A STUDY OF DIGESTION OF STARCH IN THE LARGE INTESTINE OF THE YOUNG CALF BY AN IN VITRO TECHNIQUE

by

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INTRODUCTION

The advent of milk replacers and calf starters has greatly economized calf raising, permitting the diversion of fluid milk for human consumption. Whereas lactose is the only carbohydrate consumed in significant amounts by the milk-fed calf, starch and other carbohydrates are included in milk replacers. Thus there is a shift in the type of carbohydrate from lactose to starch and other carbohydrates consumed by the young growing ruminant. Also, starch is the most economical carbohydrate in calf starters.

The digestion and utilization of starch by the young calf during its critical stages of development into a mature ruminant has been the subject of research and study since 1918. Studies on carbohydrate utilization by young calves have shown that lactose and glucose are well utilized and maltose is utilized to a limited extent. Starch and sucrose are not well utilized; however, conventional digestion trials have shown that starch and sucrose are digested to an appreciable extent. Other reports showed that starch and sucrose might be metabolized to simpler products by the action of microorganisms in the posterior part of the alimentary canal.

The techniques used in the above studies included growth studies, conventional digestion trials, blood reducing sugar responses, estimation of intestinal and pancreatic carbohydrases, apparent digestion studies using calves with ileal and cecal fistulae, and study of the metabolic products of carbohydrates

in the intestinal tract. Yet the picture is not sufficiently clear as to the metabolism and efficiency of utilization of starch in the young animal.

The possible ways by which starch could be broken down in the alimentary tract of the young bovine are by enzymes secreted by the host animal or by enzymes produced by microorganisms. Amylase activity in the intestinal tract has been reported to be low. This, taken together with the measurement of blood reducing sugar response, indicates breakdown of starch by enzymes produced by the host is limited.

The work reported in this project was an indirect approach to study postruminal digestion of starch by mixed intestinal microorganisms by an *in vitro* technique.

REVIEW OF LITERATURE

Role of Rumen in Mature Ruminant

The natural diet of the adult ruminant includes large quantities of grass, hay, or silage, which are fibrous, containing about 20% of crude fiber. The rumen is ideally suited to break down crude fiber, by virtue of its large capacity and favorable internal environment of pH, temperature and anaerobiosis. The products of crude fiber breakdown include bacterial polysaccharides, volatile fatty acids (VFA), and gases. The VFA are a major source of energy in the adult ruminant and are absorbed through the rumen wall (Barnett and Reid, 1961).

Role of Rumen in the Young Ruminant

According to Sisson (1948) the rumen and reticulum in the newborn calf put together were much smaller than the abomasum. Tamate et al. (1962) found that the reticulo-rumen developed as the animal grew and received hay and grain in addition to milk.

Sutton et al. (1963) found that the ability to absorb large quantities of acetic acid was not inherent in the rumen of the very young calf and did not develop on a diet of milk only. Propionic and butyric acids were much more slowly absorbed than acetic acid.

Wise et al. (1946) studied the nutritional role of the rumen in the young calf by performing rumenectomy. For two months immediately following the operation no abnormalities were seen.

Lupien et al. (1962) performed gastrectomy on calves 3 to 4 weeks of age by total removal of rumen, reticulum, upper third of abomasum and spleen to study digestion and blood changes in calves. The age at autopsy of calves rumenectomized at 3 to 4 weeks of age ranged between 17 to 31 weeks.

Stewart and Henning (1965) used an esophageal-abomasal anastomosis procedure to study forestomach development in calves 4 to 90 days of age. No rumen papillary development occurred in these calves which survived for about 6 months.

These experiments suggest the rumen does not have the same nutritional significance in the young animal as it does in the mature ruminant.

Digestive Role of the Intestines

In the young calf, milk passes directly into the omasum and abomasum (Schalk and Amadon, 1928). The passage and retention time of ingesta through various segments of the alimentary tract posterior to the rumen, reticulum and omasum is of significance to the digestion of nutrients. Coomb and Kay (1965), studying the rate of passage and retention time of digesta in the intestines of sheep, found that the retention time in the large intestines (10.2 to 26.5 hours) was much longer than in the small intestines (2.25 to 4.5 hours). The efficiency of digestion of organic matter and absorption of water and minerals were found to be influenced by the time that the digesta remained in the intestines. In both the small and large intestines, retention times were inversely related to dry matter intake of a particular

feed and to fecal output. They found the pattern and fluctuations in the excretion of markers from the large intestines suggested that passage through the large intestines involved some stormy mixing and intermittent release of digesta during a 24-hour period. They concluded that, on anatomical grounds, the cecum was most likely concerned in the rate of passage of digesta in the large intestines.

Benzie and Phillipson (1957), by a radiographic technique, observed that in the newborn kid, barium sulphate ($BaSO_4$) and milk fed from a bottle entered the duodenum in 5 minutes. In older animals, entry of $BaSO_4$ into the duodenum was slower. Barium sulphate tended to accumulate to a greater extent in the ileum than in the jejunum. They found $BaSO_4$ entering the large intestines in the suckling ruminant 3 to 4 hours after feeding. In a four-day-old kid barium was still present in the large intestines more than 23½ hours after feeding, though very little was found in the rest of the gut. The radiograph of a young kid showed pellet formation in the distal colon 7½ hours after feeding.

Fischer and Sutton (1949), in a review, concluded that gastrointestinal motility changes due to carbohydrate feeding might be due to indigestibility of the carbohydrate fed, its hydragogue effect, age of the animal, alteration of the intestinal flora, alteration of pH, and the nature of the carbohydrate molecule itself.

Postruminal Digestion of Carbohydrates

In the intestines, the factors responsible for digestion are pancreatic juice, bile, and intestinal juice. The extracts of submucosa in the intestinal gland zone of all domestic animals contains amylase. Pancreatic amylase hydrolyzes starch and various dextrins to maltose (Dukes, 1955).

Shaw et al. (1918) found that the digestibility of starch in 4-7 and 30-day-old calves was 20 and 90%, respectively, and suggested that starch might be included in the milk diet of young calves a few days old. This report conflicted with later studies, using refined techniques, of starch utilization in the young calf.

Johnson et al. (1940) studied growth requirements of calves by feeding purified diets from 2 to 10 days of age. Mixtures of casein, lactalbumin, sugar, butter or lard, minerals and water were fed as substitutes for milk. These workers reported that the growth of calves was below normal in most cases.

Rojas et al. (1948) found that the lactose in whole milk or modified milk was well utilized by calves fed these rations for 42 days. Even in calves that scoured, no appreciable quantities of reducing substances were detected.

Williams and Knodt (1949) fed four different types of milk replacements, all containing 5% oat flour and 7.75% dextrose, to Holstein calves from 4 days of age for 16 weeks. At the end of the period there was very little difference in all the groups in their rate of growth or general appearance as compared to the milk-fed controls.

Flipse et al. (1950) studied the nutritive value of glucose, corn syrup, and lactose as carbohydrate sources in synthetic milk fed to calves from 2-3 days after birth for a 31-day feeding period. Only calves that received lactose grew normally. The average weight gains for calves fed glucose, corn syrup, and lactose were 9.33, 8.66, and 18.66 pounds, respectively.

Larsen et al. (1956) found no rise in blood sugar following abomasal infusion of corn or starch in 8-9 month old bull calves. Following abomasal infusion of malt syrup or dextrose, there was an increase in blood sugar between 2-4 hours after feeding.

Noller et al. (1956) found that milk replacers containing material of vegetable origin were not satisfactorily utilized till the calf was approximately 25 days old. Ten to fourteen day old calves digested only 25% of the dry matter as compared to 75.4% digestibility of dry matter by calves 26 to 38 days of age.

Barhydt and Dye (1957) found that little digestion, absorption or fermentation of carbohydrates took place in the abomasum. On a milk diet, fed from a nipple, milk entered directly into the abomasum but all the lactose was digested and absorbed in the small intestine.

When lactose was included in a milk diet in place of milk fat, digestibility of dry matter was 90.3 to 95%, though the diet contained 66% lactose (Raven and Robinson, 1958). Cornstarch, when included in a milk diet, was only 50% digested and the dry matter digestibility of the entire diet was 77.4 to 81.3%.

Dollar and Porter (1959) found that the calf was unable to utilize raw starch or starch modified by cooking or enzyme action till it was 30 days old. This inability was due to insufficient secretion of digestive enzymes in the gastrointestinal tract. Lactase activity was quite appreciable; maltase and pancreatic amylase activities were low with no sucrase activity detected.

Young calves up to 6 weeks of age were found by Okamoto et al. (1959) to be able to utilize glucose and lactose but not sucrose, dextrins or starch. When glucose, sucrose, dextrins or lactose was administered to fasted calves 1, 2, 3, and 4 weeks of age, no change in blood sugar levels was seen except in the glucose- or lactose-fed calves, in which the levels after 5 hours were 2-3 times higher than the fasting levels. Sucrose plus invertase produced changes similar to glucose. Fungal amylase fed with dextrins or starch did not increase response. Pancreatic diastase (4%) plus dextrins increased the response slightly.

When various sugars were fed as warm solutions to fasting bull calves (Velu et al., 1960), glucose gave the highest blood sugar response. Fructose utilization was slight throughout the eight-week feeding period. There was little or no maltose utilization. Calves were not able to absorb sucrose throughout the test period. Corn syrup (Stalex) was utilized between 4-8 weeks of age as shown by a blood sugar rise of 60-106 mg./100 ml. at 0.5 hour and reaching a maximum of 205 mg./100 ml. at 1.5 hours after feeding. Karo corn syrup was not utilized so well. Sucrose inverted by invertase or citric acid was readily utilized.

