EFFECT OF PHLORIDZIN, INSULIN, AND BUTYRATE ON THE CONCENTRATION OF GLUCOSE AND KETONES IN THE BLOOD AND URINE OF FASTED STEERS

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SUMMARY

Seven experiments were conducted in an attempt to produce both clinical and biochemical symptoms of bovine ketosis. The treatments imposed were phloridzin, which caused a lowering of the renal threshold for glucose, phloridzin plus 800 units of insulin, which depressed the total reducing substances (T.R.S.) level in the blood during a period of increased glucose demand due to the lowered renal threshold, and the infusion of 25 g. per hour of butyrate, either by itself or with phloridzin and insulin.

When phloridzin was infused following a 48- to 72-hr. fast, 56.62 g. of glucose were voided in the urine in 8 hr. Only a moderate fall in blood T.R.S. level and a moderate rise in blood and urine ketone levels were observed.

When two intramuscular injections of 400 units of insulin were superimposed on phloridzin infusion, the blood T.R.S. level rapidly dropped to the ketotic level. The blood and urine ketone levels were less than with phloridzin alone and less glucose was voided in the urine.

The infusion of butyrate at the rate of 25 g. per hour caused a very rapid drop in the T.R.S. level of the blood. Even at this high level of butyrate infusion, the level of ketones found in the blood approached ketotic levels only if phloridzin was infused concurrently. This observation adds support to the observation and theoretical postulation that a glucose stress is a predisposing factor in ketone formation. The butyrate concentration in the blood increased to a peak early in the infusion period, followed by a reduction and the establishment of a plateau. Approximately 185 g. of butyrate were thus metabolized during an 8-hr. period without adverse effect upon the animal.

No clinical symptoms of hypoglycemic shock were observed under any of the experimental treatments applied.

One of the primary deterrents to a solution of the cause-and-effect relationships involved in bovine ketosis has been the insidiousness of the disorder and an inability of research workers to produce the syndrome at will. Bovine ketosis is defined as a metabolic disorder of high-producing cows that is characterized by low blood glucose, elevated blood and urinary ketone bodies, poor appetite, loss in milk production, and either lethargy or high excitability. Paresis or incoordination may be present and a rapid loss in condition occurs if the syndrome is not corrected (17). The death loss from bovine ketosis is negligible because spontaneous recovery usually occurs when milk production has been depressed sufficiently. Although few animals die of the disorder, milk production for the entire lactation is considerably reduced as is income from milk.

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1 The data presented are from a thesis submitted by the senior author to the Graduate College, University of Illinois, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
The currently popular ketosis-producing substances that are used in treating ketotic cows or attempting to prevent its occurrence in cows with a previous history of the upset are, (a) glucose and/or other monosaccharides, (b) propionates, glycerol, or lactates, and (c) adrenocorticotropic hormone (ACTH), adrenocorticoids, or synthetic compounds with adrenocorticoid activity. These treatments and preventive measures would each add in some manner to the amount of available carbohydrate in the system. The compounds in Group 1 add carbohydrate directly to the blood. The Group 2 compounds are made up of three-carbon units that are glucogenic if absorbed unchanged from the rumen or are converted by rumen bacteria to other three-carbon compounds that are glucogenic when absorbed. The hormones and hormone-like substances in Group 3 step up gluconeogenesis primarily from amino acids by increasing the endogenous production of adrenocorticoids via ACTH, or increasing the corticoid level by adding the hormone or hormone-like substance from an exogenous source.

A high level of milk production is usually the stressing factor postulated as the cause of ketosis in dairy cattle. Because of the curative effect of glucose and glucogenic substances, it is equally plausible and perhaps more precise to postulate that the high requirement for carbohydrate in the synthesis of lactose is the stressing agent. For example, a cow producing 25 kg. of milk containing 4.8% lactose would need 1,200 g. of carbohydrate per day for lactose synthesis alone. This amount is greater than that estimated to be available from the propionic acid formed in the rumen of a cow on full feed (17).

