Concentrations of total and dextran sulfate precipitable- (DSP-) cholesterol and triglyceride in serum from control cows or cows subjected to feed restriction plus dietary butanediol (FRBD) during weeks 1 through 4 (d 14 to 42 postpartum). Asterisks denote significant differences ($P < .05$) between treatments at that week.

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

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

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

D. DSP-triglyceride (SED = 2.3). Treatment, $P = .56$; week, $P = .29$; treatment x week, $P = .18$.

Figure 6. Concentrations of hormones in plasma from control cows or cows subjected to feed restriction plus dietary butanediol (FRBD) during weeks 1 through 4 (d 14 to 42 postpartum). Asterisks denote significant differences ($P < .05$) between treatments at that week.

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

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

Figure 7. Composition of liver (percent of wet weight) from control cows or cows subjected to feed restriction plus dietary butanediol (FRBD) during weeks 1 through 4 (d 14 to 42 postpartum). Asterisks denote significant differences ($P < .05$) between treatments at that week.

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

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

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

D. Phospholipid (SED = .2). Treatment, $P = .67$; week, $P = .04$; treatment x week, $P = .38$.

Figure 8. Conversion of palmitate to carbon dioxide or total acid-soluble metabolites (ASM) by liver slices from control cows or cows subjected to feed restriction plus dietary butanediol (FRBD) during weeks 1 through 4 (d 14 to 42 postpartum). Asterisks denote significant differences ($P < .05$) between treatments at that week. A. Palmitate to carbon dioxide (standard error of the difference (SED) between treatment means at any week = 6.4). Probabilities of greater F for effects in model: treatment, $P = .24$; week, $P = .39$; treatment x week, $P = .34$. B. Palmitate to ASM (SED = 35). Treatment, $P = .28$; week, $P = .39$; treatment x week, $P = .34$.

liver slices from control cows tended to decrease throughout (Figure 8b), whereas for FRBD cows it remained relatively constant. Rates were greater ($P < .05$) for FRBD cows at wk 4. The decrease in control cows is similar to results of others where hepatic ketogenesis in vitro decreased from d 30 to d 60 of lactation. The tendency of FRBD cows to maintain palmitate oxidation to ASM may reflect intrahepatic changes in response to greater negative energy balance and increased influx of NEFA.

Comparison with the Clinically Ketotic Cow

As already mentioned, one FRBD cow quickly became clinically ketotic, and it is instructive to compare her with the other six FRBD cows and earlier ketotic cows. The cow had milk fever at calving and was off-feed for 3 d. When pretreatment samples were taken at d 13, she had moderate fatty liver (Table 1) but seemed healthy and was producing 34.5 kg of milk per d. Concentrations of BHBA, glucose, and NEFA in blood were within normal ranges (Table 1). When clinically ketotic 9 d later, concentrations of metabolites in blood and liver were similar to those of ketotic cows. Gluconeogenic capacity of liver slices was decreased by 84% (Table 1). In contrast, measurement of in vitro gluconeogenic capacity of two subclinically-ketotic cows showed no differences from controls (data not shown). Oxidation of palmitate to CO_2 by liver slices at ketosis (Table 2) was increased over fivefold, and oxidation to ASM was increased by 26%.

Ratio of Triglyceride to Glycogen in Liver

Mehnert proposed that the ratio of lipid to glycogen in liver be used as an indicator of metabolic stress in dairy cows during early lactation, with greater ratios indicating increased metabolic disorders. Calculated ratios of triglyceride to glycogen in liver from cows of the present and earlier experiments are in Table 2. The average liver triglyceride-to-glycogen ratio for the ketotic group of Mills et al. was 4.1 before initiating FRBD, and increased to 18.3 at clinical ketosis. The ketotic group of Veenhuizen et al. had an average ratio of 2.3 before FRBD and increased to over 90.0 at 1 wk before ketosis and was 12.0 at clinical ketosis. Their control cows decreased from 1.2 to .7 during the same time. Our subclinically-ketotic FRBD cows had an average ratio of only .5 before initiation of FRBD, which increased only to .6 during treatment, before decreasing to less than .4 at wk 4. By that time, cows of Veenhuizen et al. were clinically ketotic. Our one cow that quickly developed fatty liver and ketosis had a pretreatment ratio of 3.0 and a ratio of 25.4 at ketosis. A weight ratio of triglyceride-to-glycogen in liver of greater than about 2.0 during the early postpartum period seems to have excellent potential as an indicator of susceptibility to fatty liver and clinical ketosis.

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

Item	Pretreatment (d 13)	Ketosis (d 22)
Whole blood		
BHBA ¹ , mg/dl	2.7	38.7
Plasma		
Glucose, mg/dl	54.8	38.6
NEFA ² , uequiv/l	340	1485
Liver ³		
Glycogen, %	1.9	.3
Glucose, %	.49	.31
Total lipid, %	9.4	10.9
Triglyceride, %	5.8	7.1
Total cholesterol, %	1.4	1.1
Phospholipid, %	1.5	1.3
In vitro metabolism of propionate ⁴		
CO ₂ , μ mol/h x g wet weight	.5	.4
Glucose, μ mol/h x g wet weight	18.6	3.0
In vitro oxidation of palmitate ⁵		
CO ₂ , nmol/h x g wet weight	11.7	60.5
ASM, nmol/h x g wet weight	189	238

¹ β -Hydroxybutyrate.