Netke et al. (1960) found daily gains of calves higher when

only dried skim milk was fed without addition of any carbohydrate. When starch, with or without amylolytic enzymes was fed, gains were more on the diet containing enzymes and starch. When molasses, with and without acid hydrolysis, was fed at 33.3% of the ration, calves made higher gains on the acid hydrolyzed molasses diet. The calves scoured severely on the other diet.

Huber et al. (1961a) studied the effect of age and diet on digestive enzyme pattern in calves sacrificed at intervals up to 6 weeks of age. Lactase activity was highest at one day of age, decreased by 50% at 22 days, and changed very little from 22 to 44 days. Maltose utilization by one month old calves was poor but improved by 7 weeks of age. The inefficient utilization of starch by young calves was presumed to be due to low levels of maltase and amylase. Intestinal sucrase was not present. Pancreatic amylase, as estimated by blood reducing sugar levels, was low.

Huber et al. (1961b) studied the ability of the bovine from 2 weeks to 2 years of age to utilize various carbohydrates by measuring blood reducing sugar responses. Glucose, maltose, lactose, sucrose, amylose, amylopectin, Flojel, and tapioca starch were fed as separate test meals to 5 age groups of mean ages 22, 50, 136, 227, and 600 days. Maximum increases of blood sugar from the 5 groups from youngest to oldest were (in mgs.): glucose, 134, 130, 76, 82, 50; lactose, 147, 117, 36, 37, 14; and maltose, 31, 72, 30, 34, and 17. Increases following sucrose and starch digestion were not appreciable.

The apparent digestibilities for 8 different carbohydrates

fed at 10% of the milk weight were determined in calves 12-14 weeks of age (Huber et al., 1961c). The digestion coefficients were: lactose, 94; maltose, 97; sucrose, 57; amylose, 83; amylopectin, 89; Flogel, 80; and tapioca starch, 80. Using an increase in blood sugar to study carbohydrate utilization, they obtained greatest response from lactose and maltose. Increases in blood sugar levels due to starch feeding were found to be more sustained than those for controls, as contrasted to earlier work in which only negligible increases in blood sugar were noticed.

Morrill et al. (1965) studied the degree of utilization of carbohydrates in the alimentary tract posterior to the rumen but proximal to large intestines in young calves. From the differences in digestibility of the carbohydrates determined from ileal and fecal samples, the digestion coefficients of the test carbohydrates posterior to the rumen but orad to the large intestines were estimated, which in the case of sucrose was 41%.

Putnam and Davis (1965) fed alfalfa fiber and wood cellulose to two intact and two abomasally fistulated calves 4 to 14 months of age to determine postruminal fiber digestion. They found that the postruminal digestion coefficient for alfalfa fiber and wood cellulose was only 29% in fistulated calves, whereas calves fed orally had digestion coefficients of 43 and 63%, respectively. They concluded that the potential for postruminal fiber digestion in the young ruminant was less than in the horse but greater than in swine or rabbits.

Postruminal Volatile Fatty Acid Production

Norris (1925) found much greater concentration of VFA and alcohols in feces of calves on a cereal diet than on milk. The inability to digest starch well was stated as the reason for greater production of VFA, as the lower intestinal tract became rich in carbohydrate residues, which served as a medium for development of aciduric bacteria.

Barcroft et al. (1944) reported that VFA which appeared in the blood draining the cecum of sheep was a clear indication of carbohydrate fermentation in the large intestines. They found that the mixture of the acids in the cecum was similar to that in the rumen.

Ridges and Singleton (1962) observed that four-fifths of the soluble carbohydrate fed to goats disappeared in the rumen and that some of the remaining might be fermented to VFA in the large intestines.

Morrill et al. (1965) found that an average of 43.5% of the total sucrose fed was hydrolyzed in the large intestines of calves 1 to 4.5 months of age. As this hydrolysis of sugar was not associated with increases in blood sugar, further metabolism of the products of hydrolysis to VFA was suggested.

Huber et al. (1961c) determined the apparent digestibilities of carbohydrates by collecting composite (total collection) and diurnal (manual collection by mechanical stimulation every two hours) fecal samples. The apparent digestibilities for composite and diurnal samples, respectively, were: lactose, 94, 95;

maltose, 97, 96; starch, 83, 80; and sucrose, 57, 32. The high apparent digestibility for sucrose for the composite sample was suggested to be due to fermentation of sucrose in the collection pan.

Ward et al. (1961) determined the VFA concentrations in samples of digesta from various sections of the gastrointestinal tracts of full fed heifers. The concentrations of VFA varied in the small intestines from 1.607 to 5.446 mM./100 ml. of fluid. The average total concentrations of VFA in the cecum and colon were 12.155 and 11.214 mM./100 ml. fluid, respectively.

Huber and Moore (1964) fed 4 different rations to calves from 3 to 77 days of age and then sacrificed them. On the hay-grain ration, acetic and lactic acids, with traces of formic, were the only acids found in the small intestines. Total concentrations were higher in the large intestine and cecum than in the small intestines. On the hay-grain and whole milk plus 15% lactose rations, total concentrations of VFA were higher than on whole milk or whole milk plus 5% lactose rations. This was believed to be due to passage of much undigested material into the large intestines when calves were fed the first two rations. Acetic, propionic, and butyric acids formed most of the acid in large intestines and ceca on hay-grain, whole milk, and milk plus 5% lactose rations, with acetic acid predominating. The ratios of acetic to propionic acid in large intestines and ceca of calves on the hay-grain ration were 4.6 and 4.8, respectively. Ratios were higher for calves on the milk ration.

Young et al. (1965) studied the ability of milk-fed calves

to metabolize labelled VFA administered intravenously. By measuring expired CO_2 derived from each acid they concluded that though VFA were not actively produced in the rumen, the young calf had the ability to obtain a significant portion of its maintenance energy from the metabolism of VFA.

Martin et al. (1959) compared the responses of feeding a starch-hay diet, VFA diet, and a whole milk diet to young calves. They found no rise in blood concentration of VFA in the milk-fed group in 6 hours, though a slight rise was noticed in the eighth hour. When the other two diets were fed there was marked increase of VFA in the blood.

When two rations, one containing 7 and the other 27% fiber, were fed to Holstein bull calves (Yang and Thomas, 1963), the entire alimentary tract contained 2 and 6 moles of VFA on the 7 and 27% fiber rations, respectively. The ruminal VFA accounted for 79 and 75% of the total on the 7 and 27% fiber rations, respectively. The molar percentages of acetic and propionic acids were 66 and 24 in the rumen and 75 and 17 for the lower tract, respectively.

In Vitro Techniques Used to Study Rumen Function

In vitro techniques have been extensively used in rumen metabolism studies. Similar use of in vitro techniques for study of microbial activity in the intestinal tract of ruminants apparently has not been reported, though Jesuitova et al. (1964) used an in vitro technique for the study of digestion of soluble starch in the rat intestine.

Huhtanen et al. (1954) observed that any in vitro apparatus, as the "artificial rumen," had both advantages and disadvantages. The advantages and uses listed were: convenience, economy, usefulness as a screening technique, study of the breakdown of nutrients in ruminant feeds other than fiber and the end products of digestion, study of fiber breakdown by pure cultures of rumen bacteria, and study of in vitro effect of antibiotics. The disadvantages cited were the problem of applying results from the laboratory to the intact animal and the limitation that the results obtained do not represent similar functions in vivo.

For an in vitro system to approximate the conditions in vivo, Barnett and Reid (1961) have noted that maintenance of numbers and motility of organisms, maintenance of normal rates of digestion of cellulose, starch and protein, and the ability to predict quantitatively in vivo results were the main considerations. Other important considerations listed were: Use of a suitable mineral salt solution, suitable inoculum, necessary nutrients, test substrate, an incubation temperature of 39-40°C., and control of pH and anaerobiosis.

Bezeau (1965) studied the effect of source of inoculum on in vitro digestion of five different hays. These hays formed the test substrates which were incubated with inocula obtained from rumen fistulated cows fed these hays. Inocula obtained from animals fed two alfalfa hays were more active than those from native hay. Inocula from animals fed grass hay were more active than those from the native hay. A highly significant difference existed between the activities of the inocula from

the two donor cows.

Stewart and Schultz (1958) used an *in vitro* procedure to find feed(s) which would influence the total VFA produced, especially the proportion of propionic acid. Urea caused the greatest increase in VFA, though this was not found in earlier *in vivo* work, perhaps due to small quantities used. Molasses depressed acetic acid production whereas cornmeal significantly increased propionic acid production. Feeding cornmeal and urea gave lower levels of VFA than feeding beet pulp and urea. Feeding hay did not yield VFA in such large quantities as did grass. Inoculum source did not affect individual or total VFA production.

Salsbury *et al.* (1961) obtained inocula from animals fed hay and concentrates in 10:1 (Ration A) or 11:9 (Ration B) ratios. Strained rumen fluid, obtained from animals fed either Ration A or B, was incubated with either raw or boiled starch, resulting in approximately equal VFA production. Incubation of boiled starch generally but not invariably resulted in higher lactic acid production than untreated starch. With centrifuged rumen fluid from Ration B as the inoculum, production of total VFA was low with a high production of lactic acid.

Using an *in vitro* system for the study of starch fermentation by ruminal bacteria from sheep, Moore *et al.* (1962) found maximal fermentation at pH 6.8. Differences in the amounts of starch fermented in a given time were suggested to be due to the lag phase in the activity of the microorganisms. These lag periods in microbial activity differed from donor to donor as well as among trials conducted. Accumulation of lactate was associated

with higher rates of fermentation.

