This is to certify that the

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Use of Hydrogen Gas (H_2) Excretion to Assess Small Intestinal Malabsorption in Calves.

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USE OF HYDROGEN GAS (H_2) EXCRETION TO ASSESS SMALL INTESTINAL MALABSORPTION IN CALVES

BY

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ABSTRACT

USE OF HYDROGEN GAS (H_2) EXCRETION TO ASSESS SMALL INTESTINAL MALABSORPTION IN CALVES

By

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Small intestinal carbohydrate and protein malabsorption, associated with increased end and total-expired breath H_2 excretion, was demonstrated in experiment 1. Oral administration of lactulose signi ficantly increased ($P < 0.001$) H_2 excretion over values measured before it was given. Hydrogen excreted after chloramphenicol administration was significantly increased ($P < 0.001$) from values measured after feeding milk alone. Concurrently, chloramphenicol administration significantly decreased intestinal villous length ($P < 0.001$), and D-Xylose absorption ($P < 0.05$), compared to values before treatment was given.

In experiment 2, small intestinal malabsorption and diarrhea occurred after inoculation of *Cryptosporidium sp*. End and total-expired breath H_2 excretion, and fecal production were significantly increased ($P < 0.001$, $P < 0.025$, and $P = 0.06$, respectively). These observations were limited to the diarrheal stage (Stage 2).

Results of feeding 2 diets on H_2 excretion are reported in experiment 3. Values measured for end and total-expired breath H_2 excretion were significantly higher ($P < 0.05$) while feeding diet 2 (hay and concentrate) compared to values measured for diet 1 (whole milk).

DEDICATION

To my parents, Mr. and Mrs. S. A. Holland
and my wife, Margo Steele-Holland

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Dr. Thomas H. Herdt, my major advisor, for his encouragement, guidance and support during the course of this study. I am grateful to Dr. John B. Kaneene for serving as my major advisor in Dr. Herdt's absence. His persistent guidance and unwavering support was relentless through the completion of this thesis. Appreciation and special thanks are extended to Dr. John C. Baker, Dr. Frederik J. Derksen and Dr. Howard D. Stowe, members of my graduate committee, for their stimulating, yet constructive criticism and editorial assistance during the preparation of this thesis. I am grateful to Dr. Edward C. Mather, Chairman, Department of Large Animal Sciences for securing the funds necessary for this project and for providing laboratory space and animal care facilities. Special thanks are extended to Dr. Richard B. Talbot for his encouragement, assistance and interest, and to Dr. Kent R. Refsal for his knowledge and expertise in statistics and statistical modeling. A special thanks is extended to Dr. John L. Gill, Department of Animal Science, for assistance in the statistical analysis, Dr. S. D. Grimes, Department of Pathology, for critiquing this thesis and Ms. S. Eisenhauer for clerical assistance.

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INTRODUCTION

The mucosa of the small intestine of neonatal calves is affected by a variety of infectious and noxious agents. Insults to the mucosa, associated with bacterial, viral and protozoal inflammatory diseases, cause villous atrophy and decreased villous epithelial cell function. Similarly, the oral administration of certain antibiotics and the feeding of soybean protein have resulted in villous atrophy as well as crypt hyperplasia. Accompanying villous atrophy is a decrease in brush-border enzymes which are necessary for carbohydrate and protein hydrolysis(lactase and peptidases) and the efficient operation of the sodium-dependent transport process (Na.K.ATPase). The enzymes are synthesized by and located within the microvillous membrane of intestinal epithelial cells. Disruption of the mucosa, and associated villous atrophy results in carbohydrate and protein maldigestion, malabsorption and diarrhea.

Dietary carbohydrates and proteins presented to the normal small intestine are enzymatically hydrolyzed into smaller, more readily absorbed components. In malabsorptive conditions, they remain either nondigested or nonabsorbed and pass into the large intestine where they are 1) fermented by colonic bacteria, and 2) increase the osmotic pressure within the intestinal lumen. Colonic bacteria ferment the nonabsorbed carbohydrates and proteins into organic acids and gases.

Organic acids increase the osmotic effects of the nonabsorbed carbohydrates, thereby contributing to the diarrhea and acidosis. The gases produced are carbon dioxide (CO_2), hydrogen (H_2) and methane (CH_4). A portion of the H_2 thus produced is transferred via the blood to the lungs and is excreted in the breath.

In humans, determination of pulmonary H_2 excretion has been used for the evaluation of small intestinal carbohydrate malabsorption. Under normal conditions, H_2 production within the small intestine is negligible. With complete absorption of dietary carbohydrates and protein, little change in H_2 excretion is observed. However, in small intestinal malabsorptive conditions, nonabsorbed carbohydrates and proteins pass into the large intestine where bacterial fermentation occurs, resulting in increased H_2 production.

The specific objective of these experiments was to measure end-expired breath and total-expired breath H_2 excretion as a means of evaluating small intestinal carbohydrate and protein malabsorption in preruminating calves. The primary objectives were 1) to measure end-expired breath and total-expired breath H_2 excretion resulting from induced carbohydrate and protein malabsorption, 2) to determine the changes in breath H_2 excretion resulting from Cryptosporidium-induced diarrhea, and 3) to determine the effects of diet on H_2 excretion.

EXPERIMENT 1:

Breath Hydrogen Concentration and Small Intestinal Malabsorption in Calves

SUMMARY

Breath hydrogen concentrations were measured to assess intestinal carbohydrate malabsorption in preruminanting calves. Oral administration of 1.25 g of lactulose (a nonabsorbable carbohydrate)/kg to calves produced breath hydrogen concentrations significantly higher ($P < 0.001$) than values determined after calves were fed milk and before the treatment was given. This indicates that, in the calf, fermentation of nonabsorbed carbohydrates results in increased breath hydrogen values. To induce small intestinal malabsorption, chloramphenicol was administered orally at 50 mg/kg, 2 times a day, to 5 calves for 3 days. Before therapy was started, each calf was fitted with a duodenal cannula to facilitate collection of intestinal mucosal biopsy samples during treatment. Chloramphenicol therapy significantly increased ($P < 0.001$) breath hydrogen concentrations from those measured after calves were fed milk alone. Concurrently, chloramphenicol administration significantly decreased intestinal villous length ($P < 0.001$) and D-xylose absorption ($p < 0.05$), compared with those values before treatment was given. These results demonstrate that decreased intestinal absorptive capacity is associated with an increase in breath hydrogen concentrations and that breath hydrogen may be useful in evaluating malabsorption in calves with naturally occurring enteric disease.

INTRODUCTION

Carbohydrates presented to the normal small intestine are enzymatically hydrolyzed into smaller components, which are readily absorbed. In malabsorptive states, carbohydrates remain either nondigested or nonabsorbed in the small intestine and pass into the large intestine where these are fermented by colonic bacteria.[1] The colonic bacteria ferment nonabsorbed carbohydrate and/or protein into organic acids and gases. The major gases produced are carbon dioxide (CO_2), hydrogen (H_2) and methane (CH_4),[1,2] Measurable concentrations of hydrogen and methane are not found in the atmosphere. In addition, these gases are not products of mammalian metabolism, but are derived entirely from bacterial fermentation within the colon.[2-4] A portion of the hydrogen gas produced in the colon is transferred in the blood to the lungs and is excreted in the breath.[5-7] Thus, the measurement of expired H_2 by the breath H_2 test provides a clinical index of carbohydrate malabsorption and lactase deficiency.[1,6-10]

The purpose in the present study was to describe the clinical usefulness of breath H_2 as a means of estimating carbohydrate malabsorption in healthy preruminanting calves and in calves where malabsorption was induced with chloramphenicol.

MATERIALS AND METHODS

Animals - Nine male Holstein calves were obtained at birth from cows in the same herd. The perineal areas and udders of the parturient cows had been washed with disinfectant soap, and the calves were delivered onto a clean sheet of plastic, removed immediately from the barn, and isolated from other cattle. All calves were fed their dam's

colostrum for two days. At the end of the colostrum feeding, each calf was fed raw, whole, bovine milk at a quantity equivalent to 10% of its body weight, divided into 2 equal feedings/day, for the duration of the experiment.

The calves were randomly alloted to 2 groups, group I comprised 4 calves, and group II comprised 5 calves. A physical examination, CBC and bovine serum chemistry profile were obtained for each calf. None of the calves used in these experiments showed evidence of systemic disease or diarrhea.

During the first week of life, calves in group II were surgically fitted with an open T-shaped intestinal cannula (Fig 1). A 1-week adjustment period was allowed following surgery. The experiment was begun when the calves were 3 weeks of age and weighed an average of 60.15 ± 1.37 kg (mean ± SEM).

Measurement techniques - Breath H_2 samples were obtained by 2 techniques for both groups: total-expired breath and end-expired breath. Total breath H_2 samples were obtained by placing a face mask connected by a Rudolph valve[a] to a 4-L breathing bag[b] over the external nares and mouth. The mask was held snugly in position until the bag was filled. It was compressed once more and then allowed to refill. A 60-ml syringe to which a 3-way stopcock was attached was used to obtain an aliquot of air from the bag.

To obtain end breath H_2 samples, the skin over the trachea was clipped and aseptically scrubbed. Two percent Lidocaine (10 ml) was infiltrated subcutaneously midway over the trachea. A 1- to 2-cm full thickness skin incision was made into the anesthetized area. A 5.0 cm

10-gauge needle was inserted between 2 tracheal cartilage rimgs, and into the lumen of the trachea. Twenty-five centimeters of polyethylene tubing[c] was passed through the lumen of the needle in the trachea to just beyond the bifurcation of the trachea. The 10-gauge needle was removed, and a needle adapter was inserted in the tip of the tubing which was sutured to the skin. At the end of the visible expiration, 50 ml of gas was aspirated from the polyethylene tubing. This sample should have had the approximate composition of alveolar gas.