One problem encountered in studies of bovine ketosis has been the lack of a suitable method for experimental production of the disorder. Withholding feed from lactating cows results in an immediate decline in milk production which apparently eliminates the causative stress. If a glucose stress is the immediate cause of ketosis in lactating cows, then it should be possible to produce ketosis in steers, provided that a suitable glucose stress could be imposed. In the studies herein reported, phloridzin was administered to fasted steers to provide a prolonged glucose drain.

**EXPERIMENTAL PROCEDURE**

A Holstein steer which weighed about 350 lb. was used in all seven experiments. In each case a plastic catheter was placed in the left exterior jugular vein according to the method of Ralston et al. (14). The various solutions were infused through the catheter into the blood and blood samples were withdrawn through it. Drawing blood samples in this manner allows the removal of blood without causing discomfort to the steer or exciting him in any way. Between trials that were run on consecutive days, the tube was filled with heparinized 0.9% sodium chloride and sealed at the end with heat. All infusion solutions were administered with an intravenous drip apparatus and a gravity flow blood serum bottle. In the first four experiments the solution was infused as fast
as it would flow through a 16-gauge needle through the jugular catheter. In the last three experiments the flow rate was regulated so that one liter of solution was infused each hour.

All of the solutions used were made isotonic with mannitol, which is non-metabolizable by mammalian tissue (7, 10).

Blood proteins were precipitated by the method of Somogyi (18) for both ketone and total reducing substances analyses. Total reducing substances were measured by Nelson's (11) modification of the Somogyi method (18).

Ketones, calculated as acetone, were measured by a modification of the Greenberg and Lester (8) method in which 3 instead of 2 cc. of carbon tetrachloride were used for extraction, in order to provide enough volume for the readings to be made in a Bausch and Lomb Spectronic 20 Colorimeter.

Somogyi filtrates were made on the urine samples for ketone analyses. Urinary glucose was measured in activated charcoal filtrates with Glucostat, a commercially available glucose-oxidase and chromogen preparation. In experiments in which phloridzin was infused, one to 99 dilutions were necessary to obtain color development which was in a suitable range for reading in the colorimeter.

Blood volatile fatty acids were determined by the method described in a previous publication from this laboratory by Davis, Brown, Staubus, and Nelson (4).

Experiments 1 and 3 were run after the steer had been fasted for 48 and 72 hr., respectively. Isotonic mannitol solution (56 g. per liter) containing 0.8 g. of phloridzin per liter was dripped into the jugular as rapidly as possible (approximately 1.54 liter/hour) for an 8-hr. period. Zero time and hourly jugular blood samples were collected in tubes containing heparin and sodium fluoride. These samples were immediately refrigerated at 40° F. and the proteins were precipitated within 4 hr. after completion of the experiment. Individual urinations were collected, measured, and sampled. The urine samples were immediately frozen until analyzed.

Experiment 2 was conducted the day after Experiment 1. Experiment 4 was conducted the day following Experiment 3. No feed was available to the animal between Experiments 1 and 2 or Experiments 3 and 4. In Experiments 2 and 4, 400 units of insulin were administered intramuscularly to the steer at zero time and 4 hr. after the infusion of isotonic mannitol and phloridzin was begun. Samples were taken in the same manner as in the two previous experiments.

Experiment 5 measured the response of a normal steer starved 24 hr. to the infusion of 1 liter per hour of isotonic mannitol containing 25 g. of butyric acid neutralized with sodium hydroxide. Samples were collected and stored in the same manner as in the previous experiments, except that enough blood was withdrawn at 0, 2, 4, 6, and 8 hr. to allow analyses for volatile fatty acids.

Experiment 6 measured the response of the same steer after an additional 24-hr. fast to a butyrate infusion and glucose stress mediated by the addition

Worthington Biochemical Corporation, Freehold, New Jersey.
of 0.8 g. of phloridzin per liter of infusate. Sampling proceeded as in Experiment 5.

In Experiment 7, following an additional 24-hr. fast, two subcutaneous injections of 400 units of insulin were superimposed on the treatment administered in Experiment 6. Samples homologous to those in Experiments 5 and 6 were collected.