²Nonesterified fatty acids.

³Composition given as percent of wet weight.

⁴Production of CO₂ and glucose from propionate by liver slices (13).

⁵Production of CO₂ and acid-soluble metabolites (ASM) from palmitate by liver slices.

TABLE 2. Ratio of triglyceride to glycogen in liver biopsies from ketotic and nonketotic cows in the present and earlier experiments.

Experiment and group	Cows	Stage of experiment	
		Pretreatment ¹	Ketosis ²
----- (mean ratio) -----			
Mills et al.			
Ketotic	4	4.1	18.3
Nonketotic	1	.4	1.2
Veenhuizen et al.			
Ketotic	6	2.3	12.0
Control	6	1.2	.7
Present study			
Subclinically ketotic	6	.5	.4
Control	6	.9	.3
Ketotic	1	3.0	25.4

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

²Biopsy of liver obtained at time of clinical ketosis or from d 35 to 46 postpartum.

A greater triglyceride-to-glycogen ratio in liver represents the combined effects of increased uptake of NEFA and decreased content of glycogen, and may provide a better indicator of hepatic carbohydrate status than glycogen content alone. We observed significant Pearson correlation coefficients between that ratio and energy balance ($r = -.39$, $P < .002$), concentrations in blood of glucose ($r = -.34$, $P < .005$), NEFA ($r = .51$, $P < .0001$), and BHBA ($r = .61$, $P < .0001$), and in vitro hepatic oxidation of palmitate to CO_2 ($r = .36$, $P < .003$) and ASM ($r = .24$, $P < .05$). Correlations were weaker when using either hepatic triglyceride or glycogen contents individually.

GENERAL DISCUSSION

Fatty liver and clinical ketosis were not induced as expected in this experiment. Much valuable information was gained, however. Discussion will center on the picture that is emerging about why some cows are susceptible to fatty liver and ketosis.

Cows in earlier studies that eventually became clinically ketotic showed greater negative energy balance, greater concentration of NEFA and lower glucose in plasma, greater triglyceride and lower glycogen in liver, and greater hepatic triglyceride-to-glycogen ratios in early lactation before initiation of FRBD. Such changes indicate that cows that were susceptible to fatty liver and ketosis were mobilizing more body lipid before FRBD was started than were cows that resisted induction of fatty liver and ketosis.

Although our protocol was designed to be identical to that of Veenhuizen et al., several differences did occur. First, cows in the earlier experiment may have had greater genetic potential, because they produced 10,030 kg of milk (305-d mature equivalent) in the previous lactation (+893 kg over herd mates), whereas our FRBD cows produced 8,665 kg (+218 kg). Second, dietary protein was less than the 16% fed earlier. Their greater dietary protein may have stimulated greater milk production and mobilization of body stores. Third, hay intake was estimated earlier and may have been overestimated, in which case their cows would have been in even greater energy deficit. Fourth, average age of cows at calving was less in our study (42 vs. 53 mo.). Older cows usually are higher producers and have a greater incidence of fatty liver and ketosis. Fifth, earlier cows had an additional liver biopsy taken 5 d postpartum. It seems that cows react more adversely to liver biopsy within the first week postpartum, often leading to brief decreases in feed intake and increases in concentrations of NEFA and BHBA in blood. It seems unlikely that any of these five differences per se would have caused the low incidence of induction of fatty liver and clinical ketosis by FRBD; rather, the combination of these factors likely caused a lesser reliance on body tissue to support milk production than in the earlier study.

A condition necessary for development of fatty liver and ketosis is increased hepatic uptake of NEFA from blood. Because hepatic uptake of NEFA is concentration-dependent, increased mobilization of NEFA will result in greater uptake of NEFA by liver. In our subclinically ketotic FRBD cows, concentrations of NEFA generally were less than in clinically ketotic cows of earlier studies. Maintenance of hepatic glycogen in our FRBD cows was associated with normal plasma glucose concentrations, which in turn would be associated with decreased mobilization of NEFA from adipose tissue. Thus, less NEFA would be available to the liver of our FRBD cows for esterification and ketogenesis than in cows of the earlier studies.

Two of our FRBD cows that did not develop fatty livers or clinical ketosis, however, had NEFA concentrations similar to earlier ketotic cows. This suggests that intrahepatic factors that partition flux of NEFA between esterification or oxidation and ketogenesis may be altered by early negative energy balance and tissue mobilization, before initiation of the induction protocol.

The specific nature of these intrahepatic changes cannot be determined from available data, but there are at least four possibilities. First, intermediates in the citric acid cycle, particularly oxaloacetate, may be depleted by early negative energy balance to concentrations that are insufficient to maintain activity of the cycle. Depletion of hepatic glycogen in bovine liver is associated with depletion of glycolytic and citric acid cycle intermediates and is related inversely to ketogenesis. In our study, glycogen was not depleted and may have maintained activity of the citric acid cycle, thereby decreasing ketogenesis.