A D-xylose absorption test was administered to each calf in both groups. After calves were fasted for 24 hours, D-xylose was administered orally via a nipple bottle at a dosage of 0.5 g/kg in a 5% aqueous solution. Blood was withdrawn from the jugular vein through a previously placed jugular catheter at 0, 30, 60, 90, 120, 150, 180, 240 and 300 minutes after D-xylose was administered. The heparinized blood was immediately centrifuged, and the plasma was frozen until assayed.[11]

Each calf in group II was fitted with an open T-shaped duodenal cannula fabricated from Silastic medical-grade tubing.[d] The cannula was surgically inserted into the descending duodenum approximately 30 cm caudal to the pylorus and exteriorized through the right midflank. Biopsy samples of intestinal mucosa were obtained using a suction biopsy instrument.[e] The biopsy tool was passed through the T-shaped cannulae distally along the intestine for 50 cm. This was done to avoid cannulation effects and to obtain mucosa from the proximal jejunum. The biopsy specimens were placed in 10% buffered formalin until villous length determinations were made. The fixed specimens were stained with 2% new methylene blue and examined under a dissecting

microscope equipped with an ocular micrometer. Twenty villi were measured on each specimen.[12]

Chromatographic analysis - Total breath and end breath hydrogen samples were analyzed on a gas chromatograph[f] which contained a solid state detector for the specific analysis of H_2. The chromatograph was supplied with an internal pump which provided room air as the carrier gas. However, a reference gas containing a known concentration of H_2 is required for calibration.[13]

EXPERIMENTAL PROTOCOL

Group 1 - Breath H_2 excretion was measured after whole milk feeding and lactulose[g] were fed to calves. Hydrogen samples were taken before the morning feeding and at 1-hour intervals for the next 6 hours. The calves were fasted for 12 hours, and 1.25 g of lactulose/kg was administered orally as a single dose in 2 L of water via a nipple bottle. Lactulose, a nonabsorbable carbohydrate, was administered to simulate malabsorption. Hydrogen samples were taken for lactulose feeding in the same manner as described for milk feeding. D-xylose absorption test was performed on the seventh day.

Group II - Breath H_2 samples and jejunal biopsy samples were obtained in response to whole milk feeding before and after the 3-day chloramphenicol regimen. The first breath H_2 test was done on the first day of the experiment. On day 2, the initial mucosal biopsy samples were obtained. Subsequently, chloramphenicol was administered for the next 3 days at 50 mg/kg 2 times a day. At the end of chloramphenicol adminstration (day 5), additional mucosal biopsy samples were obtained. D-xylose absorption was done on the sixth day. The second breath H_2 test was performed on the eighth day. This was done to

allow sufficient time for the bacteria to become reestablished and to avoid the H_2 produced by D-xylose fermentation within the large intes tine.

STATISTICAL ANALYSIS

Analyses were done to determine the significance of H_2 excretion resulting from lactulose feeding compared with that from whole milk feeding in group I and with that from whole milk feeding before and after chloramphenicol was adminstered to group II. Therefore, for H_2 excretion, only within group comparisons were made. Analysis of variance, using calf, treatment, and time as main effects with an interaction effect of treatment and time, was used.[14] Fisher's variance-ratio (F test) was used to determine significance between treatments for each group. To assess the effect of chloramphenicol administration on jejunal villous length, analysis of variance, using calf and time as determinant variables, was used. For this analysis, each calf served as its own control. The F test was used to test differences between villous length before and after chloramphenicol was administered.

D-xylose absorption was analyzed by analysis of variance, using a repeat measure split-plot model. Bonferroni-t statistic was used for comparing means.[15] Correlation between total breath and end breath H_2 excretion was determined by Pearson's correlation analysis.

RESULTS

Group 1 - Total breath and end breath H_2 excretion after whole milk and lactulose were fed to calves are shown in Figure 3. Total and end breath H_2 concentrations were significantly increased ($p < 0.001$) after lactulose was fed to calves when compared with those values after whole milk feeding.

Group II - Chloramphenicol administration increased breath H_2 concentrations over those measured before treatment was given ($P < 0.001$) (Fig 4) and decreased both intestinal villous length ($P < 0.001$) and D-xylose absorption ($P < 0.05$) (Fig 5 and 6, respectively). The severity of villous atrophy (Fig 5) can be associated with the administration of chloramphenicol.[16] Although histologic changes were observed in 4 of the 5 calves, it is noted that 3 of the 5 developed diarrhea and 2 had softer stools. Average villous length was longer in calf No. 5 after chloramphenicol administration.

Time trend changes on plasma xylose concentrations of groups I and II calves are shown in Figure 6. D-xylose absorption peaked at 90 minutes at a maximal concentration of 66 mg/dl for group I calves and at a maximal concentration of 45 mg/dl for group II calves. The chloramphenicol-treated calves absorbed significantly less D-xylose at 60, 90 and 120 minutes ($P < 0.05$).

Correlation analysis was done on total breath and end breath H_2 responses. Total breath and end breath were highly correlated ($r^2 = .8118$, $P = 0.001$).

DISCUSSION

The present experiments have shown that carbohydrate malabsorption in preruminanting calves can be detected by the breath H_2 test. The oral administration of 1.25 g of lactulose/kg produced breath H_2 concentrations significantly higher than the values obtained after whole milk was fed to calves. The breath H_2 response obtained with lactulose administration was indicative of colonic bacterial fermentation of the nonabsorbed carbohydrate. Lactulose (4-O-B-D-galactopyranaosyl-D-fructose) is a synthetic, nonabsorbable disaccharide.[17,18]

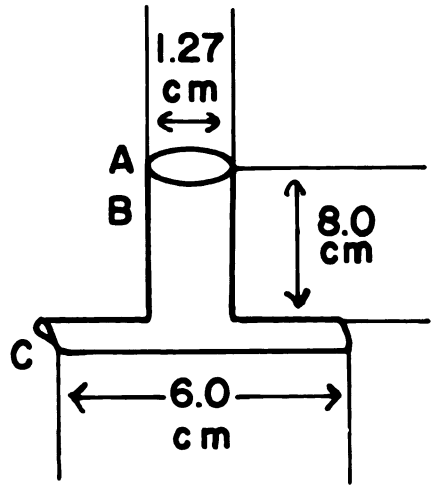

Figure 1.1: Duodenal cannula: opening for stopper (A), cannula barrel (B), open lumenal segment (C).

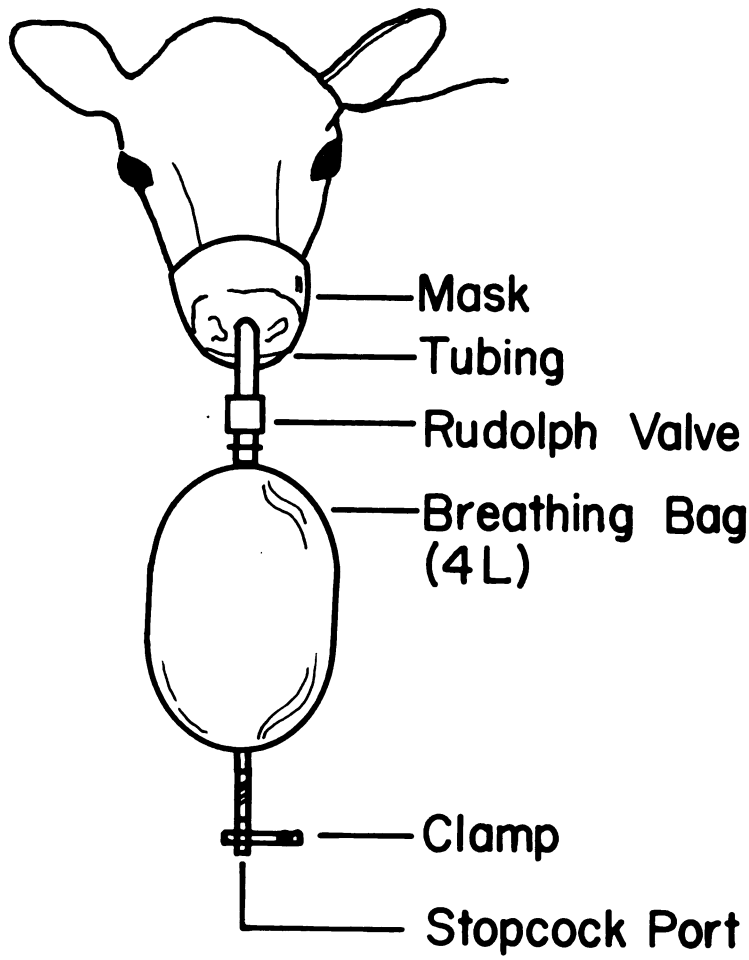

Figure 1.2: Schematic representation of mask, breathing bag, and valve system for measuring total breath hydrogen concentrations.