Cline et al. (1958) studied utilization and synthesis of valeric acid during digestion of glucose, starch and cellulose by rumen microorganisms in vitro. The type of substrate influenced concentration of valeric acid after 30 hours of fermentation. When cellulose was the substrate, no appreciable amounts of valeric acid were present.

EXPERIMENTAL PROCEDURE

Experiment I

This experiment was designed to evaluate the digestion of starch by a mixed population of microorganisms from the large intestines of the young calf by an *in vitro* technique, with the following specific purposes: (1) To determine if *in vitro* techniques are appropriate for the study of intestinal microorganisms; (2) to determine what intermediate and end products of starch fermentation could be detected by an *in vitro* technique; (3) to develop techniques for collection and handling of inoculum from the intestinal tract for *in vitro* fermentations; and (4) to determine if changes in the intestinal microbial population due to a change of diet can be detected by *in vitro* techniques.

To estimate the amount of starch fermentation at the end of the specified periods, the concentrations of total carbohydrates, reducing sugars, and volatile fatty acids (VFA) were estimated.

Feeding and Management of Experimental Animals. Three Holstein bull calves were used in this experiment. They were separated from their dams 4 days after birth and kept in metal cages with heavy metal screens. Calf 042 was fed whole milk at 10% of its body weight in two equal feedings daily till 113 days of age. From the 114th day pregelatinized starch (table 1) was included in the diet gradually till the calf was receiving 125 gm. per feeding on the 116th day. This level caused severe scouring, so the amount was reduced to 75 gm. per feeding. This diet was maintained for the duration of the experiment.

TABLE 1. EXPERIMENTAL ANIMALS AND DIETS (EXPERIMENT I)

Trial	Number of replicates	Calf	Date of birth	Date fistulated	Diet
1	4	042	3-17-65	4-6-65	Whole milk[a,b]
2	3	042	3-17-65	4-6-65	Whole milk[a,b] + Amijel[c]
3	3	066	7-22-65	7-29-65	Basal milk replacer[a,b,d]
	3	067	7-24-65	7-29-65	

[a]Each calf received 750 mg./day of a mineral supplement having the following analysis (%): calcium, 12; iron, 10; manganese, 10; zinc, 5; copper, 1; iodine, 0.3; and cobalt, 0.10.

[b]The vitamin premix provided each calf 5,000 I.U. vit. A, 400 I.U. vit. D, 10 I.U. vit. E, and 66 mg. oxytetracycline per day.

[c]Amijel. Pregelatinized Corn Starch, Corn Products Company, New York.

[d]The basal milk replacer consisted of 90% spray dried skim milk and 10% animal fat.

Calves 066 and 067 were fed whole milk till 7 days of age. The diet was then changed to basal milk replacer to provide a constant diet. The composition of milk from different cows could vary from day to day whereas uniformity of the diet fed could be maintained by feeding a milk replacer. The milk replacer was reconstituted with water (15% w/w) and fed at 10% of body weight in two equal feedings per day. The calves were on this diet throughout the remainder of this experiment.

Once daily all three calves received a supplement of minerals, vitamins, and an antibiotic (table 1).

Operative Technique. When the animals were of the following ages: 042, 19 days; 066, 7 days; and 067, 5 days; re-entrant colonic fistulae were established. The animals were anaesthetized

and a vertical incision was made from about 1 inch ventral to the third lumbar transverse process and extending 3 to 4 inches ventrally in the right paralumbar area. The peritoneum was incised and the cecum and proximal colon were brought out through the abdominal incision. A longitudinal incision of about 1½ inches was made into the lumen of the colon just distal to the ileocecal valve for the proximal cannula. A second longitudinal incision was made 3 inches distal to the first, for the distal cannula. The bases of the two cannulae were inserted through the two respective incisions and sutured into position. The barrels of the two cannulae were brought out through the skin through stab incisions which were then sutured. The cannulae were held in position with rubber washers.

The different parts of the cannulae used were similar to those used by Morrill et al. (1965), except that right angled stainless steel elbows (external diameter 16 mm. and internal diameter 14 mm.) and rubber tubing were used for connecting the exposed ends of the cannulae barrels.

Inoculum Collection. On days of in vitro fermentation trials, the stainless steel elbows were disconnected and a sterile rubber balloon was slipped on to the end of the proximal cannula. The distal end was closed with a rubber stopper. As soon as the sample was collected, the tip of the balloon was tightly tied with a string, the balloon was removed and placed in a vacuum jar containing water at 39-40°C. and immediately taken to the laboratory for use.

In Vitro Studies. In vitro fermentations were carried out

in 125 ml. Erlenmeyer flasks by a method similar to that of Baumgardt et al. (1962). Each flask contained 100 mg. soluble starch,[1] 18 ml. buffer, and 2 ml. of the material collected from the large intestines of the donor, which comprised the inoculum. The flasks were flushed with CO_2 before and after the addition of inoculum for 5 to 10 seconds, stoppered with Bunsen valves and kept in a shaking water bath at 39 to 40°C. At the end of 0, 3, 6, and 12 hours of fermentation the respective flasks were removed from the water bath and the pH of the contents was measured. After fermentation was stopped by adding 1 ml. 4 N H_2SO_4, the samples were stored in a freezer till analysis was made. The number of flasks used for each period of fermentation was: 3, (0 hour); 2, (3 hours); 2, (6 hours); and 3, (12 hours).

Analysis of Samples. Two flasks each from 0 and 12 hours and one each from 3 and 6 hours fermentation were analyzed for total carbohydrate by the method of Dubois et al. (1956) as modified by Marier and Boulet (1959). The samples were thawed at room temperature and to the entire contents of each flask 10 ml. of 2N NaOH were added to dissolve the starch. After 1 hour, 10 ml. of 2N HCl were added for complete neutralization (Carroll and Cheung, 1960). This procedure was followed because earlier results had shown that removal of aliquots of the sample for analysis before alkali treatment gave erroneous results. By taking the entire contents of the flask the percent recovery of

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[1]Soluble Starch, Difco Certified, Difco Laboratories, Detroit, Michigan.

soluble starch was 101.7 ± 4 (S.D.).

The concentrations of VFA and lactic acid were determined by the method of Wiseman and Irwin (1957) with the following modifications (Waldren, 1962). Reagent grade acetone and normal hexane (Phillips 66 high purity) were used instead of Skellysolve B. The concentrations of solvents used for elutions were: [percent (V/V) acetone in hexane] for butyric acid, 1; for propionic acid, 5; for acetic acid, 15; for formic acid, 30; and for lactic acid, 20.

To prevent gradual removal of water from the column by dry eluents, concentrations of solvents above 15% were equilibrated with 50% sugar solution but without the addition of saturated barium hydroxide solution and cresol red indicator. The cap material used contained 8 parts celite[2] to 12 parts sodium sulfate.

The titrant used was isopropanol-potassium hydroxide reagent prepared by dissolving 25 gm. potassium hydroxide pellets in 400 ml. isopropanol by warming in a steam bath. The supernatant solution was decanted and cooled to obtain the isopropanol-potassium hydroxide solution which contained approximately 50 mg./ml. potassium hydroxide. This formed the stock solution. For titration, 12 ml. of the stock solution, 488 ml. methanol and 500 ml. isopropanol were mixed to yield approximately 0.01 N potassium hydroxide, which was then titrated with standard potassium acid phthalate solution with cresol red indicator, in an atmosphere of nitrogen. This formed the standard titrant.

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[2]Johns-Manville Celite, Analytical Filter Aid.

Analysis of reducing sugars was done by the method of Somogyi (1945, 1952).

For the analysis of variance for total carbohydrates and reducing sugars, a split plot design was used, Snedecor, 1956).

Experiment II

The results obtained in Experiment I suggested that a change in diet caused a change in the metabolites produced in the large intestine, perhaps due to a change in the microbial population. Experiment II was therefore designed to study the effect of three different diets on intestinal microbial activity in fermenting raw, pregelatinized, and soluble starches *in vitro* and to compare the digestibilities of raw and pregelatinized starches *in vivo*.

Feeding and Management of Experimental Animals. The two animals used in this experiment (066 and 067) were the same used previously in Experiment I. Three trials were conducted using these two animals in each trial. The diets fed and the number of replicates carried out are given in table 2.

The basal diet comprised of a mixture of 90% spray dried skim milk and 10% animal fat. In addition to the basal diet they were fed either raw or pregelatinized starch introduced into the diet at 5% of the total dry matter fed. Inoculum from each animal was obtained for *in vitro* studies during the last 3 days of Trials 1, 3, and 4. Fecal collections for determining *in vivo* digestion were made during the last 5 days in Trials 1, 2, 3, and 4.

TABLE 2. EXPERIMENTAL ANIMALS AND DIETS (EXPERIMENT II)

Trial	Number of days	066 Diet	066 Replicates	067 Diet	067 Replicates
1	10	Basal milk replacer[a,b,c] + Amijel[d]	3	Basal milk replacer[a,b,c] + raw starch[e]	3
2	10	Basal milk replacer[a,b,c]	-	Basal milk replacer[a,b,c]	-
3	10	Basal milk replacer[a,b,c] + raw starch[e]	3	Basal milk replacer[a,b,c] + Amijel[d]	3
4	10	(Died)		Basal milk replacer[a,b,c]	3

[a]The basal milk replacer consisted of 90% spray dried skim milk and 10% animal fat.

[b]Each calf received 750 mg./day of a mineral supplement having the following analysis (%): calcium, 12; iron, 10; manganese, 10; zinc, 5; copper, 1; iodine, 0.3; and cobalt, 0.10.

[c]The vitamin premix provided each calf with 5000 I.U. vit. A; 400 I.U. vit. D; 10 I.U. vit. E; and 66 mg. oxytetracycline per day.