Second, controls linking carbohydrate status with ketogenesis may activate ketogenesis to a greater degree in cows mobilizing body tissue at high rates. In nonruminants, ketogenesis and content of glycogen in liver are linked by malonyl-CoA, an intermediate in fatty acid synthesis that is a potent inhibitor of CPT I. Malonyl-CoA is a regulator of fatty acid oxidation in sheep liver as well. Although some controls of fatty acid oxidation and ketogenesis in dairy cows have been studied, little is known about regulation in cows in negative energy balance or during development of fatty liver or ketosis.

The third possibility is that hepatic capacity for triglyceride synthesis may increase during early energy deficit. Cows with high rates of tissue mobilization after parturition may increase phosphatidate phosphohydrolase, which could allow greater rates of triglyceride deposition when NEFA are increased further during FRBD. Increased deposition of triglyceride in ruminant liver is associated with increased ketogenesis, and fatty liver developed before clinical ketosis in earlier cows subjected to FRBD.

Fourth, butanediol or ketone bodies may potentiate increased mobilization and hepatic esterification of NEFA. A major opportunity provided by our FRBD model is that intrahepatic changes can be studied in individual cows as they move through development of different degrees of fatty liver and ketosis.

The triglyceride-to-glycogen ratio shows promise as a tool for predicting susceptibility to fatty liver and clinical ketosis. Cows could be screened at 7 to 14 days postpartum for elevated triglyceride-to-glycogen ratios (perhaps above 2.0) to detect cows likely to be susceptible. Subjecting cows with low postpartum triglyceride-to-glycogen ratios to FRBD, as in the present experiment, would provide a model of subclinical ketosis. The ability to compare subclinical to clinical ketosis in the same controlled experiment may enable determination of the unknown metabolic events that "trigger" clinical ketosis. Future experiments must verify utility of the ratio and more closely define the critical ratio above which cows are susceptible to induction of fatty liver and clinical ketosis.

In summary, these results point to changes in the late-prepartum or early-postpartum period as being critical in determining susceptibility to fatty liver and ketosis. From an experimental approach, negative energy balance and mobilization of body tissue must be maximized during this time to induce fatty liver and ketosis. From a practical standpoint, our results reemphasize the importance of efforts to prevent excessive loss of body tissue during the early-postpartum period to decrease incidence of fatty liver and ketosis. Such efforts should include prevention of other peripartum and postpartum diseases, maximizing intake of high-quality rations, and minimizing stresses that lead to catecholamine-induced increases in release of NEFA from adipose tissue. The utility of the FRBD model as a tool to determine sequential metabolic events in development of fatty liver and ketosis has been strengthened. Fatty liver and ketosis will not be induced indiscriminantly in all cows given the protocol; susceptibility seems to depend on a predisposition that develops very early in lactation. The challenge now is to use the FRBD protocol and other experimental techniques to answer many remaining questions about metabolic origins of fatty liver and clinical and subclinical ketosis.

SECTION IV: Clinical Chemistry of Cows with Fatty Liver and Ketosis

The cows used in the experiments reported in this section were obtained from farms and slaughter houses, and they were cows with proven cases of fatty liver and ketosis. Efforts were concentrated on the clinical chemistry picture, particularly the lipid and lipoprotein fractions. It seems that lipoproteins may be involved in the process of liver fattening, with HDL and LDL/VLDL ratio changing significantly.

It is well to be reminded of the changes in the relative proportions in lipid fraction of the liver when cows develop fatty liver. This is illustrated graphically in Figure 1, which shows that in normal cows phospholipids and cholesterol are the two primary fractions, whereas in fatty liver, triglyceride is the overwhelming fraction with some increase in the cholesterol ester fraction.

Figure 1. Relative distribution of lipid fractions extracted from normal and fatty liver.

Table 1 involves cows that were suspected of having fatty liver or that had a history of fatty liver and were suspected to have metabolic problems. A blood sample was taken from each of the cows once before calving and again at 10-12 days after calving and studied for abnormal levels of blood constituents. Throughout the table (parts A, B, and C) the data before calving were grouped by days before calving, and the data after calving were categorized by lactation number.

Part A of Table 1 is a summary of blood lipid constituents. The most pronounced event taking place is the decrease of the relative ratios of LDL + VLDL/total cholesterol with the increasing age of the cows, i.e., increasing the number of lactations. This change is particularly evident for the fifth and sixth lactation. There is a significant increase in ketone bodies (i.e., β -hydroxybutyrate) for second or later lactation cows, with the first lactation heifers having relatively lower values. There also is a distinct decrease in concentration of triglycerides in blood after calving and initiation of milk production.