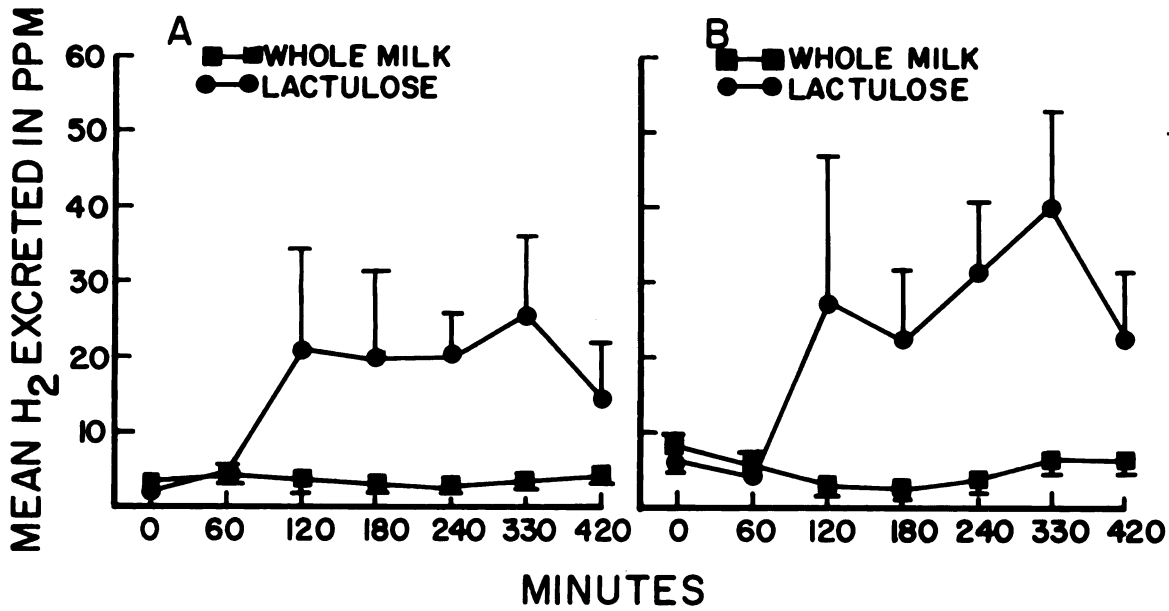

Figure 1.3: Mean total breath (A) and end breath H_2 (B) excreted in ppm for each time. Hydrogen excreted after lactulose was fed to calves was significantly increased ($P < 0.001$) when compared with that after whole milk was fed to calves in both total breath and end breath measurements. Parts per million (PPM) equals 1 molecule of H_2 in 999,999 molecules of air.

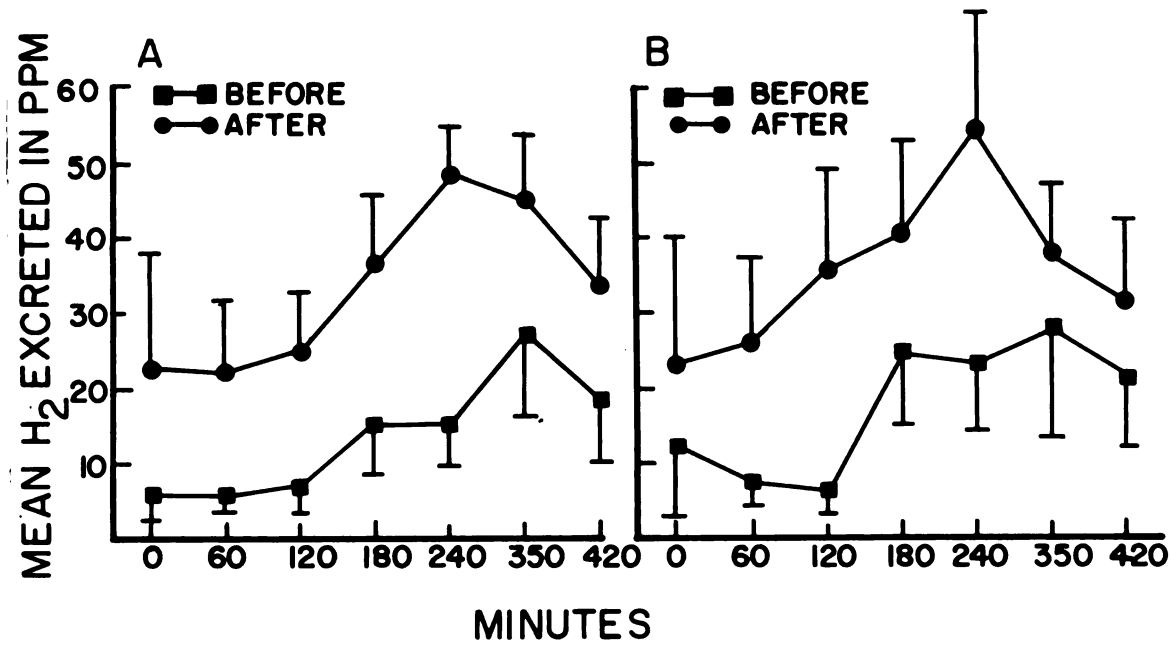

Figure 1.4: Mean total breath (A) and end breath H_2 (B) excreted in ppm for each time. Hydrogen excreted after whole milk was fed to calves and after chloramphenicol was administered was significantly increased ($\underline{P} < 0.001$) when compared with that after whole milk was fed to calves and before chloramphenicol was administered. Parts per million (PPM) equals 1 molecule of H_2 in 999,999 molecules of air.

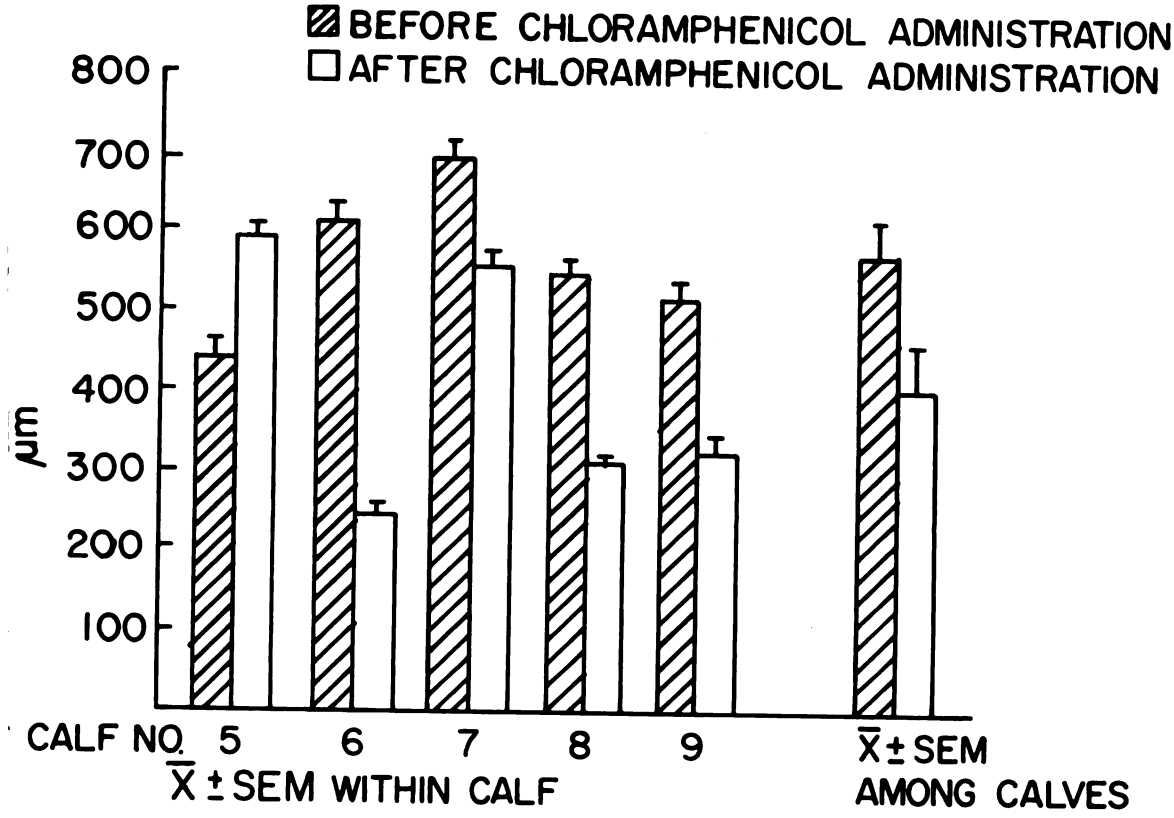

Figure 1.5: Jejunal villous length (n=20, r=100) before and after chloramphenicol was administered. Mean villous length was significantly decreased after chloramphenicol was administered ($P < 0.001$).