[d]Amijel. Pregelatinized cornstarch. Corn Products Company, New York.

[e]Raw starch, Buffalo, Corn Products Company, New York.

Collection and Analysis of Samples. The collection of inocula and the procedure for in vitro fermentation studies were the same as described in Experiment I except that raw and pregelatinized starches were also used in this experiment as substrates, following the same procedure as for soluble starch. Fermentation flasks for 3, 6, and 12 hours fermentation were used in duplicate and for 0 hour in triplicate.

During in vivo digestion trials, feces were collected in plastic bags as described by Noller et al. (1956) with the

following modifications. All the straps used were double straps, riveted together at each end. The strap on the underside of the calf was omitted; instead a double strap with a snap fastener connecting the straps was attached across the thighs below the perineum by means of branding cement.

The plastic bag for fecal collection was attached to the animal by first passing the tail through the tail opening. The flap was then passed in between the double strap on the back and fixed to the upper strap by means of small staples. The lower flap of the plastic bag was passed in between the lower straps across the thighs and fixed taut by fastening the snap fastener, which held the bag tightly in place.

Morning and evening and at any other time of visit to the calf barn the fecal bags were removed, labelled, and placed in a freezer.

Fecal samples for each digestion trial were thawed, mixed well, and composite samples were dried and analyzed for proximate composition by the A.O.A.C. (1955) method. Samples of feed were also analyzed for proximate composition.

Analysis of samples for total carbohydrates was as described in Experiment I.

For the analysis of variance for total carbohydrates a randomized block design, with unequal number of observations, was used for the comparison of diets and animal variation, (Snedecor, 1956).

RESULTS

Experiment I

Total Carbohydrates. Results of total carbohydrates always showed a rapid disappearance of substrate from 0 to 6 hours and gradual disappearance from 6 to 12 hours of fermentation (figures 1, 2, 3, and 4). The amount of carbohydrates remaining at each of the four successive sampling periods was significantly ($P<0.01$) less than that at the preceding period (table 3). The decrease in total amounts was nearly proportional at each of the successive sampling periods. The differences among calves were also significant, suggesting that inocula from different donors gave rise to different fermentation rates. A significant calf x fermentation period interaction indicated a differential response of calves in the fermentation periods. This may have been due to the difference in age of the experimental animals. The amount of soluble starch used in each flask for fermentation was 100 mg., but analysis of 0 hour flasks always gave values higher than 100 mg. for total carbohydrates after addition of inoculum. This was probably due to the carbohydrates present in the inoculum added. The range of values obtained (100.3 to 119.7 mg.) suggested that the total carbohydrate content of inoculum varied from day to day.

Reducing Sugars. Theoretically, the reducing sugar content of 0 hour flasks should have been low; the amounts detected could have been due to the reducing sugar in the inoculum added or due to the reducing activity of the soluble starch used as the substrate. The reducing sugar content of the starting substrate was

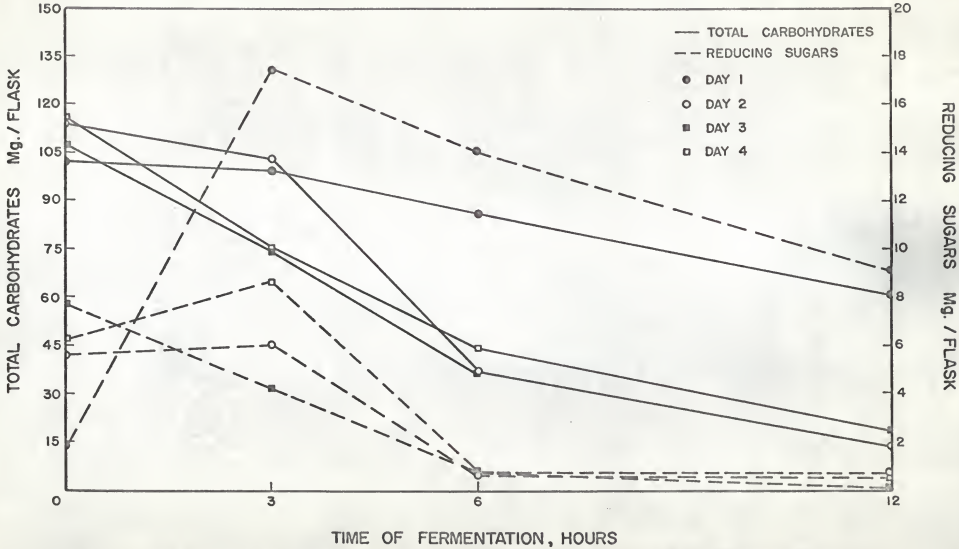

Fig. 1. Carbohydrates remaining after various periods of <u>in vitro</u> fermentation of soluble starch. Inoculum Donor 042; Diet, whole milk.

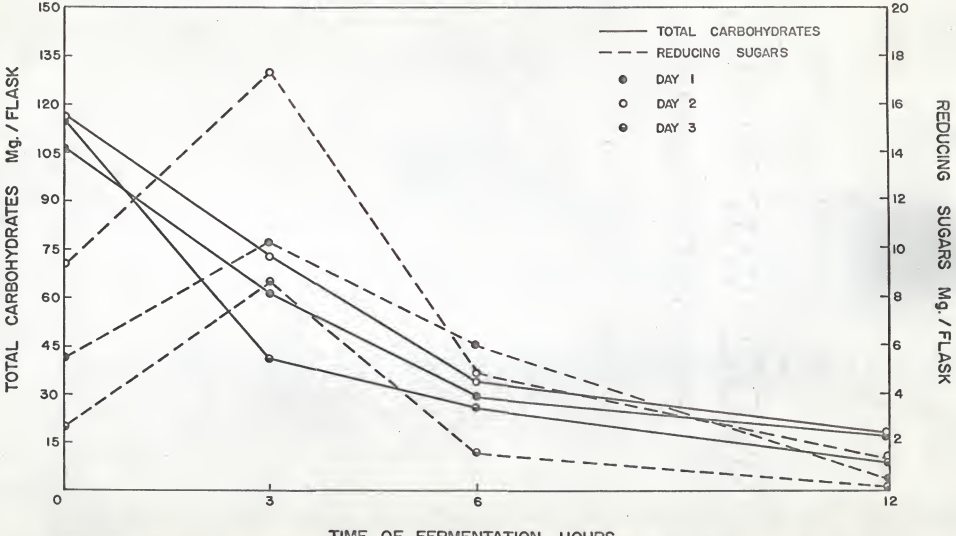

Fig. 2. Carbohydrates remaining after various periods of <u>in vitro</u> fermentation of soluble starch. Inoculum Donor 042; Diet, whole milk plus pregelatinized starch.

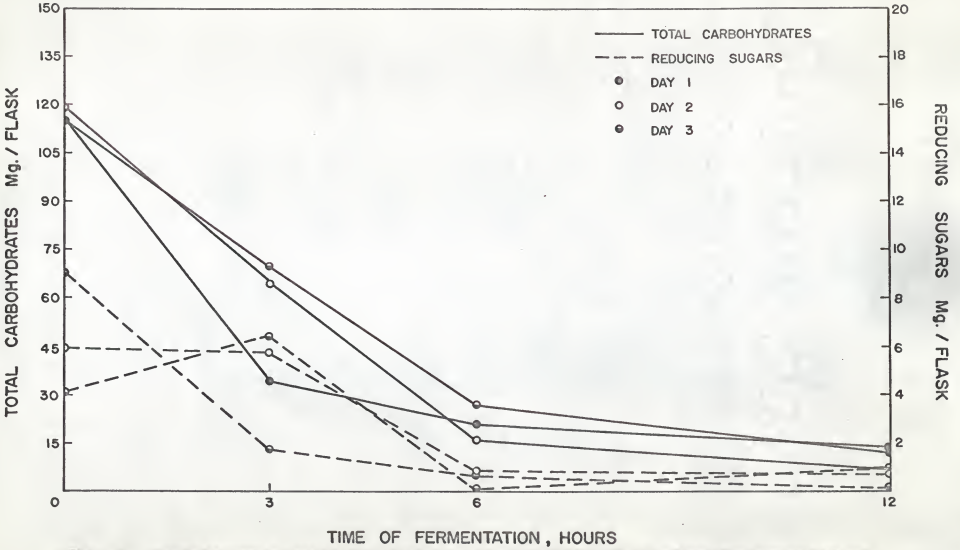

Fig. 3. Carbohydrates remaining after various periods of <u>in vitro</u> fermentation of soluble starch. Inoculum Donor 066; Diet, basal milk replacer.

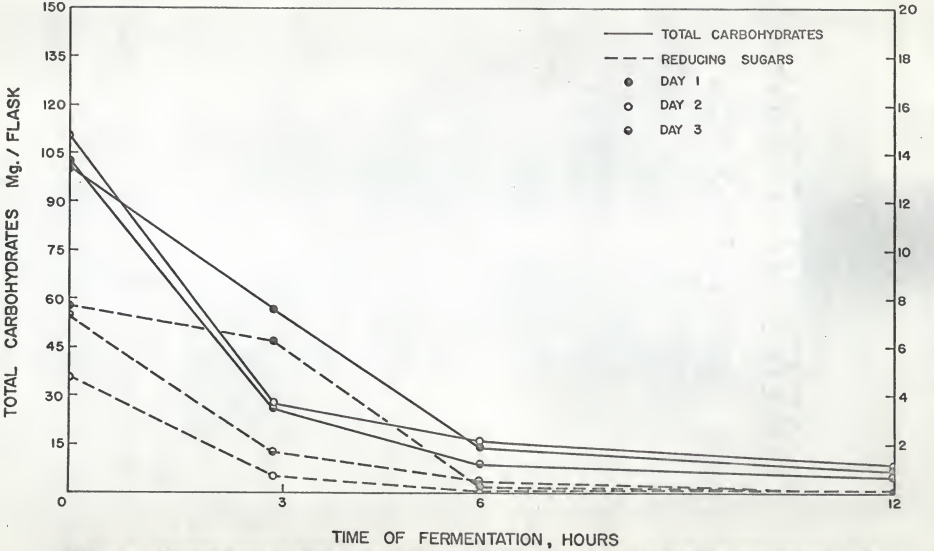

Fig. 4. Carbohydrates remaining after various periods of in vitro fermentation of soluble starch. Inoculum Donor 067; Diet, basal milk replacer.