Part B of Table 1 presents results of assays of different enzymes present in blood. After calving, there is a marked increase in the serum levels of aspartate aminotransferase, creatine phosphokinase, and lactate dehydrogenase. These increases are probably from muscle damage or from the stress of milking. Similarly there is an increase in sorbitol dehydrogenase, which is a liver enzyme. Part C of Table 1 deals with several metabolites in the blood. By far the most pronounced changes are those seen in blood glucose following calving. There is a major decrease in glucose 10-12 days after calving, especially for second or third lactation cows.

The data in Table 2 are from normal cows and are presented to follow normal changes in blood lipid fractions before and after calving and also to allow comparisons of the data in Table 1A from cows that were suspected of having metabolic disorders. The values in Table 2 show that there is a decline in all lipid fractions and consequently total lipids as cows move toward calving day. Concentrations of all lipid fractions increase thereafter, except for triglycerides which remain quite constant. The relative levels of LDL+VLDL versus total cholesterol is stable before and after calving. Immediately around calving time "fraction X" is at its highest relative levels. When values for LDL+VLDL/total cholesterol for normal cows in Table 2 are compared with values in Table 1A for cows suspected of having fatty liver, it is clear that the latter values are lower. Calving 5 and 6 show low levels of LDL+VLDL.

TABLE 1. Blood Parameters of Dairy Cows Before Parturition and 10-12 Days Postpartum.

Part A: Lipids in the blood

Days before calving:		β -hydroxy- butyrate	High density lipoproteins	Total cholesterol	LDL+ VLDL mg/dl	Phospho- lipids	Triglyceride	LDL + VLDL Total Chol. %
-14 to -9	X	6.3	100	116	19.1	133.0	28.2	16.2
	SD	1.5	16.9	18	7.2	16.1	9.6	4.5
	n	16	15	16	16	11	16	16
-8 to -4	X	6.6	92.7	107	16.3	127	30.6	14.9
	SD	1.8	14.1	16.1	7.3	21.7	9.0	5.2
	n	16	15	16	16	8	15	16
-3 to -2	X	6.8	103	125	21.1	158	37.6	15.1
	SD	2.2	22.1	35	10.9	44.9	12.6	4.9
	n	6	7	6	6	6	6	6
-1	X	10.0	75.3	85.0	12.0	118.5	37.0	14.0
	SD	2.4	11.9	12.1	2.9	31.5	11.1	2.6
	n	3	3	3	3	2	3	3
Lactation:								
First	X	12.0	110.0	134	19.3	140	17.6	14.0
	SD	15.0	56.5	63.2	13.3	35.4	5.1	5.5
	n	13	12	13	12	9	12	12
Second	X	32.7	108	132	22.3	167	19.7	17.1
	SD	14.2	34	38	10.0	44	9.1	7.5
	n	12	12	12	12	12	12	12
Third	X	38.6	82.	98	12.5	134	13.0	12.8
	SD	20.5	23.	26.2	9.2	34	3.0	6.6
	n	12	12	12	12	12	10	12
Fourth	X	28.3			11.3			11.8
	SD	16			7.7			8.1
	n	11			11			12
Fifth	X	33.7			6.4			6.6
	SD	17.8			2.8			2.
	n	12			11			11
Sixth	X	26.8			6.4			8.4
	SD	18.3			2.0			3.5
	n	7			7			7

Table 1 (continued)
Part B: Enzymes in the blood

Days before calving:		Aspartate amino transferase	Glutamate amino transferase	Amylase	Lactic dehydrogenase	Alkaline phosphatase	Creatine phosphokinase	Sorbitol dehydrogenase
		 units per liter						
-14 until -9	$\bar{X}$	41	17.8	152	1410	76		6.0
	SD	11	5.6	81	520	12		3.0
	n	8	7	9	7	9		7
-8 until -4	$\bar{X}$	40	22.1	183	1392	89	48	5.1
	SD	5	3.9	57	326	13	18	2.6
	n	11	12	12	11	12	12	8
-3 until -2	$\bar{X}$	50	27.4	159	1413	106	51	6.1
	SD	13	4.3	70	150	17	19	3.1
	n	4	4	3	4	4	4	3
-1	$\bar{X}$	66	28.2	168	1781	101	-	-
	SD	1.0	2.6	42	6	16	-	-
	n	2	2	2	2	2	-	-
Lactation:								
First	$\bar{X}$	59	27.3	170	1727	159	140	11.6
	SD	19	13	82	434	66	155	6.8
	n	9	11	12	9	12	9	10
Second	$\bar{X}$	77.7	23.7	208	1644	99	87	10.2
	SD	30	6.0	69	508	41	72	3
	n	9	11	12	12	12	9	11
Third	$\bar{X}$	103	31.8	213	1994	87	111	23.3
	SD	71	20.5	86	1087	19	77	30
	n	9	11	11	11	11	10	12

Table 1. (continued)
Part C: Metabolites in the blood.