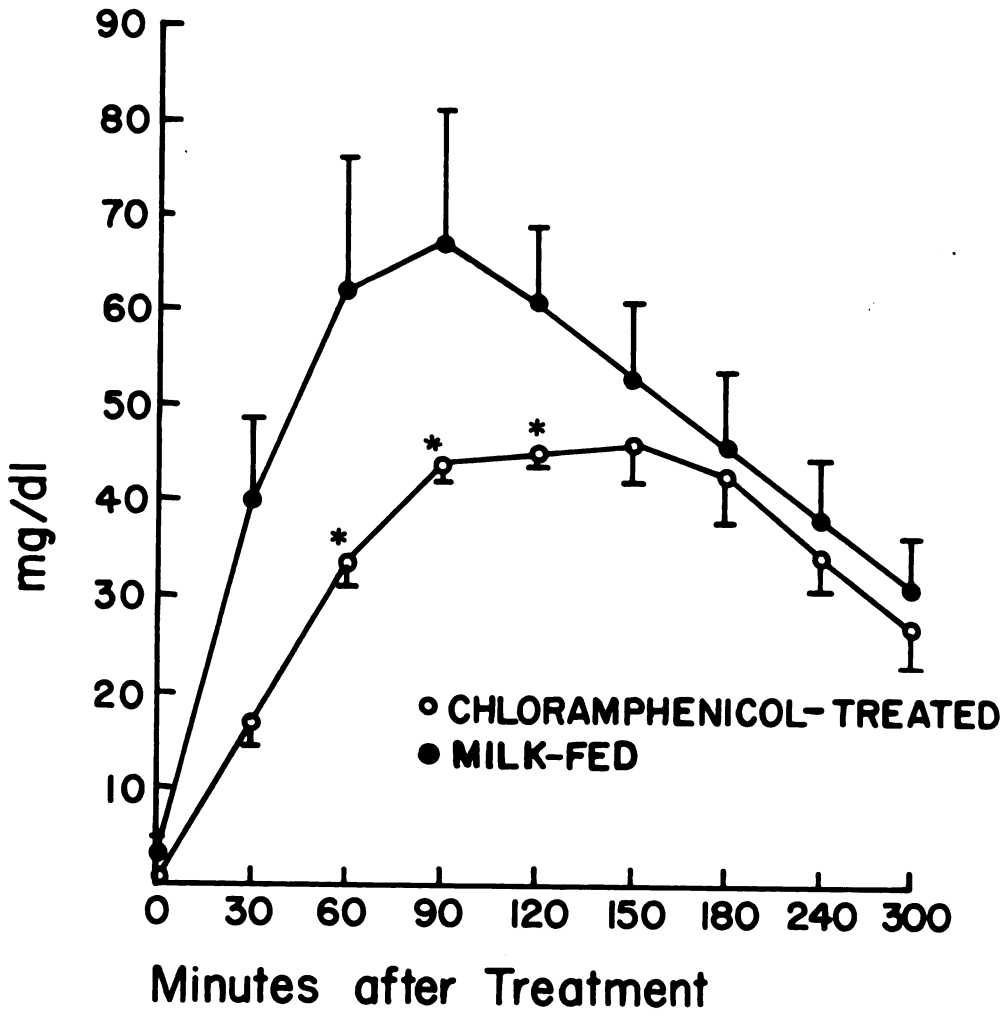

Figure 1.6: Plasma D-xylose concentrations in milk-fed only calves (group 1) and milk-fed, chloramphenicol-treated (group 2) calves. The chloramphenicol-treated calves absorbed significantly less D-xylose at 60, 90, and 120 minutes ($\underline{P} < 0.05$).

It had a 2-fold purpose in this experiment. First, it was used to induce physiologic malabsorption. It passes through the small intestine nonabsorbed, reaching the colon where it is fermented by colonic bacteria to short-chain organic acids and gases.[6,17] Second, it confirmed the presence of H_2-producing bacteria in the gastrointestinal tract of preruminant calves.

In the calves in group II, carbohydrate malabsorption was induced by the oral administration of chloramphenicol. The oral administration of chloramphenicol has been shown to decrease the number and height of the villi and microvilli, resulting in a decrease in the total surface area for absorption within the small intestine.[16] In this experiment, chloramphenicol administration induced small intestinal malabsorption as observed by the increase in breath H_2 excretion and decrease in D-xylose absorption. The decreased D-xylose absorption after chloramphenicol was administered was presumably the result of decreased synthesis of mitochondrial proteins which are necessary for the efficient operation of the sodium dependent cotransport process.[16,19-21]

Malabsorption and diarrhea in persons[22] and calves[23] have been reported in association with the oral administration of antibiotics. Similarly, factors such as diet changes,[24-26] infective agents[27,28] and age[24,29] have been cited as influencing lactase activity. Lactase activity reflects the degree of villous atrophy. The results of the present study indicate that the malabsorption defect was, in part, attributable to villous atrophy.

Variability in the concentrations of H_2 excreted by the calves in the 2 groups occurred; it was more pronounced among calves in group

II. The first breath H_2 test results were higher and less consistent in group II than those in group I.

This inconsistency may have been affected by the invasive procedures used. The cannulae, necessary for acquiring the biopsy specimens, may have affected peristalsis and the bacterial population of the intestine. However, the second breath H_2 test results were significantly increased ($P < 0.001$) over the first test. This effect was anticipated, since villous atrophy developed as expected with chloramphenicol administration and was accompanied by malabsorption and an increase in H_2 excretion. It has been cited that the oral administration of antibiotics will decrease the colonic flora and hydrogen production.[8,30,31] Therefore, a decrease in H_2 excretion might have been expected, since chloramphenicol has a broad spectrum of activity against both gram-negative and gram-positive bacteria. In the present experiment, H_2 excretion was significantly increased after the antibiotic was administered.

Total breath and end breath H_2 excretion were obtained to determine which was the most sensitive measure of carbohydrate malabsorption. End breath H_2 excretion approximates the H_2 concentration in alveolar gas. Total breath H_2 excretion reflects dilution by dead space volume within the conducting airways, face mask and gas bag. Solomons[32] reported a 30% decrease in H_2 concentration with an H_2 collecting system similar to the one used in the present experiments. Results from the present study indicate that both total breath and end breath H_2 excretions are accurate predictors of carbohydrate malabsorption in calves. In these experiments, total breath and end breath

H_2 concentrations were highly correlated, indicating H_2 gas dilution due to dead space volume is not a major factor in obtaining reliable data.

The present investigation demonstrates that the breath H_2 test is useful in evaluating induced malabsorption in the preruminanting calf. The decreased intestinal absorptive capacity is reflective of increased H_2 concentrations in the expired air.

FOOTNOTES:

a Rudolph Valve No. 1400, Han's Rudolph Inc, Kansas City, MO.
b North American Drager, Telford, PA.
c No. 240 Intramedic Polyethylene tubing (inside diameter, 1.67 mm; outside diameter, 2.42 mm), Clay-Adams, Division of Becton, Dickinson & Company, Parsippany, NJ.
d Silastic Medical Grade tubing (inside diameter, 0.95 cm; outside diameter, 1.27 cm), Dow Corning Corp, Medical Products Division, Midland, MI.
e 4.7-mm Multipurpose Biopsy tube, Quinton Instruments Co, Seattle, WA.
f Model 12 Microlyzer, Quin Tron Instrument Co Inc, Milwaukee, WI.
g Lactulose (cephulac syrup), Merrell Dow Pharmaceuticals Inc, Cincinnati, OH.
h Anacetin, Bio-Ceutic Laboratories Inc, St. Joseph, MO.

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EXPERIMENT 2:

Changes in Breath H_2 Excretion with *Cryptosporidium*-Induced Diarrhea in Calves.

SUMMARY

Breath hydrogen gas (H_2) concentrations were measured in five healthy (control) calves and in seven calves incoulated with *Cryptosporidium sp.*(experimental). Each calf was fitted with a rectal cannula and a fecal collecting bag. The control calves were maintained free of detectable infectious agents. The experimental calves were given 10^7 to 10^8 *Cryptosporidium* oocysts orally in one dose. Time trend changes in H_2 excretion after feeding and changes in the weight of feces produced were measured at three stages: before *Cryptosporidium* inoculation (Stage 1), three days after the onset of diarrhea (Stage 2), and after resolution of diarrhea (Stage 3). There was a treatment-by-stage interaction, where end-expired breath and total-expired breath H_2 concentrations were significantly increased ($P < 0.001$ and $P < 0.025$, respectively). Mean end breath and total breath H_2 excreted by the experimental calves were higher ($P < 0.025$ and $P = 0.06$) for Stage 2, than the same response measured for the control calves. End breath and total breath H_2 excreted by the experimental calves for Stages 1 and 3 did not differ ($P > 0.1$) from the controls either within treatments or between stages. *Cryptosporidium* infection increased fecal production ($P = 0.06$) during Stage 2.

The results indicate that *Cryptosporidium* infection caused diarrhea during Stage 2. The increased H_2 production was the result of

decreased small intestinal absorption of carbohydrates and proteins with the concurrent colonic fermentation of the malabsorbed products.

INTRODUCTION

Cryptosporidium spp., an enteropathogen, has been cited as a cause of diarrhea in several animal species and man.[1-13] In young calves it most commonly affects the distal small intestine, primarily the mid-to-lower jejunum and ileum.[1-10] Lesions in the large intestine have also been observed.[1,2,4,5]

Experimentally inoculated calves develop a profuse, watery diarrhea 2 to 4 days after inoculation [5,6] with concurrent shedding of oocysts and abnormal feces for 5 to 12 days.[4,5,8] The infection causes mucosal damage, as indicated by the passage of feces containing mucus, flecks of blood and fibrin.[6,8] Histologic changes in the mucosa include atrophy, fusion, blunting and distortion of villi and crypt hyperplasia.[1,2,4,5] Since the villous epithelium contains disaccharidase and peptidase enzymes,[14-17] disruption of the mucosa by Cryptosporidium could result in deficiencies of these enzymes. The decreased enzyme activity, as a result of mucosa damage, directly affects digestion and absorption of carbohydrates and/or protein.[3,4]

Nonabsorbed carbohydrates and proteins pass into the large intestine where colonic fermentation occurs. Products of this colonic bacterial fermentation include organic acids and gases.[18-20] The major gases produced are carbon dioxide (CO_2), hydrogen (H_2) and methane (CH_2).[18-20] These gases are absorbed into the blood stream and excreted by the lungs. Therefore, measuring H_2 excretion provides a means of detecting colonic H_2 production and thus, carbohydrate malabsorption.[18,19,21,22]

The objective of this study was to determine if Cryptosporidium infection in calves caused carbohydrate and protein malabsorption.

The breath H_2 test was used to assess H_2 excretion resulting from deceased small intestinal absorption of carbohydrates and proteins.

MATERIALS AND METHODS

Animals - Twelve newborn male Holstein calves were used in the experiment. Seven were assigned to the experimental group and five to the control group. The calves were isolated from other cattle and maintained in individual metabolism cages. A physical examination, CBC and serum chemistry profile were obtained on each calf. All calves used in this experiment were healthy and did not have any evidence of systemic disease. Each calf was fitted with a rectal cannula and a fecal collecting bag[a] (Fig 1). The feces were collected daily and weighed.

Diet - The calves were fed their dam's colostrum for two days. Following colostrum feeding, they were fed raw, whole, bovine milk at 10% of their respective body weights. The diet was divided into two daily feedings.

Cryptosporidium inoculation - Cryptosporidium oocysts, which were obtained from a naturally infected calf, were given orally to a newborn calf. Feces containing Cryptosporidium oocysts, as determined by the carbolfuschin, direct-staining technique,[23] were collected from this calf and stored at 4°C in aqueous 2% potassium dichromate in a volumetric ratio of 2:1 (dichromate to feces) until required for inoculation. Immediately prior to inoculating the experimental calves, the fecal-dichromate solution was centrifuged and the fecal pellet was added to 8% peracetic acid at 1 part fecal suspension to 10 parts peracetic acid. The feces were exposed to this solution for 30 minutes,

which included centrifugation time. The suspension was washed three times with saline and resuspended in phosphate buffered saline before administration. Each calf was given 10^7 to 10^8 oocysts in milk via a nipple bottle. Oocysts were quantified by direct microscopic techniques.