TABLE 3. ANALYSIS OF VARIANCE OF TOTAL CARBOHYDRATES AND MEAN LEVELS OF TOTAL CARBOHYDRATES AT DIFFERENT FERMENTATION PERIODS (EXPERIMENT I)

Source of variation	d.f.	Mean square
Replicates	2	24.70
Calves	3	1041.10*
Error A	6	125.87
Fermentation period	3	23625.82**
Calf x fermentation period	9	195.74*
Error B	24	83.17

Fermentation period	Mean levels
hours	mg.
0	111.9[a]
3	58.9[b]
6	25.7[c]
12	12.3[d]

*$P < .05$

**$P < .01$

[a,b,c,d]Means having different superscripts are significantly different ($P < .01$) from each other.

not determined and hence no corrections were made in the values presented in figures 1, 2, 3, and 4. Analysis of variance (table 4) showed that highly significant ($P < .01$) differences for fermentation periods and fermentation period x calf interaction existed. This may have been due to calf 042 being older than calves 066 and 067. A comparison of mean values for reducing sugars revealed no significant difference between the amounts remaining at 0 and 3 hours after fermentation, though reducing sugars were highest 3 hours after fermentation. Likewise, the

difference in the mean values for reducing sugars remaining after 6 and 12 hours fermentation was not significant. The value for reducing sugars was significantly ($P < .05$) lower for both 6 and 12 hours as compared to 0 and 3 hours. The interaction between calves and fermentation would also suggest that there was a differential response of calves to fermentation.

TABLE 4. ANALYSIS OF VARIANCE OF REDUCING SUGARS, AND MEAN LEVELS OF REDUCING SUGARS AT DIFFERENT FERMENTATION PERIODS (EXPERIMENT I)

Source of variation	d.f.	Mean square
Replicates	2	3.36
Calves	3	23.94
Error A	6	9.18
Fermentation period	3	122.59**
Calf x fermentation period	9	11.48**
Error B	24	3.17

Fermentation period	Mean levels
hours	mg.
3	6.5[a]
0	6.3[a]
6	1.4[b]
12	0.4[b]

**$P < .01$

[a,b]Means having different superscripts are significantly different ($P < .05$) from each other.

Total Volatile Fatty Acids and Lactic Acid. The concentration of total VFA in the fermentation flasks showed a gradual increase from 0 hour reaching a maximum usually at 12 hours

(table 5). This increase in the concentration of VFA was in marked contrast to the steady but consistent fall in the levels of total carbohydrates or to the almost complete disappearance of reducing sugars after 12 hours of fermentation. The predominant VFA was acetic acid. Except on one day in Trial 1, Experiment I, the variation in concentration of acetic acid (27.35 to 39.30 μM/ml.) compared to the variation in propionic acid production (2.40 to 14.45 μM/ml.). The levels of formic acid decreased from the 6 to 12 hour fermentation period on 2 days and increased on 2 other days when donor 042 was on a whole milk diet. On a pregelatinized starch diet, formic acid levels increased from 0 to 12 hours. In the cases of 066 and 067 on the basal milk replacer diet, the production of formic acid increased from 0 to 12 hours fermentation. Valeric and succinic acids were found in very low concentrations. Lactic acid (table 5) was present only in Trial 2 when pregelatinized starch was added to the diet of the donor animal. The addition of pregelatinized starch resulted in increased production of acetic acid with a trend for decreased levels of propionic acid, resulting in a marked change in the C_2:C_3 ratio as compared to that in Trial 1. As fermentation proceeded there was a slight decrease of pH in the 12 hour fermentation flasks compared to 0 hour flasks. The VFA content of inocula was not determined.

Experiment II

In Vitro Digestion of Total Carbohydrates. The method of Carroll and Cheung (1960) used for dissolving starch prior to the

TABLE 5. THE IN VITRO PRODUCTION OF VOLATILE FATTY ACIDS AND LACTIC ACID FROM SOLUBLE STARCH

Day	Sampling period	pH	Micromoles per milliliter							Total VFA produced per mg. soluble starch
			C_4^a	C_3	C_2	C_1	Total VFA	Lactic acid	$C_2:C_3$	
	hours									micromoles
			Trial 1, calf 042, whole milk diet							
1	0	6.0	2.12	2.41	2.76	1.36	8.65	-	1.1	
	3	6.1	1.76	2.43	4.41	1.51	10.11	-	1.8	
	6	6.0	2.44	3.20	7.45	1.42	14.51	-	2.3	
	12	5.9	3.00	5.40	16.30	0.82	25.52	-	3.0	8.1
2	0	7.2	0.58	0.66	6.69	0.49	8.42	-	10.1	
	3	-	0.86	0.69	14.74	0.57	16.86	-	21.3	
	6	-	1.57	6.44	23.38	1.29	32.68	-	3.6	
	12	-	2.20	11.10	31.90	3.71	48.91	-	2.8	8.1
3	0	7.2	0.41	0.15	2.32	1.97	4.85	-	15.4	
	3	6.7	1.05	0.65	14.17	8.52	24.39	-	21.8	
	6	6.8	1.91	6.98	22.23	9.22	40.34	-	3.1	
	12	6.8	1.50	9.50	28.90	1.13	41.03	-	3.0	8.1
4	0	8.3	0.58	0.30	3.00	0.24	4.12	-	10.0	
	3	6.9	0.61	2.84	9.78	0.29	13.52	-	3.4	
	6	6.8	1.80	8.89	23.54	1.11	35.34	-	2.6	
	12	6.9	1.60	9.90	26.00	1.19	38.69	-	2.6	7.0

TABLE 5 (CONT.)

Day	Sampling period	pH	Micromoles per milliliter							Total VFA produced per mg. soluble starch
			C_4[a]	C_3	C_2	C_1	Total VFA	Lactic acid	C_2:C_3	
	hours									micromoles
			Trial 2, calf 042, whole milk + Amijel[b] diet							
1	0	7.0	0.56	0.42	2.67	1.97	5.62	5.13	6.3	
	3	6.9	0.65	0.91	16.56	3.82	21.94	20.57	18.1	
	6	6.8	0.48	2.21	22.54	5.82	31.05	25.60	10.1	
	12	6.8	2.30	3.20	39.30	7.30	52.10	22.87	12.2	10.2
2	0	7.1	0.63	0.58	4.30	1.54	7.05	2.84	7.4	
	3	7.0	0.74	2.48	16.17	4.83	24.22	12.81	6.5	
	6	6.9	1.36	5.66	26.45	4.77	38.24	23.02	4.6	
	12	6.9	2.20	6.10	37.25	5.00	50.55	14.19	6.1	8.8
3	0	7.3	0.00	0.11	3.18	1.30	4.59	0.64	28.9	
	3	7.1	0.25	0.89	20.40	2.80	24.34	8.09	22.9	
	6	6.9	0.25	0.89	22.38	2.78	26.30	8.53	25.1	
	12	7.2	1.70	6.60	33.00	3.10	44.40	8.45	5.0	7.5
			Trial 3, calf 066, basal milk replacer[c] diet							
1	0	7.1	0.52	0.26	7.68	0.93	9.39	-	29.5	
	3	7.3	1.32	7.24	19.29	4.09	31.94	-	2.6	
	6	7.0	1.47	9.53	24.69	3.07	38.76	-	2.5	
	12	6.8	0.18	4.80	28.20	4.70	37.88	-	5.8	5.5
2	0	7.3	0.55	0.23	4.13	0.42	5.33	-	17.9	
	3	-	1.67	21.18	14.81	1.50	39.16	-	0.69	
	6	7.0	2.56	9.16	27.49	1.89	41.10	-	3.0	
	12	6.8	2.75	9.15	30.40	1.10	43.40	-	3.3	6.7

TABLE 5 (CONCL.)

Day	Sampling period	pH	Micromoles per milliliter						$C_2:C_3$	Total VFA produced per mg. soluble starch
			C_4[a]	C_3	C_2	C_1	Total VFA	Lactic acid		
	hours									micromoles
3	0	7.3	0.81	0.40	5.82	0.58	7.61	-	14.5	
	3	7.3	2.20	16.53	15.42	2.68	36.83	-	0.93	
	6	6.9	2.74	2.21	28.48	1.21	34.64	-	12.8	
	12	6.8	1.45	5.45	28.90	1.60	37.40	-	5.3	5.7
			Trial 3, calf 067, basal milk replacer[c] diet							
1	0	7.3	1.59	0.16	4.38	-	6.13	-	27.3	
	3	7.3	2.29	1.51	13.36	-	17.16	-	8.8	
	6	7.1	3.28	7.41	26.51	-	37.20	-	3.5	
	12	-	2.05	2.40	27.67	-	32.12	-	11.5	5.6
2	0	8.0	1.64	1.26	5.15	-	8.05	-	4.0	
	3	6.9	2.83	1.51	24.12	-	28.46	-	15.9	
	6	-	3.60	11.65	31.13	-	46.38	-	2.6	
	12	-	2.20	14.45	29.25	-	45.90	-	2.0	7.3
3	0	7.4	0.87	0.39	5.16	0.89	7.31	-	13.2	
	3	7.0	1.31	7.37	4.33	2.36	15.37	-	0.58	
	6	6.8	2.58	8.37	27.90	0.84	39.69	-	3.3	
	12	-	2.60	3.00	27.35	2.30	35.25	-	9.1	5.7

[a] C_4, Butyric; C_3, propionic; C_2, acetic; C_1, formic acid.
[b] Amijel. Pregelatinized cornstarch, Corn Products Company, New York.
[c] Basal milk replacer consisted of 90% spray dried skim milk and 10% animal fat.

estimation of total carbohydrates did not prove satisfactory in the case of raw and pregelatinized starches. Hence, the data obtained for these starches (Appendix tables 1-5) were unreliable. Therefore, in this experiment, only the effect of different diets on the in vitro digestibility of soluble starch is presented.