Days Before Calving		Creatinine (mg/dl)	Urea (mg/dl)	Glucose (mg/dl)	Albumin (g/dl)	Protein (g/dl)	Calcium (mg/dl)	Inorganic P (mg/dl)
-14 until -9	$\bar{X}$	1.37	28.7	55	3.28	7.36	9.80	4.55
	SD	0.19	10.4	8	0.20	0.33	1.09	0.98
	n	6	8	6	8	8	8	4
-8 until -4	$\bar{X}$	12.1	31.7	60.3	3.16	7.13	9.62	5.00
	SD	0.31	7.5	6.7	0.28	0.41	0.79	0.78
	n	11	12	12	12	12	12	8
-3 until -2	$\bar{X}$	1.15	31.7	57.8	3.28	7.15	10.0	5.30
	SD	0.15	11.9	5.1	0.28	0.45	0.89	0.85
	n	4	4	4	4	4	4	3
-1	$\bar{X}$	1.1	36.7	62.5	2.90	7.3	9.2	-
	SD	0	2.9	3.5	0.28	0.4	0.2	-
	n	2	2	2	2	2	2	-
Lactation:								
First	$\bar{X}$	1.19	39.0	53.6	3.06	7.16	9.15	5.02
	SD	0.64	12.6	8.9	0.28	0.46	1.29	1.86
	n	8	12	5	12	12	12	9
Second	$\bar{X}$	1.11	37.0	36.9	3.16	7.38	9.04	4.61
	SD	0.43	12.3	9.1	0.16	0.43	0.86	1.33
	n	11	11	9	12	12	12	11
Third	$\bar{X}$	0.99	32.3	39.0	3.08	7.26	9.5	4.94
	SD	0.17	13.4	12.2	0.29	0.48	0.73	1.40
	n	8	10	9	11	11	10	9

Table 2. LIPID FRACTIONS IN BLOOD OF NORMAL DAIRY COWS AT DIFFERENT TIMES BEFORE AND AFTER CALVING.

Days from calving	Total Fat	Phospho-lipids	Total Cholesterol	HDL	LDL	Triglyceride	VLDL ₁	LDL+VLDL ₁	LDL+VLDL ₁ Total Chol	"Fraction X" ²
< 25	608 ± 22	198 ± 7	158 ± 6	133 ± 9	28 ± 2	25.6 ± 1.1	5.1 ± 0.2	25	16	189 ± 9
(-24) - (-12)	435 ± 33	177 ± 22	120 ± 12	93 ± 10	22 ± 2	26.0 ± 3.2	5.2 ± 0.6	27	23	111 ± 27
(-11) - (-9)	353 ± 19	119 ± 11	110 ± 10	95 ± 15	13.1 ± 2	27.4 ± 3.0	5.5 ± 0.6	15	14	122 ± 12
-5	365 ± 34	121 ± 10	96 ± 8	85 ± 3	26 ± 9	24.7 ± 4.3	4.9 ± 0.9	11	11	112 ± 32
-4	374 ± 18	124 ± 25	105 ± 19	81 ± 17	19 ± 2	27.6 ± 2.3	5.5 ± 0.5	24	23	151 ± 14
-3	371 ± 27	105 ± 11	87 ± 11	65 ± 12	18 ± 3	23 ± 4.2	4.6 ± 0.8	22	25	97 ± 11
-2	402 ± 28	130 ± 8	109 ± 12	85 ± 6	17 ± 5	35 ± 5.2	7.0 ± 1.0	24	22	129 ± 20
-1	393 ± 45	113 ± 21	99 ± 14	79 ± 13	15 ± 5	23 ± 4	4.5 ± 0.7	20	20	168 ± 21
0	342 ± 18	101 ± 8	86 ± 6	70 ± 5	13 ± 3	16 ± 2	3.2 ± 0.4	19	22	139 ± 12
1	344 ± 28	99 ± 7	85 ± 6	71 ± 3	12 ± 3	12.5 ± 0.9	2.5 ± 0.2	14	16	150 ± 18
2	354 ± 18	101 ± 10	85 ± 7	73 ± 6	9 ± 4	13.7 ± 1.7	2.7 ± 0.3	12	14	154 ± 7
3	382 ± 21	119 ± 8	93 ± 6	76 ± 5	14 ± 2	13.2 ± 0.9	2.6 ± 0.2	17	18	157 ± 12
4	417 ± 29	131 ± 15	103 ± 11	82 ± 9	18 ± 3	15.6 ± 1.8	3.1 ± 0.4	21	20	166 ± 9
5	431 ± 57	134 ± 23	113 ± 20	94 ± 19	16 ± 3	13.2 ± 1.5	2.6 ± 0.3	19	17	171 ± 18
6	374 ± 21	110 ± 6	87 ± 7	75 ± 7	11 ± 1	15.1 ± 1.3	3.0 ± 0.2	12	14	166 ± 9
7 - 9	401 ± 20	120 ± 8	95 ± 6	77 ± 3	15 ± 5	14.2 ± 0.6	2.8 ± 0.1	18	19	172 ± 12
10 - 12	439 ± 32	131 ± 8	100 ± 6	83 ± 6	14 ± 3	15.7 ± 0.8	3.1 ± 0.2	17	17	191 ± 19
13 - 15	417 ± 28	126 ± 9	97 ± 7	81 ± 6	13 ± 2	14.8 ± 1.1	3.0 ± 0.2	16	16	185 ± 17
16 - 18	532 ± 35	156 ± 12	126 ± 10	100 ± 7	23 ± 7	17.5 ± 1.1	3.5 ± 0.2	26	21	233 ± 17
19 - 21	528 ± 53	167 ± 19	136 ± 14	91 ± 10	41 ± 11	18 ± 0.8	3.6 ± 0.2	45	33	218 ± 21
22 - 24	520 ± 43	154 ± 12	119 ± 10	85 ± 6	31 ± 7	15.8 ± 1.2	3.2 ± 0.2	34	29	232 ± 21
25 - 31	709 ± 61	179 ± 26	145 ± 17	106 ± 15	35 ± 9	15.4 ± 1.3	3.1 ± 0.3	39	27	266 ± 39
32 - 37	673 ± 41	191 ± 15	159 ± 15	121 ± 13	35 ± 8	17.9 ± 1.4	3.6 ± 0.3	38	24	287 ± 13
> 38	777 ± 86	291 ± 17	236 ± 16	198 ± 13	34 ± 7	19.3 ± 2.1	3.9 ± 0.4	38	16	215 ± 27