EXPERIMENTAL RESULTS

Figure 1 shows the effect of phloridzin and phloridzin plus insulin administration on the concentration of T.R.S. in the blood. After a starvation period of 96 hr., the steer was able to maintain the total reducing substances (T.R.S.) in the jugular blood within the normal range. On the first day of the experiment the mean zero time T.R.S. was 47.4 mg.% and on the following day it was slightly higher, 50.2 mg.%. During the course of an 8-hr. infusion of isotonic mannitol and phloridzin there was a progressive drop in T.R.S., but even at the end of the period, the animal was still maintaining a level of 30.4 mg.%, which is above the level that is generally considered to be indicative of ketosis. However, under the influence of both phloridzin and 400 units of insulin, the blood T.R.S. level dropped rapidly from 50.2 to 11.0 mg.% at the end of 3 hr. After reaching this low, some recovery occurred at 4 hr. and continued, in spite of the intramuscular injection of 400 additional units of insulin, to the fifth hour, after which a slow decline began. However, it should be noted that from the third hour on, the blood T.R.S. level was within a range that is usually associated with severe ketosis.
The average magnitude of the glucose stress mediated by phloridzin was 55.81 and 30.81 g. in Experiments 1 and 3 and 2 and 4, respectively. It is apparent that the amount of glucose which was filtered out and excreted in the urine was somewhat dependent upon the level present in the blood which passes through the kidney. Although the T.R.S. data cited in Figure 1 are from jugular blood, it is probably indicative of the level present in the arterial blood passing through the kidney in relative if not absolute terms. Therefore, it is not surprising that a greater amount of glucose was voided in the urine when phloridzin was used alone than was voided when insulin injections were superimposed on phloridzin. The experimental glucose drain resulting from phloridzin and phloridzin plus insulin treatment is equal in magnitude to the more normal glucose stress of a 1,200-lb. cow producing 28.76 and 16.28 lb. of milk, respectively, assuming 100% conversion of glucose to lactose by the mammary gland. This production should also be considered to be existing after from three to five days of fasting.

Figure 2 shows the concentration of ketone bodies, measured as acetone, observed in jugular blood throughout Experiments 1 through 4. It is readily apparent that although the concentration of ketone bodies did not exceed normal fasting levels, even with an artificial glucose stress, the injections of insulin, in spite of lowering the blood T.R.S. level, appeared to reduce the buildup of blood ketone bodies.
This difference in buildup of ketone bodies in the jugular vein blood was not due to a differential rate of spillage into the urine, because the cumulative excretion into the urine averaged 554 mg. in Experiments 1 and 3 and 520 mg. in Experiments 2 and 4. Such spillages are insignificant and reflect the lack of ketone body buildup in the blood.

To eliminate the possibility that this rather surprising lack of ketone body formation was due to a lack of ketogenic substances, the steer was placed on a normal ration with a high proportion of grain for a month and then isotonic mannitol and butyric acid, which is highly ketogenic, were infused together, with phloridzin and with phloridzin and subcutaneous injections of insulin.

It is interesting to note in Figure 3 that in all three experiments in which 25 g. of butyric acid were infused per hour the T.R.S. dropped to ketotic levels in 3 hr. or less. In fact, the curve for butyric acid alone seems to drop from its higher initial T.R.S. level at the same rate as the curve for butyric acid plus phloridzin plus insulin. Following the initial drop, the T.R.S. level stabilized between 15 and 20 mg.%. 

Butyric acid alone caused an insignificant excretion of glucose into the urine. The excretion due to the inclusion of phloridzin in the infusate and insulin injections gave totals of 56.62 and 38.13 g., respectively, as compared with 54.40 and 30.81 g. when no butyrate was administered. Such close agreement is somewhat surprising in light of the lower blood T.R.S. levels during the infusion of butyrate.