Data for fermentation of soluble starch using inoculum from 066 while on raw and pregelatinized starch diets are shown in figure 5. Fermentation proceeded at a significantly ($P < .01$) faster rate when the diet was raw starch as compared to pregelatinized starch (table 6). In the case of 067 (figure 6), in vitro digestibility of soluble starch was significantly ($P < .01$) greater using inoculum from a pregelatinized starch diet than from a raw starch diet (table 6).

When in vitro digestibility of soluble starch was compared (figure 7), the digestion of soluble starch was slightly greater with inoculum from 067 than 066 when both were on a pregelatinized starch diet. However, with raw starch as the diet of these donors, there was a marked difference both in the rates of fermentation and total amounts fermented (figure 8). Analysis of variance of total carbohydrates (table 7) revealed that for diet 1, both fermentation period and fermentation period x calf interaction showed significant ($P < .01$) variation. The nonsignificant mean square for calves fed diet 1 suggested that both calves responded similarly to the diet. For diet 2, however, the animals showed a significant ($P < .01$) difference in the rate of fermentation of substrate (table 7).

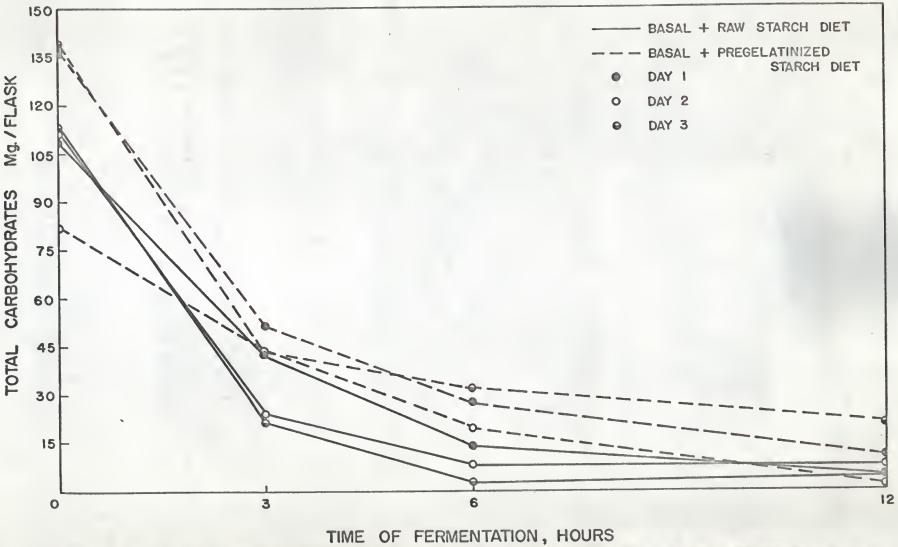

Fig. 5. Carbohydrates remaining after various periods of <u>in vitro</u> fermentation of soluble starch. Inoculum Donor 066 on two different diets.

TABLE 6. ANALYSIS OF VARIANCE OF TOTAL CARBOHYDRATES, AND MEAN LEVELS OF TOTAL CARBOHYDRATES AT DIFFERENT FERMENTATION PERIODS (EXPERIMENT II)

Source of variation	Calf 066		Calf 067	
	d.f.	Mean square	d.f.	Mean square
Fermentation period	3	35784.97**	3	32683.00**
Diet	1	2607.81**	1	4477.32**
Fermentation period x diet	3	1044.99**	3	1794.39**
Error	38	19.59	40	240.30

Fermentation period	Mean levels	Mean levels
hours	mg.	mg.
0	122.6[a]	131.6[a]
3	34.3[b]	66.5[b]
6	16.8[c]	39.1[c]
12	12.0[d]	21.4[d]

**$P < .01$

a,b,c,d Means in the same column having different superscripts are significantly different ($P < .05$) from each other.

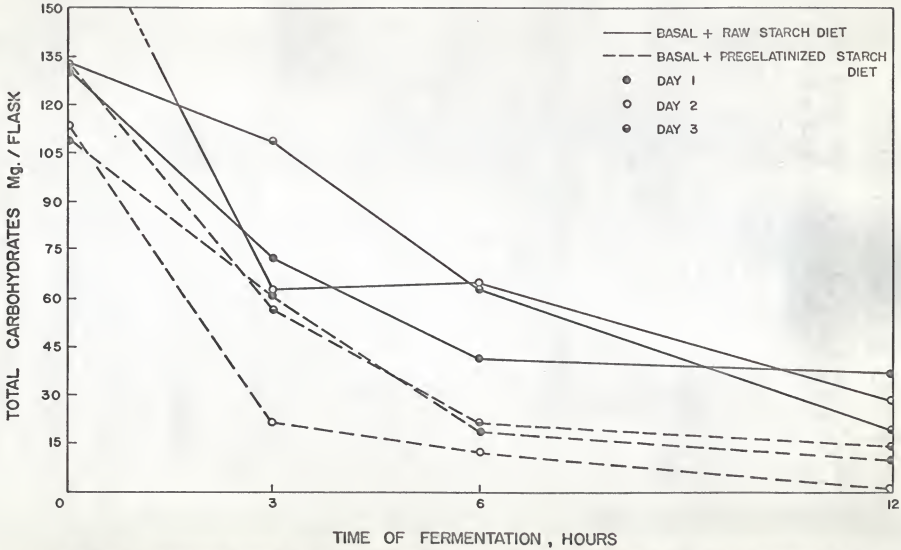

Fig. 6. Carbohydrates remaining after various periods of in vitro fermentation of soluble starch. Inoculum Donor 067 on two different diets.

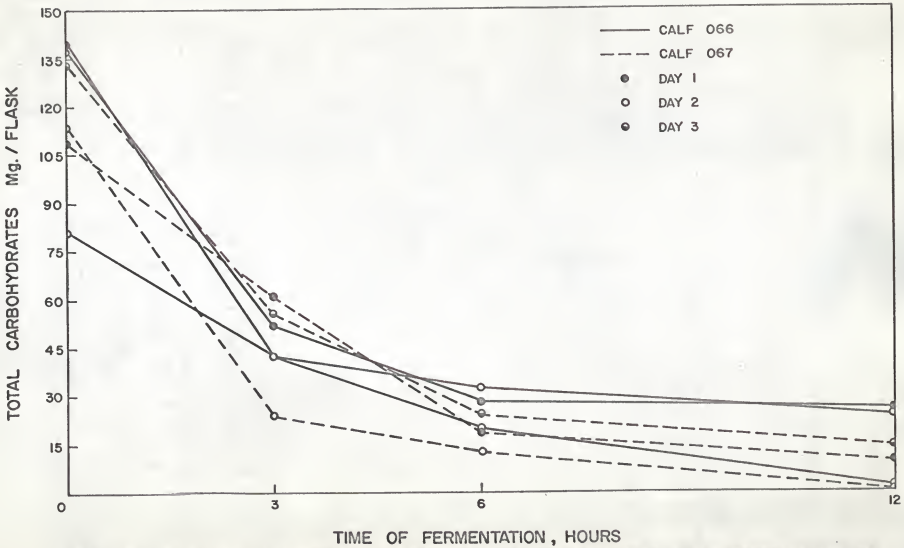

Fig. 7. Carbohydrates remaining after various periods of *in vitro* fermentation of soluble starch. Inoculum Donors 066 and 067; Diet, basal milk replacer plus pregelatinized starch.

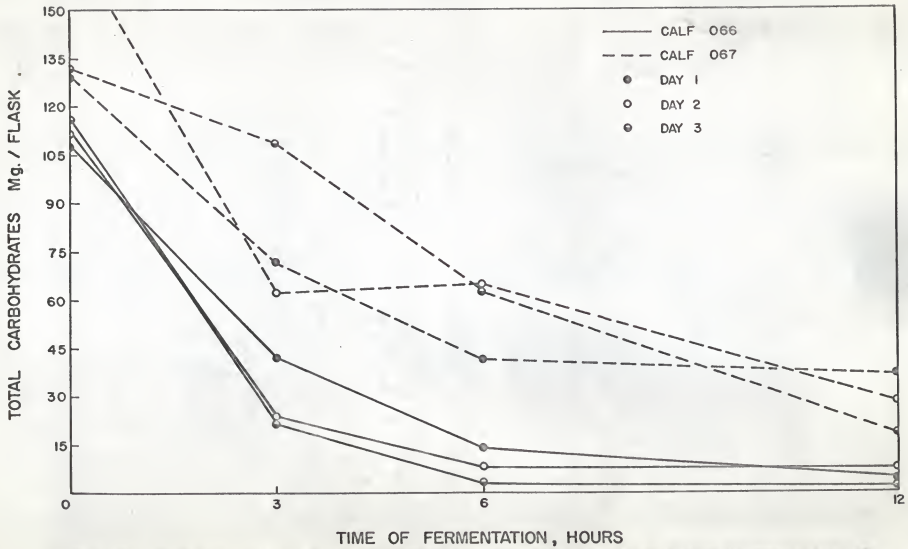

Fig. 8. Carbohydrates remaining after various periods of in vitro fermentation of soluble starch. Inoculum Donors 066 and 067; Diet, basal milk replacer plus raw starch.