¹ This fraction was calculated and not determined directly.² Fraction X is defined as total lipids minus cholesterol, phospholipids, and triglycerides.

Figure 2. The relationship between the ratio of LDL-VLDL/total cholesterol to color intensity of precipitated lipoproteins as the incidence of fatty liver changes.

We were able to confirm fatty liver by determining the relative levels of LDL+VLDL, which was done by precipitation and determining the cholesterol fraction. Furthermore, the response of cows to treatment could be predicted and correlated. Very low HDL in the serum related to poor response and the need for many treatments. An interesting observation was made that whenever LDL+VLDL levels were low to very low, the color of the precipitates of lipoproteins were white, whereas if the levels were high to normal the precipitates of LDL+VLDL were yellow. The cause and origin of the yellow color is not clear, but it may be related to phospholipids, which are also constituents of the lipoproteins. There were cases where the lipoprotein profile was measured during the dry period before calving, and whenever LDL/VLDL was low, there was a higher incidence of fatty liver. This points to the possible role of lipoproteins in the onset of fatty liver and their possible use as a marker. Whether this characteristic is genetic or not needs to be studied further.

Table 3 contains the data for parameters related to the color intensity of precipitated lipoproteins. (The color scale is from 11 for dark yellow to 1 for white.) The most pronounced change is the decrease in the percentage of LDL+VLDL, which is correlated to color intensity. This is paralleled to total cholesterol and phospholipid. The biggest decrease was seen in LDL+VLDL (89%), while the decrease for triglyceride was only 35%. These changes may point out basic changes in the composition of the lipoproteins. The ratio of LDL+VLDL/total cholesterol versus color intensity of precipitated lipoproteins is also shown graphically in Figure 2.

The correlations between several parameters were calculated. For LDL+VLDL/total cholesterol versus color intensity of precipitated lipoprotein the r was 0.85 when calculated for a linear relationship and 0.90 for an exponential relationship. For LDL+VLDL/total cholesterol versus phospholipids the r value was 0.87 and versus β -hydroxybutyrate it was -0.64. There also were good correlations between color intensity of the precipitated lipoproteins and ketone bodies. The origin and type of the yellow color is not clear, but it probably relates to phospholipids. As with other experiments, a high correlation was seen between relative levels of LDL+VLDL and color intensity of the precipitate with ketosis and fatty liver. In other words, a low ratio of LDL+VLDL/total cholesterol is parallel to a higher degree of ketosis and liver fattening.

The lipoproteins LDL and VLDL have a role in the induction and etiology of liver fattening. Low LDL+VLDL is associated with fatty liver induction and presence (Table 4). The association may be related to the role in lipid transportation.

Table 3. SERUM LEVELS OF LIPID FRACTIONS IN RELATION TO COLOR INTENSITY OF THE LIPOPROTEIN PRECIPITATED.

Color Scale	n	β -hydroxy- butyrate	Total Cholesterol	HDL Cholesterol	Phospho- lipids	Triglycerides	LDL+ VLDL	LDL+VLDL Total Chol.
			-----mg per 100 ml	-----	-----	-----	-----	%
11	5	22.2	159	123	186	19.2	33.9	22.3
10	4	32.6	110	89	144	19.7	21.3	17.9
9	4	31.3	114	99	140	13.1	14.	12.1
8	5	31.0	90	81	92	12.2	10.4	11.7
7	13	37.8	104	92	133	14.5	10.9	10.3
6	11	26.8	94	82	118	17.8	9.9	11.0
5	8	34.8	95	90	107	19.2	7.6	8.1
4	5	42.4	67	60	86	17.3	3.8	5.9
3	4	56.2	69	62	94	14.7	6.5	10.0
2	11	30.3	95	90	110	15.8	6.3	6.6
1	5	15.7	59	56	83	12.5	3.6	6.2
% change		--	-63%	54%	-83%	-35%	-89%	-72%

TABLE 4. SUMMARY OF THE INTERRELATIONS BETWEEN LIPOPROTEIN AND FATTY LIVER.