The ability of the steer to utilize high levels of ketogenic substances is shown in Figure 4. In spite of the infusion of 25 g. of butyric acid per hour,
the blood ketone level did not exceed the normal range in the case of butyrate alone or butyrate plus phloridzin plus insulin. Only in the case of butyrate plus phloridzin did the concentration exceed normal. Even then it rose only to the lower limit of that which is considered ketotic.

The cumulative urinary excretion of ketone bodies in Experiments 5, 6, and 7 amounted to 1.76, 3.54, and 3.12 g., respectively. The similarity in the cumulative amounts of ketone bodies excreted in the urine is a bit surprising. Under the current theory of unselective filtration of substances by the glomeruli of the kidney and selective resorption by the tubules, one would expect total excretion to follow the concentration in the blood quite closely. However, under the conditions of these experiments very nearly the same amounts were excreted either with or without insulin and 50% as much was excreted with butyrate alone.

Since such a large amount of butyric acid was infused in each experiment, the concentrations of the volatile fatty acids in jugular blood were measured at 2-hr. intervals. These concentrations are plotted in Figure 5. Propionate was not present in the jugular blood in measurable quantities at any time during the butyrate experiments. The acetate level was very similar in all three tests and approximated normal fasting levels, so the acetate values are plotted as a composite value for all three experiments.

In all three butyrate experiments, there was a considerable increase in the butyrate level of the blood. The zero-time sample in the first two experiments showed a negligible amount which is normal and showed no residual carryover from Experiments 5 to 6. In Experiment 7 there appeared to be some slight carryover, but it amounted to only 1.32 mg.%. However, in all cases, 2 hr. after
the infusion began the concentration of butyrate began to increase. This deviation from normal can be attributed to two possible causes. The first possibility is that 25 g. of butyrate per hour is more than the animal normally would absorb and, thus, the threshold of the liver’s ability to remove and metabolize butyrate has been exceeded. The second possibility is that under normal conditions all of the butyrate which is available comes from the rumen and goes directly to the liver; whereas, under the conditions of these experiments butyrate was introduced into the jugular vein and thus was mixed throughout the circulating blood before the liver had an opportunity to metabolize it. However, such an explanation is not consistent with the decrease in concentration observed during the last 2 hr. while the rate of infusion remained constant. Such a decrease is to be expected only under conditions of increased rate of metabolism. Why such a response should occur and how it is mediated is not clear.

**DISCUSSION**

The observations made in these seven experiments indicate that the bovine’s ability to maintain homeostasis or nearly normal physiological conditions is tremendous. The animal is apparently able to conserve and store carbohydrate in spite of extended periods of fasting and integrate other energy-yielding
compounds from both exogenous and endogenous sources into metabolic pathways in such a manner that, even under the artificial stresses of fasting and phloridzin imposed in these experiments, the deviations from normal are small.

Application of the principles established in monogastric animals to polygastric (ruminant) animals would imply a rapid disappearance of liver glycogen (5) and, thereby, a deficiency of readily available glucogenic substances under the conditions imposed in these experiments. However, the animal was apparently able to maintain near-normal blood levels of T.R.S. in spite of a sizable glucose drain (55.81 g. in 8 hr.) superimposed upon 48 to 72 hr. of fasting. After another 24-hr. period of fasting, plus an additional glucose drain, the animal could resist insulin shock and exhibit less blood ketones than were present the previous day. Such observations are direct evidence that the animal can maintain near-normal functioning of its nervous tissue at a T.R.S. level which would produce insulin shock in monogastric animals (15) and still have a readily available source of glucogenic substances, because no severe drop in blood T.R.S. levels was observed following a sudden glucose drain (phloridzin). Although no direct evidence was available in these experiments, to pinpoint the source of the glucogenic substance necessary to prevent a drop in T.R.S. level, liver glycogen is the most likely source, due to the rapidity with which it can be mobilized. Since this postulation requires the assumption of considerable glycogen stores in the liver, even after 72 hr. of fasting, which is a marked deviation from results observed in monogastric animals, it would be interesting to investigate it further via liver biopsy and glycogen analysis during similar experiments.