TABLE 7. ANALYSIS OF VARIANCE OF TOTAL CARBOHYDRATES, AND MEAN LEVELS OF TOTAL CARBOHYDRATES AT DIFFERENT FERMENTATION PERIODS (EXPERIMENT II)

Source of variation	Diet 1 (Pregelatinized starch)		Diet 2 (Raw starch)	
	d.f.	Mean square	d.f.	Mean square
Fermentation period	3	32041.30**	3	34872.60**
Calves	1	13.39	1	13667.00**
Fermentation period x calves	3	627.60**	3	2344.14**
Error	36	80.90	41	238.42

Fermentation period	Mean levels	Mean levels
hours	mg.	mg.
0	126.1[a]	127.9[a]
3	45.4[b]	54.6[b]
6	22.6[c]	34.2[c]
12	17.8[c]	16.5[d]

**$P < .01$

a,b,c,d Means in the same column having different superscripts are significantly different ($P < .01$) from each other.

The results of the *in vivo* digestion trials (table 8) did not reveal any marked difference in the digestibility of raw and pregelatinized starches. The digestibility of the NFE of the basal milk replacer was higher in both animals than that of either raw or pregelatinized starch diets.

TABLE 8. DIGESTIBILITIES OF RAW AND PREGELATINIZED STARCHES *IN VIVO*

Experimental animals and diets		Input	Output	Digested	Digestion coefficient
		gm.	gm.	gm.	%
	Trial I				
066C	Basal[a] + Amijel[b]	173.36	15.21	158.15	91.2
067	Basal[a] + R.S[c]	173.67	14.79	158.85	91.5
	Trial II				
066C	Basal[a]	190.86	5.34	185.52	97.2
067C	Basal[a]	190.86	9.19	181.67	95.2
	Trial III				
066C	Basal[a] + R.S[c]	207.91	12.48	195.43	94.0
067C	Basal[a] + Amijel[b]	207.33	10.28	196.05	94.5
	Trial IV				
067C	Basal[a]	196.81	6.98	189.83	96.4

[a]Basal milk replacer containing 90% spray dried skim milk powder + 10% animal fat.

[b]Amijel, Pregelatinized cornstarch. Corn Products Company, New York.

[c]R.S, Raw starch, Buffalo, Corn Products Company, New York.

DISCUSSION

One of the purposes of this research was to determine whether *in vitro* techniques are appropriate to study intestinal microorganisms. The results obtained revealed that they are. The disappearance of soluble starch during 12 hours of fermentation on all days of the experiments showed that under the conditions of the *in vitro* procedure adapted, mixed intestinal microorganisms in the young calf were capable of breaking down starch into simpler metabolic products.

The second purpose of this study was to develop techniques for the collection and handling of inoculum for *in vitro* investigations. Information on this aspect was lacking in the literature reviewed. Any method that might be followed had to meet certain specifications so that while handling the inoculum, no alteration was brought about in the numbers, motility, anaerobiosis, and temperature of the material collected. To minimize changes in the numbers and motility of the microorganisms, the inoculum was used within 30 minutes after collection. Anaerobiosis was maintained by tying up the neck of the balloon before it was disconnected from the cannula. The temperature of the inoculum was maintained by transporting the balloon in a vacuum jar.

A third purpose was to determine some of the gross products of starch fermentation by the intestinal microorganisms of the calf. The analytical procedures followed for the determination of total carbohydrates, reducing substances and VFA were found suitable for the quantitative estimation of these major groups.

The applicability of these procedures further demonstrated the adaptability of the in vitro technique to study starch fermentation.

Fermentation of soluble starch resulted in a steady accumulation of VFA as the end product. Reducing sugars, apparently resulting from the degradation of starch, did not tend to accumulate but disappeared rapidly, indicating that the shorter chain reducing sugar molecules were more readily attacked by intestinal microorganisms than the longer chain preliminary products of starch breakdown.

Phillipson and McAnally (1942) noted that VFA in fermentation flasks were stable only for 7½ hours, with a decrease in concentration due to rapid multiplication of acid-splitting bacteria. In the experiment reported here there was a steady accumulation of VFA during 12 hours fermentation. Elsden (1945) incubated glucose with rumen contents and found production of large quantities of VFA and lactic acid in 24 hours. During the next 48 hours of fermentation, lactic acid disappeared with a corresponding increase in VFA. This is in conflict with the report of Phillipson and McAnally (1942) who used 100 ml. rumen ingesta and 10 ml. of pure solutions of VFA as substrates. Probably the ratio of substrate to inoculum used, buffer, anaerobiosis and diet of the donor influenced the character of fermentation and products of metabolism.

Elsden (1945) found that the rate at which glucose was fermented in the rumen depended upon the diet of the animal. During in vitro fermentation of glucose, propionic acid was the

main VFA produced as compared to acetic acid which was the predominant VFA *in vivo*. This was, however, not the case in the present study. In all trials in Experiment I, acetic acid was produced in a much larger quantity than any other VFA. The total weight of VFA was calculated on the basis of the molecular proportions and weights of individual VFA produced. The total VFA produced *in vitro* was 500.8 mcg./mg. soluble starch from donor 042 on a whole milk diet at 60 days of age. On a pregelatinized starch diet when donor 042 was 90 days old, the *in vitro* value was 539.4 mcg. VFA/mg. soluble starch. This indicates increased VFA production due to a change in diet or age or both. The *in vitro* rates of fermentation, using inoculum from donors 066 and 067 on a basal milk replacer diet when both animals were 14 days old, were 368.7 and 396.2 mcg. of total VFA/mg. soluble starch fermented, respectively. Moore *et al.* (1960) reported that the milliequivalents of VFA produced at 6, 12, and 24 hours of fermentation averaged 8.35 $\pm$ 0.85 when rumen microorganisms were used for the study of *in vitro* fermentation of powdered cornstarch. This compares with the value of 7.1 milliequivalents of total VFA produced per gram of soluble starch in the experiment reported herein.

The appearance of lactic acid in Trial 2, Experiment I, indicated that with a change in the age and diet of the donor, there was a change in the intestinal microorganisms. While the total VFA increased from 0 to 12 hours, lactic acid production, which reached a peak at 6 hours, began to decrease from 6 to 12 hours of fermentation. A similar finding was reported by Elsden

(1945) when glucose was fermented in vitro. The level of lactic acid decreased after reaching a peak in 24 hours. This decrease was associated with a corresponding increase in VFA, indicating that lactic acid gave rise to VFA. In another study they confirmed this by using lactic acid as a substrate for in vitro fermentation by microorganisms from rumen contents of sheep. Lactic acid gave rise to acetic, propionic, and butyric acids.

The acetic acid:propionic acid ratios ranged between 2.6 to 3.0 in Trial 1 and 5.0 to 12.2 in Trial 2 (Experiment I). This change in the ratios may have been due to increased lactic acid production which resulted from a change in the diet or age of the animal. Bruno and Moore (1962) reported that the production of lactic acid in rumen ingesta in vitro was stimulated by the addition of glucose and heated starch. Waldo and Schultz (1956) reported in vivo production of lactic acid when glucose was fed to fistulated steers. On normal rations they found no lactic acid before feeding concentrates, but the level of lactic acid rose to a peak 1 hour after feeding concentrates and then fell rapidly to pre-feeding levels. Huber and Moore (1964) reported lactic acid production as great as that of acetic acid in the large intestines of calves fed milk plus 15% lactose. Lactic acid was not found posterior to the small intestines in calves on other rations.

In the present study, no attempt was made to remove metabolic end products from the fermentation flasks, which under in vivo conditions would have been absorbed. Therefore, VFA accumulated in the fermentation flasks. With the accumulation

of VFA there was a slight fall in the pH of the medium in the 12 hour flasks as compared to 0 hour flasks. Phillipson (1942) found a fall in pH in the rumen of the sheep which was generally associated with a rise in VFA.

Experiment II was conducted to study further the effect of change of diet on changes in intestinal microorganisms. When pregelatinized starch was fed to the experimental animals, in vitro digestion of soluble starch was slightly greater using inoculum from 067 (93.4%) than with inoculum from 066 (88%). Correspondingly, the trend in the in vivo digestibilities of pregelatinized starch in these animals was similar; 067 had a higher digestibility (94.5%) than 066 (91.2%). The differences in in vitro digestion, however, were not statistically significant. When the donor animals were on a raw starch diet, in vitro digestion of soluble starch was greater with inoculum from 066 (95.6%) than with inoculum from 067 (81.1%). This difference was statistically significant ($P < .01$). The difference in in vivo digestion of raw starch was of a similar nature being greater for 066 (94%) than for 067 (91.5%). This difference between animals might be due to an individual variation in the kinds and numbers of microorganisms naturally present in the large intestines, or due to the effect that changing of diets has on large-intestinal microorganisms. This might suggest that the sequence in which various feeds are fed to an animal might have a noticeable effect on the characteristics of intestinal microorganisms.

Differences in fermentation of soluble starch by inocula from the same animal but on different diets (figures 5 and 6)

were statistically significant ($P < .01$). These differences may also have been due to changes in the diet that resulted in changes in intestinal microorganisms.