Item	Normal cows Cows with fatty liver	
	(characteristic or response)	
<u>LDL+VLDL</u> total cholesterol	increased to above 15	decreased to below 10
color of precipitate of serum lipoproteins	yellow	white
ketone bodies	decreased	increased
response to treatment of ketosis	good	poor

Cows with a low ratio of LDL+VLDL to total cholesterol showed a higher frequency of fatty liver formation. Furthermore, it seems that cows with a low ratio in their dry period will develop fatty liver, and when fatty liver is developed the response of those cows to treatment is poorer, i.e., more treatments are needed. We have used the ratio as a marker to tell the veterinary practitioner about progress and chances for effective treatment with good success.

Table 5 summarizes a study in which blood was taken from cows before and after slaughter. The cows had some degree of ketosis and eventually at a later date were slaughtered. At slaughter, liver samples were taken and the degree of fattening later was determined histologically. The results show there was an increase in β -hydroxybutyrate, and decreases in HDL, total cholesterol and the ratio of LDL+VLDL/total cholesterol. Correlation coefficients were calculated for various parameters and are as follows:

β -hydroxybutyrate vs. glucose	$r = 0.65$ (n=39)
β -hydroxybutyrate vs. amylase	$r = 0.14$
degree of liver fattening vs. β -hydroxybutyrate	$r = 0.67$ (n=15)
degree of liver fattening vs. HDL	$r = -0.60$ (n=15)
degree of liver fattening vs. total cholesterol	$r = -0.70$ (n=15)
degree of liver fattening vs. LDL+VLDL/total cholesterol	$r = -0.84$ (n=14)
degree of liver fattening vs. phospholipids	$r = -0.69$ (n=13)
degree of liver fattening vs. triglycerides	$r = -0.35$ (n=14)

TABLE 5. Summary of Changes in Some Blood Constituents with Different Degrees of Fatty Liver

Degree of liver fattening		β -hydroxy-butyrate	Total Cholesterol	Phospho-lipids	<u>LDL+VLDL</u> Total Chol.	HDL
0 - 1	$\bar{X}$	12.5	122	140	24.1	96
	SD	10	67	63	20.4	50
	n	16	16	14	16	16
1 - 2	$\bar{X}$	11	135	100	18.0	111
	SD	9	75	43	14.1	65
	n	7	8	5	8	8
3 - 4	$\bar{X}$	24.0	70	115	7.3	63
	SD	25.5	34	45	3.7	32
	n	6	6	4	6	6

The general message of Section IV is that there is a strong relationship between fatty liver and the lipoprotein profile in the blood of cows. While the lipoprotein picture of the mature cow is entirely different from that of humans, its role in lipid mobilization and metabolism is very similar. In humans the major lipoprotein is LDL+VLDL, with HDL being the major fraction, while in cows the LDL+VLDL fractions are minor and HDL is the major fraction. Normally HDL comprises about 80% of the cholesterol in cow serum, but under pathological fatty liver it consists of over 90%. This change is seen mainly in fatty liver and not in other metabolic diseases. We did one trial to establish a method for detecting fatty liver based upon an immunological-chemical reaction but it failed because of a lack of specific antibodies against the cows apolipoproteins. (Both human and goat antibodies were tested but there was no cross reaction.) If a reactive antibody were available, mass screening of cows for the characteristics of fatty liver induction under conditions of metabolic stress could be done.

In summary, our studies looked into the biochemical aspects of fatty liver, comparing blood and liver of normal cows and cows having fatty liver. We have shown that there were significant changes in the liver during fattening as well as changes in the serum. Of major interest is the change seen in the lipoprotein profile. These changes seem to be related to the onset of liver fattening, which can be

detected even at the dry period prior to the process of liver fattening. Possibly these changes can serve as a marker for fatty liver susceptibility and response to treatment. The cause for lower LDL+VLDL lipoproteins is not clear and requires further investigation.

F. DESCRIPTION OF COOPERATION:

One very meaningful aspect of cooperation was exchange visits of the principal scientists to each other's laboratories. Dr. Young visited Dr. Bogin's laboratory during the first two weeks of May 1988. Dr. Bogin reciprocated during July of 1989. The research approaches of the two groups are very different, with Dr. Bogin's group focusing on field cases and Dr. Young's group using a model fatty liver and ketosis that is induced experimentally. The exchange of ideas and detailed discussion of results obtained were extremely helpful during each visit and helped greatly to get a broader understanding of fatty liver.

There also was a valuable exchange of research materials. The Iowa State group supplied Dr. Bogin with a reagent kit for an effective assay of free fatty acids in blood, with a barrel of 1,3-butanediol for some induction experiments, and with an antibody for goat LDL and VLDL. There was no exchange of blood or liver samples for different assays because each laboratory was well equipped to do the assays described in the original proposal.