It is unlikely that the adrenal medullary response activated via the hormone epinephrine was functioning during the course of the experiments. The mechanism is brought into play by a psychic stimulation (3) and causes the fight-or-run response. However, the major reserve of glucogenic substance which this hormone can tap is liver glycogen and should, thereby, be limited by the conditions cited above. Another factor which seems to argue against this mechanism being brought into play in the experiments reported here is the calmness with which the steer submitted to the treatments applied, the ease with which blood samples were drawn via the jugular catheter, and the lack of unusual deviations in the hourly blood samples within an experiment and between similar experiments.

Attempts to assay for urinary nitrogen increases as a measure of protein mobilization and degradation due to adrenocorticosteroids were vitiated by the large volume of urine excreted. The urinary output ranged up to 13.73 liters per 8-hr. period and so diluted the urinary nitrogen that direct measurements were impossible. This high rate of urine output did not cause serious dehydration of the animal, since it was nearly balanced by the infusion of isotonic mannitol solutions. In the experimental period in which 13.73 liters of urine was voided, 11.50 liters of mannitol was infused into the jugular vein. Only during those experiments run when the environmental temperatures were above 80°F. did the steer show signs of thirstiness, or accept water from a bucket.
The response of the steer to insulin is quite unusual, since it parallels the response in diabetic humans. This animal failed to show any of the symptoms of insulin shock and had lower levels of ketone bodies in his blood after insulin treatment than during administration of phloridzin alone, even though the T.R.S. concentration was lower. This observation implies that the body tissues were more capable of metabolizing ketones or that ketone formation was less at this low T.R.S. level than at a higher, more normal level. Since only extra-hepatic tissue is considered capable of metabolizing ketones (6, 7), and the major pathway of oxidation is considered to be via the tricarboxylic acid cycle, a carbohydrate sparker in the form of oxalacetic acid must be present in order to oxidize C₂ pieces resulting from the cleavage of ketone bodies. The most logical explanation for this somewhat contradictory situation is that the insulin probably decreased the threshold level required for the passage of glucose across the cell membranes and thus built up the reserve of glucose or glucogenic substance in the cells. Due to this reserve, the animal was actually more able to metabolize ketone bodies as fast as they were available at a low blood level of T.R.S. than at a normal level. However, as this reserve was dissipated the ketones began to build up at a rate faster than that observed with phloridzin.

The low levels of ketones in the urine reflect the low levels in the blood and reaffirm the ability of the kidney to resorb utilizable substrate up to a given threshold. A sizable portion of the spillage observed from low blood ketones may have been due to the high filtration of water and mannitol from the blood and a mechanical interference with resorption due to the volume of liquid passing through the kid-ney tubes.

The sudden and sharp decline in blood T.R.S. values following the infusion of isotonic mannitol and butyrate, and the closeness with which it parallels the decline observed under the influence of both insulin and butyrate, suggests that the drop in blood T.R.S. level may be mediated through the release of endogenous insulin from the pancreas. The possibility, of course, is no more than suggested by the data obtained in these trials, but as soon as a good test for insulin levels in the blood is available, it would be an interesting phenomenon to investigate. The similarity in the blood ketone and butyrate concentrations is also evidence which would tend to point toward this possibility, because T.R.S. levels dropped, ketone levels failed to rise as rapidly, and blood butyrate did not build up as high, implying a more rapid transfer of glucose across cell membranes and an increased ability to metabolize butyrate.