Measurement techniques - End-expired breath H_2 samples were collected at the end of visible expiration by way of polyethylene tubing[b] that was placed into the trachea. Twenty-five centimeters of polyethylene tubing were aseptically placed into the trachea and was passed to a point just beyond the bifurcation of the trachea. A needle adapter was inserted and the polyethylene tubing was sutured to the skin.

A face mask connected by a Rudolph valve[c] to a 4-L breathing bag[d] was used to collect total-expired breath H_2 samples. The mask was held over the mouth and nares until the collapsed bag was filled. The bag was compressed once more, then allowed to refill. Hydrogen samples were drawn from the 4-L bag and polyethylene tubing into 60 ml syringes equipped with three-way stopcocks. Hydrogen samples were collected before the morning feeding and at 60, 120, 180, 240, 330 and 420 minutes after feeding. Hydrogen samples were collected for each time period for each stage for the two groups.

Hydrogen analysis - Hydrogen content of each sample was determined by a gas chromatographic technique.[24]

Fecal collection - Each calf was fitted with a rectal cannula. The cannulae were fabricated out of 20 ml syringe cases and rubber tubing. They were surgically placed into the rectum. Each calf was given 0.1 mg/kg xylazine[e] IM and caudal epidural anesthesia was achieved

with 2 ml of 2% lidocaine hydrochloride.[f] Ten simple interrupted sutures were placed circumferentially around the anus. The sutures extended through the skin and external anal sphincter muscle into the rubber tubing surrounding the cannula. Umbilical tape was used to add additional support dorsally. It was tied to a tension-supported horizontal mattress suture placed over the dorsal sacrum.

EXPERIMENTAL PROTOCOL

Prior to inoculating the experimental calves, the breath H_2 test was performed on each calf within both groups. Calves in the experimental group were inoculated with Cryptosporidium oocysts at 3-4 days of age. Fecal samples were obtained daily from the inoculated calves and examined for the presence of oocysts. Feces from all the calves were periodically collected for bacteriological and virological examination. A second breath H_2 test was performed 3 days after the onset of diarrhea in the experimental group and a paired response was obtained from the controls. Following resolution of diarrhea, which was determined by the absence of Cryptosporidium oocysts and the return of normal fecal characteristics, a third breath H_2 test was performed on both groups.

STATISTICAL ANALYSIS

Analysis of variance using a repeat measure split-split plot design[25] was used to test effects of treatment, stage and time. Treatment effects were tested in the main plot and stage and time effects with appropriate interactions were tested in subplots. The F-test was used to determine the significance of each effect. When a significant

effect was identified, Bonferroni t-statistic was used for making comparisons among means.[26]

Changes in the weight of feces produced were analyzed by analysis of variance using a repeat measure split-plot design.[25] Treatments were tested in the main plots. Stage and treatment-by-stage interaction were tested in the subplots. The F-test was used to determine the significance of each effect.

Extreme variation was observed in the weight of feces produced by the experimental calves during Stage 2. Unequal variances were judged to be an important consideration for the comparison that was to be made between the two groups. Therefore, a t-like test statistic[26] for one sided alternates was used to make a single comparison between means for the experimental calves and the control calves for Stage 2, only.

RESULTS

During the experiment, the control calves did not develop diarrhea and remained free of detectable infectious agents. After inoculation, the experimental calves developed diarrhea and started shedding oocysts 5 to 6 days later. Feces collected from these calves were free of other detectable pathogens for the three stages.

Mean end breath H_2 (Fig 2) and total breath H_2 (Fig 3) concentrations are shown for the three stages and seven time periods. There was a significant treatment-by-stage interaction for both end breath ($P < 0.001$) and total breath ($P < 0.025$) H_2 excretion. Mean end breath and total breath H_2 excreted by the experimental calves were higher ($P < 0.025$ and $P = 0.06$, respectively) for Stage 2, than

the same response measured for the control calves. End breath and total breath H_2 excreted by the control calves did not change among stages ($P > 0.1$). End breath and total breath H_2 excreted by the experimental calves did not differ ($P > 0.1$) from control calves at Stages 1 and 3. Also, end breath and total breath H_2 excreted by the experimental calves during Stages 1 and 3 did not differ ($P > 0.1$).

Mean $\pm$ SEM changes in the weight of feces produced by the calves for each treatment during each stage are shown in Figure 4. During Stage 2, mean daily production of feces by the experimental calves ranged from 45.70 to 1839.70 g. Feces produced by the control calves ranged from 44.80 to 127.67 g. Cryptosporidium infection increased ($P = 0.06$) the weight of feces produced by the experimental calves during Stage 2 when compared to the control calves. Feces produced by the experimental calves did not change between Stages 1 and 3 ($P > 0.1$) and feces produced by the control calves did not change among stages ($P > 0.1$).

DISCUSSION

This study quantifies the changes observed in breath H_2 excretion resulting from Cryptosporidium- induced diarrhea. The ability of the breath H_2 test to detect H_2 excretion resulting from small intestinal malabsorption was demonstrated by the strong treatment-by-stage interaction for both end breath and total breath H_2 excretion. This effect was limited to Stage 2. The increased H_2 excretion was probably due to the malabsorption and fermentation of lactose, although colonic fermentation of nonabsorbed proteins cannot be excluded as a cause of increased breath H_2 excretion. This was expected because the Cryptosporidium infection caused malabsorption and diarrhea.

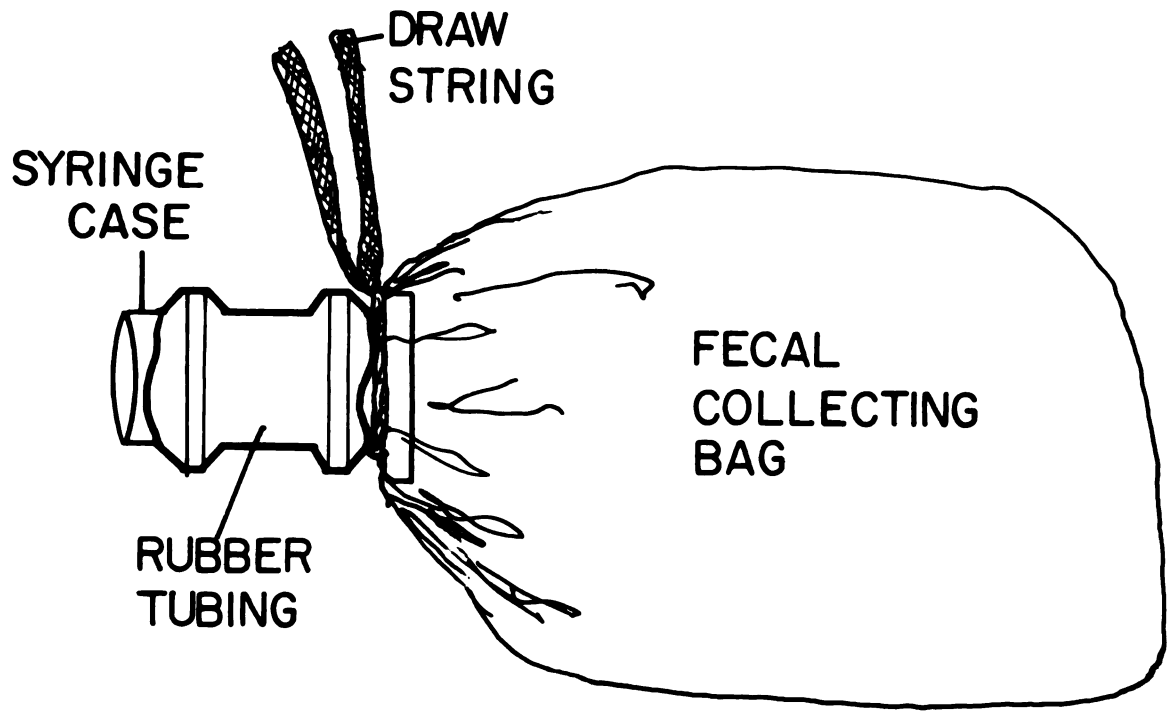

Figure 2.1: Schematic drawing of rectal cannula and fecal collecting bag.