The most valuable benefit of the cooperation to date, however, is providing the basis for future collaboration. For example, the Iowa State group will utilize the information generated about relationships of lipoproteins to fatty liver by the Israeli group to test the authenticity of the model fatty liver and ketosis they are studying. The Israeli group will utilize the information obtained by the Iowa State group on the sequence of events leading to fatty liver and the subsequent ketosis to field-test when is the ideal time to test early postpartum cows for fatty liver or susceptibility to fatty liver.

G. EVALUATION OF RESEARCH ACHIEVEMENTS:

In our opinion, the research achievements and progress during this project have been excellent. With respect to the first two objectives, we believe we have shown that the FRBD (feed restriction plus dietary 1,3-butanediol) model is valid and can be a valuable tool in gaining complete understanding of fatty liver and lactation ketosis in dairy cows. We have learned much about the sequence of events leading to fatty liver and ketosis. Foremost is the indication that the triglyceride to glycogen ratio of the liver can be an important tool in determining susceptibility to fatty liver and ketosis. Susceptibility seems to increase as the ratio increases above 2 and to

decrease as the ratio decreases below 2. To be of benefit to dairy producers we need to find a way to get a reasonably accurate estimate of this ratio without having to take puncture biopsies of the liver. Future research will explore possible estimators that will have a very high correlation with the triglyceride-to-glycogen ratio.

Much information also has been gained relating to objectives three and four. Many parameters have been measured in blood obtained from cows on the farm and at slaughter and in liver tissue samples obtained at slaughter. Several blood constituents seem to have a high correlation with the degree of liver fattening but the most promising one for a diagnostic measure is the ratio of LDL+VLDL to total cholesterol. If antibodies can be developed to these constituents, a possible diagnostic procedure might be developed that would be of major benefit to veterinarians in charge of high-producing dairy herds.

H. LIST OF PUBLICATIONS:

Ph.D. Dissertations

- ✓ Veenhuizen, J. J. 1987. Progressive changes in metabolism of cows during induction of ketosis and treatment of ketosis with intestinal administration of glucose. Ph.D. dissertation. Iowa State University.
- ✓ Drackley, J. K. 1989. Factors contributing to development of fatty liver and ketosis in lactating dairy cows. Ph.D. dissertation. Iowa State University.

Refereed Journal Publications

- Drackley, J. K., M. J. Richard, and J. W. Young. 1990. In vitro production of β -hydroxybutyrate from 1,3-butanediol by bovine liver, rumen mucosa, and kidney. J. Dairy Sci. 73:679-682.
- Veenhuizen, J. J., M. J. Richard, T. P. Sanderson, L. D. Miller, and J. W. Young. 1990. Metabolites in blood and liver during development and early treatment of experimental fatty liver and ketosis in cows. J. Dairy Sci. (Ready for submission.)
- Drackley, J. K., J. J. Veenhuizen, M. J. Richard, and J. W. Young. 1990. Fatty liver and ketosis are not induced by either feed restriction or dietary 1,3-butanediol as separate treatments. J. Dairy Sci. (Ready for submission.)

Drackley, J. K., M. J. Richard, D. C. Beitz, and J. W. Young. 1990. Subclinical ketosis in dairy cows in response to feed restriction and dietary 1,3-butanediol. *J. Dairy Sci.* (Ready for submission.)

Abstracts of Papers at National meetings

- ✓ Veenhuizen, J. J. and J. W. Young. 1987. Progressive changes in gluconeogenesis in liver during an experimental ketosis. *Fed. Proc.* 46:1179.
- ✓ Veenhuizen, J. J., J. W. Young, M. J. Richard, J. K. Drackley, K. B. Atkins, and A. D. McGilliard. 1987. Effects of induction and treatment of lactation ketosis on milk yield, fatty liver, and blood metabolites in cows. *J. Dairy Sci.* 70 (suppl. 1):106.
- Sanderson, T. P., L. D. Miller, J. J. Veenhuizen, J. K. Drackley, and J. W. Young. 1987. Histopathology of liver biopsies from dairy cows with induced lactation ketosis. Abstracts of papers presented at the 68th Annual Meeting of the Conference of Research Workers in Animal Disease.
- ✓ Drackley, J. K., M. J. Richard, B. D. Strang, R. O. Harrison, D. M. Amaral, and J. W. Young. 1988. Neither feed restriction nor 1,3-butanediol separately will induce fatty liver or ketosis in high-producing dairy cows. *J. Dairy Sci.* 71 (suppl. 1):177.
- ✓ Drackley, J. K., J. W. Young, M. J. Richard, and D. C. Beitz. 1989. Palmitate metabolism by liver from lactating cows. *FASEB J.* 3:A1256.
- ✓ Drackley, J. K., M. J. Richard, D. C. Beitz, and J. W. Young. 1989. Lipid metabolism during an induced subclinical ketosis in dairy cows. *J. Dairy Sci.* 72:543 (suppl. 1).
- ✓ Strang, B. D., T. R. Smith, L. R. Hansen, and J. W. Young. 1990. Response of blood glucose to epinephrine challenges in high-producing normal and obese cows in early lactation. *J. Dairy Sci.* 73 (suppl. 1):135.