A sharp deviation from the response reported by others (1, 2, 16) to oral or intraruminal drip of butyrate was observed when the butyrate was infused into the jugular vein. In the experiments reported here, the ketone levels in the blood did not exceed normal levels, except in the case of butyrate super-imposed on phloridzin. This contrasts sharply with levels of 35 to 70 mg/100 ml. which are reported following the introduction of proportionally smaller amounts of butyrate into the rumens of sheep and goats. This could be a species difference, but this is not probable. The high levels of ketones found in ketotic cows would indicate that the bovine can accumulate ketone bodies under certain conditions. The primary difference in the two methods of butyrate
administration seems to be concerned with the rumen. Pennington (12, 13) reports that, in studies in which ruminal epithelium is incubated with butyrate as the sole substrate, large quantities of ketones are formed. Numerous workers (9, 17) have projected this observation and state that butyrate is converted to β-hydroxybutyrate during its passage through the rumen wall. This is hardly enough evidence to establish such a mechanism as operative in an intact animal whose ruminal epithelium is exposed to all of the substrates available in the circulating blood. Rumen perfusion data (19) show that when the concentration of butyrate in rumen fluid is increased to high levels by the injection of butyric acid, low levels of ketones appear in the perfusate, but these are quite insignificant when compared to the amount of free butyrate which is absorbed into the blood. In the course of the perfusion the ruminal tissue uses considerable quantities of glucose from the perfusate, casting some doubt on the reliability of single substrate incubations when projected into intact systems.

The buildup of butyrate in the jugular blood is also a sharp deviation from results observed when butyrate is dripped into the rumen. If butyrate is absorbed unchanged in the intact animal, as it is in the perfused rumen, it would be expected that it would be transported to a large degree as the sodium salt due to the pH of blood and sodium being the major extracellular base. This would imply little or no difference in the form in which the butyrate would be available at the tissue level and gives little cause for this difference. The most obvious differences are amount of butyrate supplied and its route of entry into the system.

For the amount to be important, it would have to be postulated that the system was unable to metabolize butyrate at the rate at which it was infused during the early part of the experiment but, during the period, was able to increase its metabolic capacity for butyrate and, thus, by the end of the period, reduce the blood butyrate level from its peak to a level which it could maintain when butyrate metabolism was maximum. It also appears that such a maximum rate of butyrate metabolism can be established faster even at a similar level of blood T.R.S. if the glucose drain is nonexistent or reduced somewhat as in the case of Experiments 5 and 7. Another interesting difference between the methods is the route of travel of the butyrate after its introduction into the body. In the case of the ruminal drip, all newly introduced butyrate would be transported directly to the liver via the portal vein, whereas with jugular infusion the butyrate would be distributed randomly throughout the circulatory system and would not be presented directly to the liver. Under normal conditions butyrate does not appear in large amounts in the blood that is coming from the liver and the predominant V.F.A. present in peripheral blood is acetate. If no increase in jugular blood butyrate follows its intraruminal drip, then this system apparently has a large capacity and removes butyrate completely. However, this difference in route of passage does not explain the early peak concentration of butyrate followed by a reduction to a lower level at which it stabilized. This would imply an increase in the rate of butyrate metabolism.
The fact that this steer was able to maintain normal function throughout an 8-hr. infusion period in which a total of 200 g. of butyrate was infused is an interesting observation concerning the bovine's ability to utilize butyrate. Since less than 15 g. of the infused butyrate could be accounted for as blood butyrate and ketones or urinary ketones, it must be assumed that the remainder of the dose infused (185 g.) was metabolized during the period. No direct evidence concerning the form in which it became available to the tissues was obtained in this experiment. However, if it became available as activated 2-carbon fragments (acetyl coenzyme A), it was rapidly utilized in this form and no significant amount of it was inactivated and released as free acetate into the blood because blood acetate remained low throughout the experiments. However, this does not rule out the release of inactivated 2-carbon fragments by the liver, transport by the blood to extrahepatic tissue, and reactivation and utilization there at a rate fast enough to prevent an increase to normal levels in the blood.

Similar reasoning would imply that if butyrate is oxidized to aceto-acetic acid (7) and metabolized as such, its removal from the blood is rapid enough that there is no large increase in blood ketones.

The most pertinent observation from these experiments, when considered as a whole, is that a simple glucose stress of the magnitude induced in these experiments is not sufficient to cause the clinical symptoms of bovine ketosis, even when superimposed on up to 96 hr. of fasting and high infusion rates of a ketogenic substance (butyrate). Therefore, experimental production of clinical symptoms of bovine ketosis is dependent upon either more severe glucose stress or some other key trigger mechanism.

REFERENCES