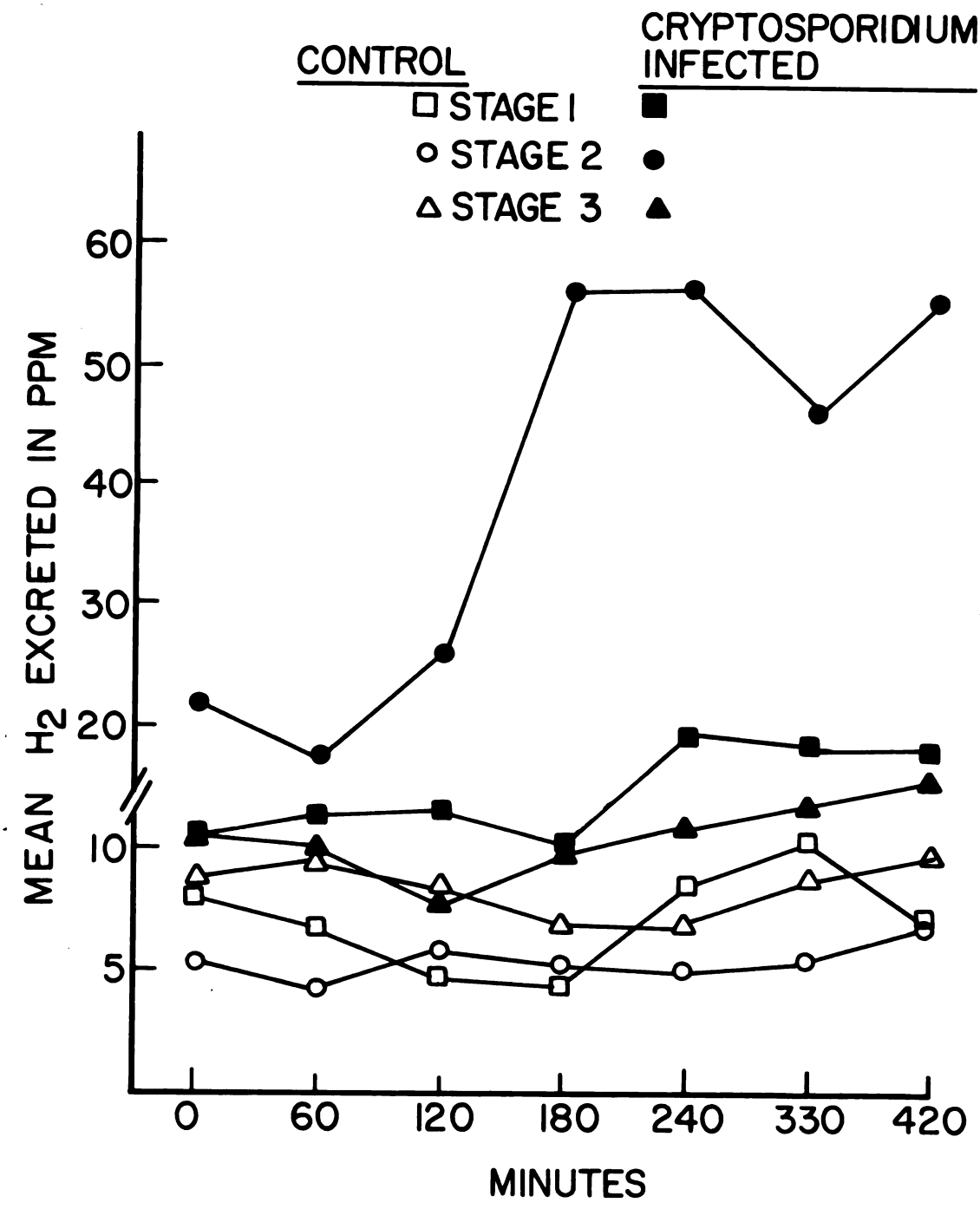

Figure 2.2: Mean end-expired breath H_2 excretion. Mean end breath H_2 excreted during Stage 2 by the experimental calves was significantly higher ($p < 0.025$) than other stages.

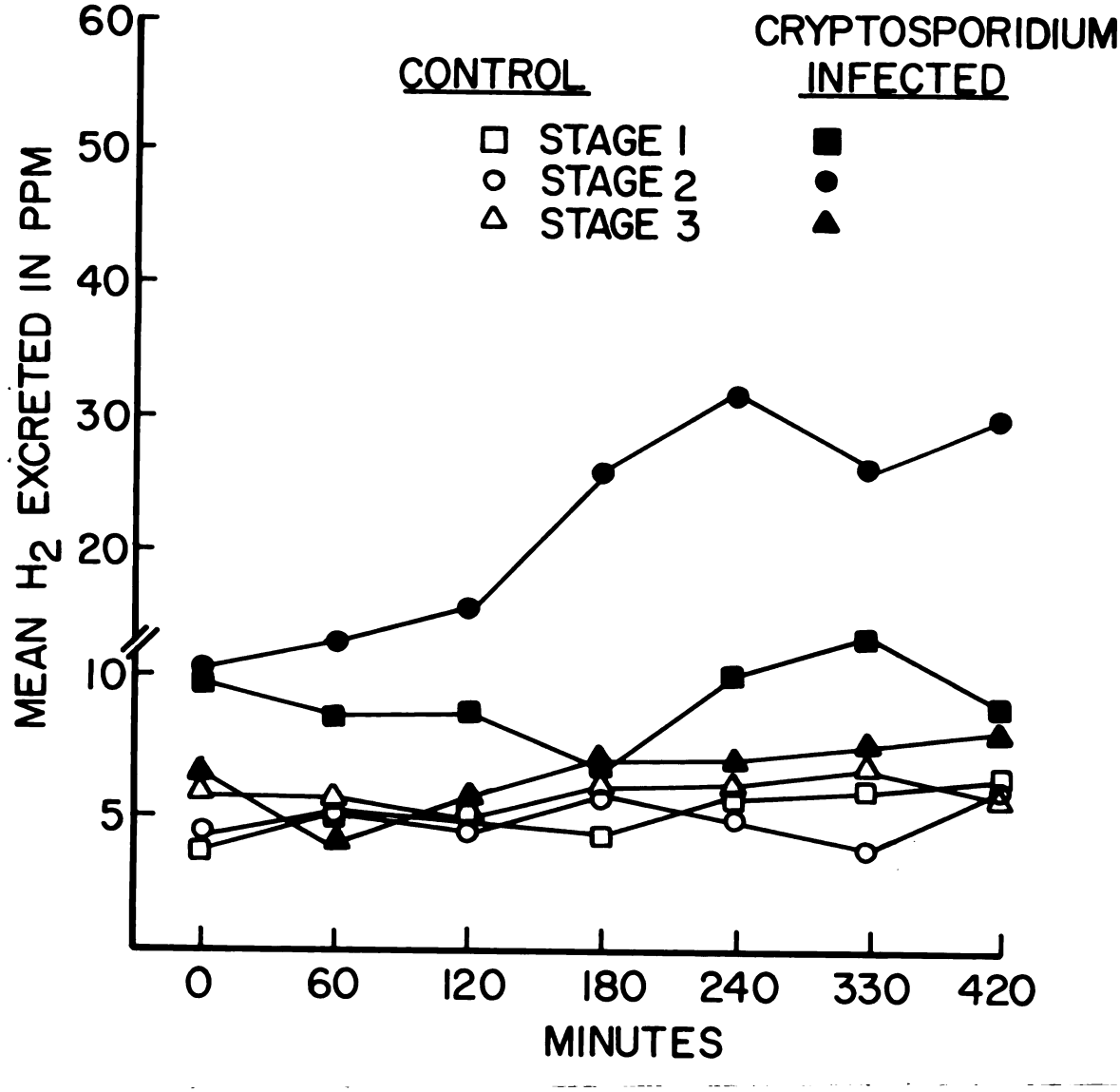

Figure 2.3: Mean total-expired breath H_2 excretion. Mean total breath H_2 excreted during Stage 2 by the experimental calves was higher ($p = 0.06$) than other stages.

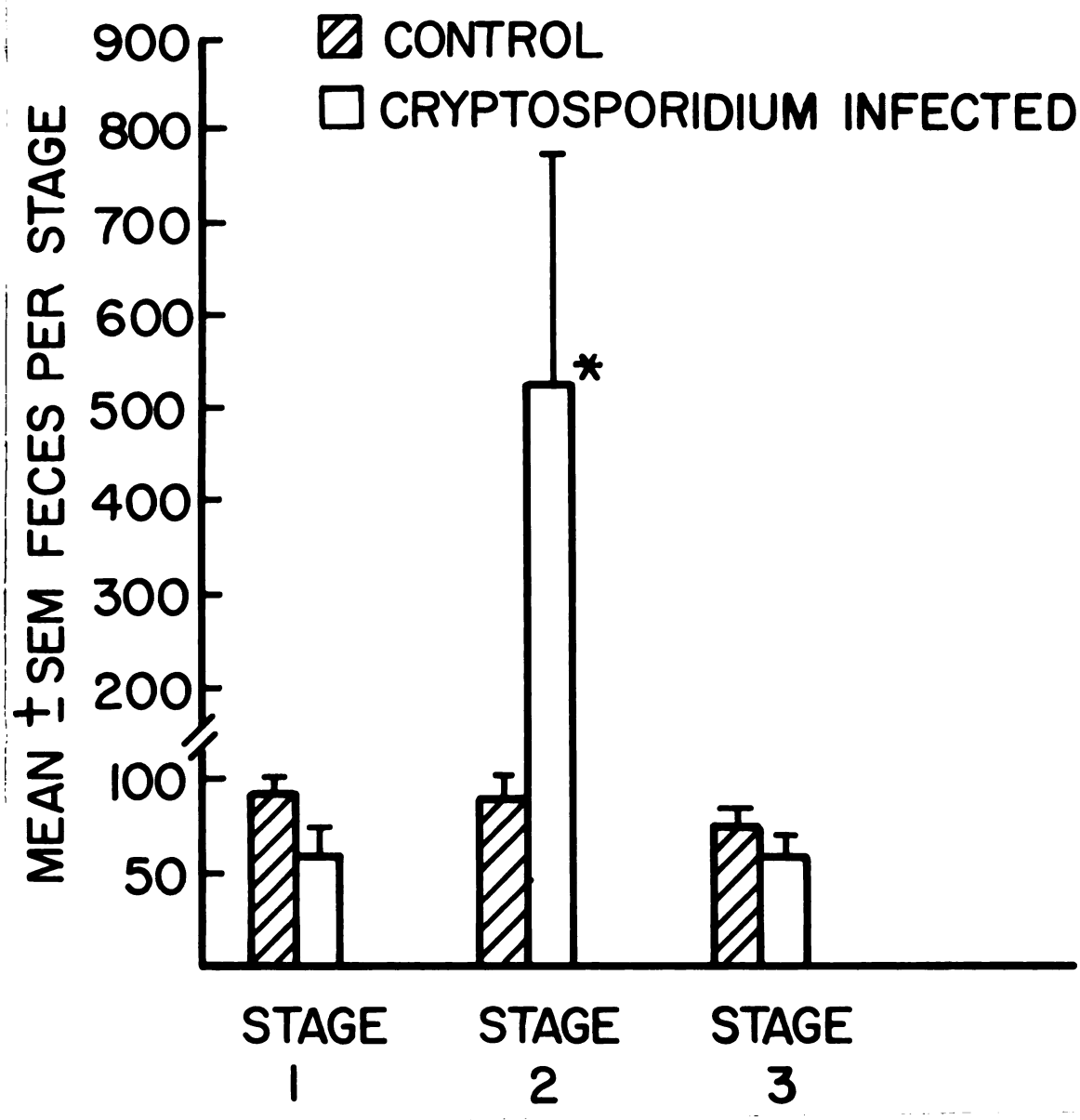

Figure 2.4: Mean ± SEM wet fecal weights for the three stages. *Cryptosporidium infection increased (p = 0.06) fecal production during Stage 2.

End breath and total breath H_2 excretion did not change among stages for the control calves. Therefore, the breath H_2 concentrations excreted were probably that of normal colonic bacterial fermentation of the residual products of whole milk digestion.

End breath and total breath H_2 excreted by the experimental calves for Stages 1 and 3 did not differ from the controls, either within treatments or between stages. The concentration of H_2 excreted by the experimental calves during Stage 1 was comparable to Stage 3. There was also a concurrent reduction in diarrhea. The return to preinoculation H_2 excretion and fecal production indicated a return to normal function.

Feces produced by the experimental calves during Stage 2 were dramatically increased over that of the control calves. There were no significant differences observed in fecal production within treatments or between Stages 1 and 3. All seven calves given Cryptosporidium oocysts developed diarrhea. They remained alert, active and continued to consume their daily allotment of milk. Although all the calves developed diarrhea, variation in the quantity of feces produced occurred. The lack of strong statistical significance for the comparison made between the experimental and control calves is reflective of the unequal variances for the two groups.

In this experiment, total-expired breath ($P = 0.06$) was not as sensitive a measure as end-expired breath ($P < 0.025$). The concentration of total breath H_2 excreted was influenced by the dead space that existed within the conducting airways and gas collecting system. Hydrogen, measured by the end-expired technique, was obtained at the end of visible expiration. This sample should have contained an

approximate composition of alveolar gas, thus avoiding some of the dead space contamination that occurred with the total-expired breath H_2 technique.

Reduced intestinal absorptive capacity associated with an increase in breath H_2 excretion in the preruminant calf has been demonstrated.[27] In that experiment, malabsorption was simulated by the oral administration of a nonabsorbable carbohydrate and induced by the oral administration of chloramphenicol. The nonabsorbable carbohydrate passed through the small intestine unabsorbed to the large intestine where colonic bacterial fermentation resulted in increased breath H_2 excretion. The oral administration of chloramphenicol increased breath H_2 excretion over that measured pretreatment. It also reduced intestinal villous length and D-xylose absorption. In this experiment, the breath H_2 test was useful in evaluating malabsorption and diarrhea resulting from a naturally occurring enteric disease. Cryptosporidium infection caused temporary malabsorption and diarrhea as evidenced by the significant increase in H_2 excretion and fecal production during Stage 2 of this experiment.

FOOTNOTES

a Steri-Lok Number 8252 Polyethylene Bags (22.8 cm x 43.1 cm) Medical Products Division/3M St. Paul, MN

b No. 240 Intramedic Polyethylene tubing (inside diameter, 1.67 mm; outside diameter 2.42 mm), Clay-Adams, Division of Becton, Dickinson and Co., Parsippany, NJ

c Rudolph Valve No. 1400, Han's Rudolph Inc, Kansas City, MO

d North American Drager, Telford, PA

e Xylazine, Haver-Lockhart, Bayvet Division Cutter Lab. Inc, Shawnee, KA

f Lidocaine Injectable, AMFAC Veterinary Supply Co, Atlanta, GA

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EXPERIMENT 3:

The effects of Diet on Breath H_2 Excretion in Calves

SUMMARY

The effects of feeding 2 different diets on end-expired breath and total-expired breath H_2 excretion was determined. Five healthy calves were fed only raw, whole bovine milk (diet 1) for 4 weeks. Subsequently, the same calves were fed water, hay and concentrate (diet 2) for 4 weeks. Overall, end breath and total breath H_2 excreted while on diet 2 were significantly higher ($P < 0.05$) than on diet 1.

The results demonstrate that H_2 excretion was increased by feeding diet 2. The increased excretion of H_2 was probably related to the production and excretion of H_2 resulting from bacterial fermentation within the rumen and gastrointestinal tract. This effect was complemented by the development of the rumen as a fermentative organ and to the establishment of H_2-producing bacteria within the rumen and gastrointestinal tract.

INTRODUCTION

Breath H_2 measurements have been used in the two previous experiments to demonstrate an increase in H_2 excretion resulting from diarrhea or induced malabsorption in preruminanting calves. The concentration of H_2 excreted was reflective of large intestinal bacterial fermentation of the nonabsorbed dietary products. Those studies were performed in preruminanting calves fed lactose as the primary carbohydrate.

When the preruminanting calf is fed roughage and concentrate, it makes the transition from the monogastric system to that of the ruminant. Therefore, the present experiment was undertaken to determine what effects roughage and concentrate feeding would have on H_2 excretion compared to whole milk feeding in normal calves.

MATERIALS AND METHODS

Animals - Five newborn male Holstein calves were used in the experiment. The calves were obtained from a local dairy at birth. All calves were isolated from other cattle and were maintained in individual stalls.

Diets - The calves were fed colostrum for two days. After colostrum feeding, raw, whole bovine milk (diet 1) was fed. Whole milk was fed at 10% of the calf's body weight divided into 2 equal feedings per day. Diet 1 was fed for 4 weeks. Diet 2 consisted of water, alfalfa hay[a] and concentrate.[a] The calves had free choice water and alfalfa hay. The nonmedicated concentrate was provided at 1.0 kg 2 times a day for 4 weeks.

Measurement techniques - Twenty-five centimeters of polyethylene tubing[b] were placed into the trachea and passed distally beyond the bifurcation. End-expired gas samples were taken from the polyethylene tubing. This was done to collect end alveolar gas.

Total breath H_2 samples were collected by using a snug-fitting face mask that covered the external nares and mouth. A nonrebreathing valve[c] was attached to the face mask and gas collection bag.[d] The mask was held in position until the bag was filled. The bag was manually compressed, then allowed to refill. Sixty milliliter syringes equipped with 3-way stopcocks were used to collect H_2 from the polyethylene tubing and breathing bag.

Hydrogen samples were collected before the morning feeding of milk or concentrate and at 60, 120, 180, 240, 330, and 420 minutes after feeding. Hydrogen content of each sample was determined by a gas chromatographic technique.[1]

STATISTICAL ANALYSIS

Analyses were performed to determine the significance of H_2 excretion resulting from whole milk feeding compared to water, alfalfa hay, and concentrate feeding. Analysis of variance using calf, diet, and week as main effects with an interaction effect of diet and week was used. The F test was used to determine significance between treatments. When a significant treatment effect was identified, Scheffe's Interval was used to make postdata comparisons.[2]

RESULTS

Each calf was healthy with no evidence of diarrhea for the two 4-week feeding periods. One calf developed a tracheal stricture during

week 3 of diet 2 and was removed from the experiment. Therefore, for week 4 of diet 2, data are X ± SEM of 4 calves.

End breath and total breath H_2 excreted for diets 1 and 2 are shown in Figures 1 and 2, respectively. End breath and total breath H_2 excreted while on diet 2 were significantly higher ($P < 0.05$) than for diet 1. End breath H_2 excreted at weeks 2 and 3 were significantly higher ($P < 0.05$) than for diet 1. Total breath H_2 excreted during weeks 2, 3, and 4 were significantly higher ($P < 0.05$) than diet 1. Neither end breath or total breath H_2 excreted during week 1 for either diet differed significantly ($P > 0.05$).

DISCUSSION

The results of this experiment indicate that H_2 excretion was increased while feeding diet 2. Calves fed diet 2 excreted significantly more H_2 than the same calves fed diet 1. This effect was observed for weeks 2 and 3, and weeks 2, 3, and 4 for end breath and total breath H_2, respectively. Hydrogen excreted at week 1 for both diets did not differ.

Feeding raw, whole bovine milk (diet 1) resulted in H_2 excretion at concentrations that did not change among the 4 week period. This is attributable to maintaining the preruminant (monogastric) pattern of digestion. In bottle feeding whole milk, the liquid meal bypasses the reticulo-rumen and enters the abomasum.[3,4] This is accomplished by the reflex-closure of the esophageal groove.[3]

With the introduction of roughage and concentrate, ruminal development is accelerated.[3,5] The transition from the monogastric system to that of the ruminant depends on the diet provided. The

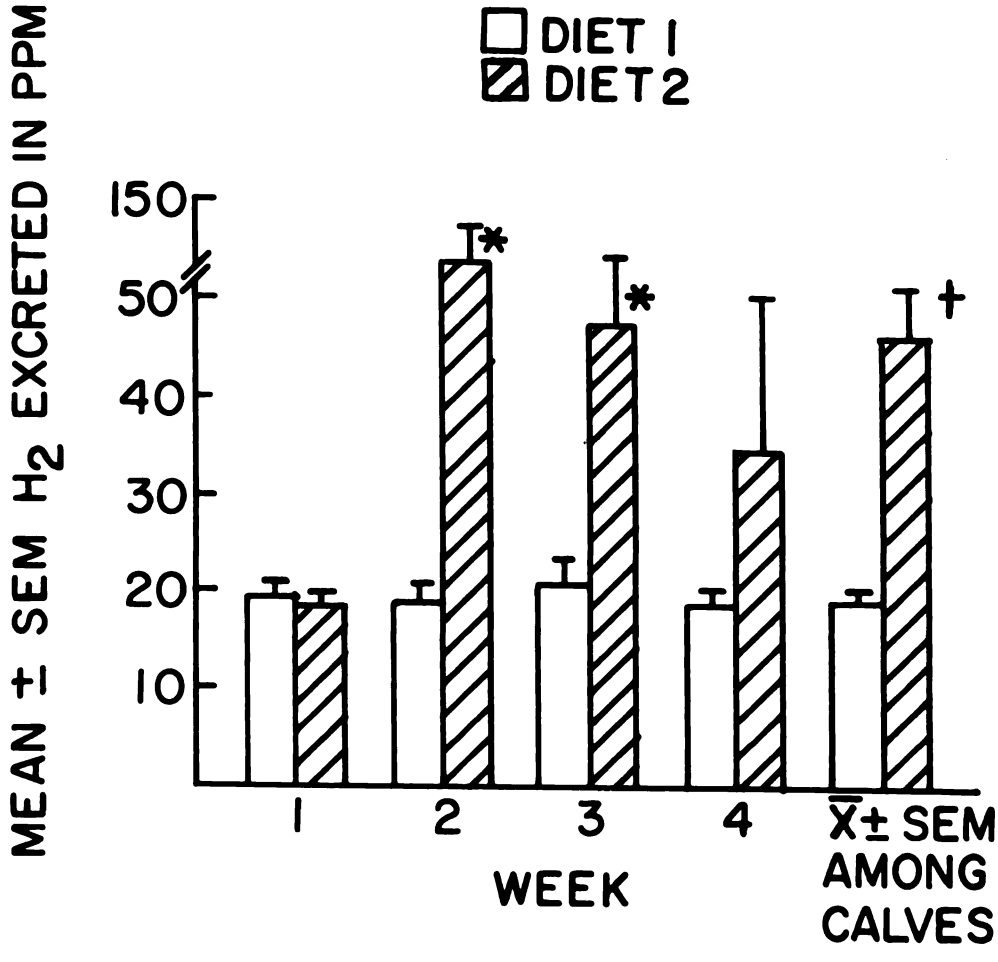

Figure 3.1: Mean ± SEM end-expired breath H_2 excretion. *End breath H_2 excreted for weeks 2 and 3 were significantly higher ($P < 0.05$) for diet 2. +Overall X ± SEM end breath H_2 excreted among calves was significantly higher ($P < 0.05$) for diet 2.

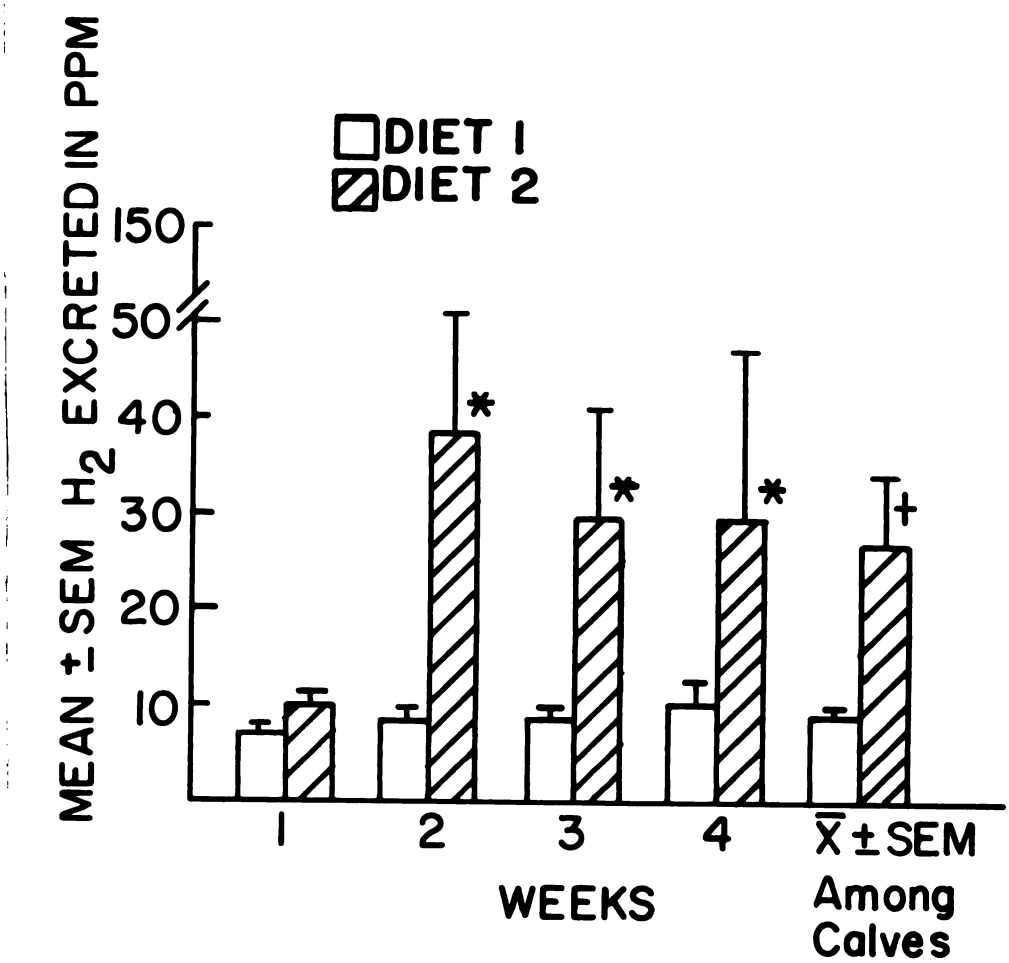

Figure 3.2: Mean ± SEM total-expired breath H_2 excretion. *Total breath H_2 excreted for weeks 2, 3, and 4 were significantly higher ($P < 0.05$) for diet 2. +Overall $\bar{X}$ ± SEM total breath H_2 excreted among calves was significantly higher ($P < 0.05$) for diet 2.

rumen, once functional, provides a favorable environment for microbial activity. Dietary carbohydrates such as sugars and starches are degraded into volatile fatty acids and gases.[6-8] The 2 major gases produced are carbon dioxide and methane.[7,8] Hydrogen is produced by some organisms and reacts with carbon dioxide to produce methane.[7,8]

The excretion of H_2 at weeks 2,3, and 4 for diet 2 was increased over that of diet 1 for the same weeks. The rumen, as an organ of fermentation and storage, serves as a constant supply of feed materials for digestion within the gastrointestinal tract. Microbial fermentation of residual feed materials within the rumen and intestinal tract produced increased amounts of H_2. The specific site of H_2 production was not determined in this experiment. However, it has been reported that H_2 is infrequently found or is present in small amounts in rumen gas samples.[8,9]

Hydrogen excreted at week 1 of diet 2 did not differ from week 1 of diet 1. Limited rumenal development or the absence of H_2 producing organisms might have contributed to this observation since the calves were off milk completely at this time.

The two previous experiments have demonstrated that increased H_2 excretion occurs when diarrhea or small intestinal malabsorption is present. Those experiments were performed on preruminanting calves fed whole milk. Therefore, lactose was the principle carbohydrate. The results of this experiment demonstrate how high concentrations of H_2 can be excreted by clinically normal calves fed hay and concentrate.

FOOTNOTES

a 22% (crude protein) concentrate, 15% (crude protein) alfalfa hay obtained from MSU dairy facilities.
b No. 240 Intramedic tubing, (inside diameter, 1.67 mm; outside diameter, 2.42 mm) Clay-Adams, Division of Becton, Dickinson and Co, Parsippany, NJ.
c Rudolph Valve No. 1400, Han's Rudolph Inc, Kansas City, MO.
d North American Drager, Telford, PA

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CONCLUSIONS

End-expired and total-expired breath H_2 excretion were measured in these experiments to determine whether H_2 excretion could be utilized to monitor malabsorption of carbohydrates and proteins in calves.

Lactulose was administered to determine what effects a nonabsorbable carbohydrate had on H_2 excretion. Hydrogen excretion after lactulose administration was significantly higher than values determined after milk was fed. This indicated that, in the calf, fermentation of nonabsorbed carbohydrates resulted in increased H_2 excretion.

Hydrogen excretion resulting from impaired absorption and decreased mucosal cell function was demonstrated by the oral administration of chloramphenicol in experiment 1 and the oral inoculation of Cryptosporidium oocysts in experiment 2. Chloramphenicol induced villous atrophy, thereby causing small intestinal carbohydrate and protein malabsorption. Villous atrophy was demonstrated by decreased D-xylose absorption and a significant decrease in villous length after chloramphenicol administration. It was shown that calves inoculated with Cryptosporidium sp exhibited malabsorption and diarrhea. After Cryptosporidium inoculation, there was a significant increase in H_2 excretion and fecal production. This effect was transitory, since H_2 excretion and fecal production returned to preinoculation values.

Feeding hay and concentrate caused significantly more H_2 excretion compared to feeding whole milk. The increased H_2 excretion was probably related to development of the rumen as an organ of fermentation and to the persistent fermentation of residual dietary products within the gastrointestinal tract.

The assessment of H_2 excretion in these experiments demonstrated that increased H_2 concentrations were excreted 1) when a nonabsorbable carbohydrate was administered, 2) when small intestinal malabsorption was induced by the oral administration of a noxious drug and the inoculation of a known enteric pathogen, and 3) after feeding a hay and concentrate diet. Therefore, the breath H_2 test may be useful in evaluating small intestinal malabsorption of carbohydrates and proteins in young calves fed milk alone. However, it is an inappropriate test to use on calves fed hay and concentrate. The end-expired and total-expired interval sampling techniques were an appropriate means of obtaining H_2 samples. Although the face mask used permitted considerable dead space contamination, it provided a crude, yet reliable means of obtaining H_2 samples. The end-expired technique was more cumbersome to use, yet provided more consistent values.

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