In this study, only a small number of trials were conducted in which few experimental animals were used, which limited any conclusions to be made. There are several problems which need to be overcome in future studies:

(1) There is much to be desired in the standardization of a suitable technique for inoculum collection. In the present study, collections were made between 10 to 12 o'clock in the morning. Information concerning activity of collections made at other times of the day is lacking. Therefore, it is suggested that studies of rate of flow, pH, and dry matter content of intestinal contents be made to arrive at what may be termed a standard inoculum. In this study, very few measurements of this nature were made.

(2) For want of a suitable buffer, one used by Baumgardt et al. (1962) in his "artificial rumen" procedure was used in this experiment. Information was not available from the literature as to the proper composition of a suitable buffer to be used to preserve the motility, numbers, and activity of intestinal microorganisms. Therefore, more information on the pH and its variations during the time of day that collections of inoculum are to be made is needed and a suitable buffer prepared accordingly.

(3) Suitable preparatory procedures for analysis of total carbohydrates in in vitro fermentation flasks containing raw and pregelatinized starches are needed. Soluble starch could be dissolved in sodium hydroxide in 2 hours time or even less, but similar homogenous solutions of pregelatinized or raw starches could not be obtained in 2N sodium hydroxide, when all three starches were similarly treated.

Originally it was planned to conduct simultaneous in vitro and in vivo studies on the counts, characteristics, and species of intestinal microorganisms. Preliminary work in this direction included some in vivo studies using various nutrient media for the study of the growth of cecal microorganisms and counts of the species present. Information is needed on the types and numbers of microorganisms present in the intestines of the calf at different ages and on different diets. Similar estimates under in vitro conditions may yield valuable information regarding the nature, behavior, and response of intestinal microorganisms to various test substrates under study. From results reported in this thesis it appears that in vitro procedures may be used advantageously for the study of intestinal digestion in the young calf.

SUMMARY

An *in vitro* technique was developed for the study of digestion of starch by intestinal microorganisms in the young calf. Two experiments were conducted using three male calves with reentrant colonic fistulae as donors for the microorganisms. Experiment I was designed to study some gross products of *in vitro* fermentation of starch and the effect of change of diet on the intestinal microbial activity. In Experiment II, the effect of change of diet on *in vitro* digestibilities of raw, pregelatinized and soluble starches and the *in vivo* digestibilities of raw and pregelatinized starches were studied.

In Experiment I, there was a steady decline in the levels of total carbohydrates during the 12 hours of fermentation by intestinal microorganisms, accompanied by a steady accumulation of volatile fatty acids (VFA). During the fermentation there was a trend for the reducing sugars to reach a peak in 3 hours and then decrease from 0 to 12 hours at a rate much faster than total carbohydrates. The steady increase in VFA with a corresponding decrease in total carbohydrates and almost complete disappearance of reducing sugars in 12 hours fermentation indicated that VFA were the end products of starch digestion. Acetic, butyric, propionic, and formic acids were the main VFA produced, with acetic acid being produced in the largest quantity. In Trial 1, when the donor animal was on a whole milk diet, the *in vitro* production of lactic acid was so low that it could not be estimated. In Trial 2, when pregelatinized starch was included in

the diet of the animal, lactic acid was produced in appreciable quantities with a tendency for increased production of total VFA, especially acetic acid. Lactic acid production reached a peak in 6 hours and decreased from 6 to 12 hours. This fall in level of lactic acid was associated with a subsequent increase in acetic acid and VFA. This increase in VFA may have been due to fermentation of lactic acid to VFA.

Inocula from the two donors used in Experiment II responded differently in the in vitro digestion of soluble starch. With one animal as donor, in vitro digestion of soluble starch was greater on a raw starch diet than on a pregelatinized starch diet. With the second animal as donor, the reverse was true. With raw starch included in the diet there was a marked difference in the in vitro digestion of soluble starch between the two donors; with pregelatinized starch in the diet the difference between donors was not significant. The difference in response of the same animal on two different diets or between two different animals on the same diet might be due to changes in the diet or the sequence in which these diets were fed.

Trials conducted to determine the in vivo digestibilities of raw and pregelatinized starches showed no marked differences. The digestibility of the NFE of the basal milk replacer was higher in both animals.

Calculation of the quantitative conversion of soluble starch to total VFA showed that changes in the diet, age, or both, of the young calf influenced conversion of soluble starch to VFA.

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APPENDIX

APPENDIX TABLE 1. IN VITRO DIGESTION OF RAW AND PREGELATINIZED STARCHES (DONOR 066, BASAL MILK REPLACER + PREGELATINIZED STARCH DIET)

Sampling period	Total Carbohydrates					
	8-19		8-20		8-21	
	Raw starch	Amijel	Raw starch	Amijel	Raw starch	Amijel
hours	mg.	mg.	mg.	mg.	mg.	mg.
0	112.5	139.0	119.0	98.6	84.6	125.0
0	108.3	102.5	119.0	123.6	70.8	134.6
0	76.0	100.8	119.0	120.8	66.6	123.6
3	45.8	29.1	30.8	23.6	23.6	30.8
3	18.3	25.0	41.6	27.5	-	-
6	25.0	7.0	34.6	12.5	20.8	22.5
6	33.3	7.0	52.5	8.3	22.5	18.0
12	27.5	2.7	2.7	2.7	12.5	15.3
12	29.1	4.1	2.7	2.7	11.0	19.3

APPENDIX TABLE 2. IN VITRO DIGESTION OF RAW AND PREGELATINIZED STARCHES (DONOR 067, BASAL MILK REPLACER + RAW STARCH DIET)

Sampling period	Total Carbohydrates					
	8-19		8-20		8-21	
	Raw starch	Amijel	Raw starch	Amijel	Raw starch	Amijel
hours	mg.	mg.	mg.	mg.	mg.	mg.
0	66.6	85.8	200.0	158.0	67.8	151.0
0	68.0	91.6	211.0	156.6	62.5	104.0
0	25.0	83.3	-	-	60.0	97.5
3	62.5	83.3	87.5	65.3	58.3	104.0
3	45.8	85.8	50.0	66.6	52.5	110.8
6	43.0	41.6	59.7	55.8	58.3	45.8
6	40.3	30.8	52.6	58.3	57.0	50.0
12	37.5	37.5	16.6	27.6	25.0	-
12	47.5	30.5	11.0	16.6	18.0	14.1

APPENDIX TABLE 3. IN VITRO DIGESTION OF RAW AND PREGELATINIZED STARCHES (DONOR 066, BASAL MILK REPLACER + RAW STARCH DIET)

Sampling period	Total carbohydrates					
	9-9		9-10		9-11	
	Raw starch	Amijel	Raw starch	Amijel	Raw starch	Amijel
hours	mg.	mg.	mg.	mg.	mg.	mg.
0	50.0	101.3	0	74.2	14.2	75.0
0	66.7	91.7	2.7	67.5	114.2	68.0
0	79.2	91.7	1.3	75.0	29.1	51.3
3	34.0	23.7	2.7	7.0	32.0	23.7
3	25.0	25.0	0	8.3	22.5	19.3
6	8.3	20.8	0	5.5	9.7	7.0
6	8.3	8.3	0	0	14.2	11.0
12	2.5	8.3	1.3	4.2	7.0	0
12	4.2	4.2	0	0.7	2.7	5.8

APPENDIX TABLE 4. IN VITRO DIGESTION OF RAW AND PREGELATINIZED STARCHES (DONOR 067, BASAL MILK REPLACER + PREGELATINIZED STARCH DIET)

Sampling period	Total carbohydrates					
	9-9		9-10		9-11	
	Raw starch	Amijel	Raw starch	Amijel	Raw starch	Amijel
hours	mg.	mg.	mg.	mg.	mg.	mg.
0	25.0	102.5	79.2	105.8	135.8	120.8
0	22.5	104.1	79.2	94.3	133.3	118.3
0	69.1	100.8	91.7	57.0	129.1	122.5
3	18.3	73.7	39.2	25.0	87.5	40.0
3	19.1	75.8	37.5	11.0	41.7	35.0
6	12.5	19.2	10.8	4.2	25.0	16.7
6	29.1	25.8	-	-	23.7	18.3
12	2.5	8.3	1.3	1.3	8.3	8.3
12	0	8.3	2.5	1.3	8.3	10.0

APPENDIX TABLE 5. IN VITRO DIGESTION OF RAW AND PREGELATINIZED STARCHES (DONOR 067, BASAL MILK REPLACER DIET)

Sampling period	Total carbohydrates					
	9-20		9-22		9-24	
	Raw starch	Amijel	Raw starch	Amijel	Raw starch	Amijel
hours	mg.	mg.	mg.	mg.	mg.	mg.
0	118.3	108.3	83.3	122.5	119.2	130.8
0	110.0	177.5	75.0	123.7	68.3	126.6
0	83.3	104.2	129.2	112.5	-	143.3
3	44.2	44.2	45.8	52.7	25.0	29.2
3	47.5	35.0	95.8	54.2	37.5	43.3
6	35.8	44.2	27.5	26.6	29.2	15.0
6	39.2	18.3	62.5	15.3	19.2	16.7
12	27.5	12.5	19.2	7.0	15.0	10.0
12	26.7	8.3	25.0	8.3	10.0	8.3

A STUDY OF DIGESTION OF STARCH IN THE LARGE INTESTINE OF THE YOUNG CALF BY AN *IN VITRO* TECHNIQUE

by

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An *in vitro* procedure for the study of intestinal digestion in the young calf is reported. Three male calves fistulated with re-entrant colonic fistulae served as donors for inocula containing microorganisms from the large intestines.

In one experiment, three trials were conducted to study some major groups of products of breakdown of soluble starch and the effect that different diets had upon changes in production of these major groups of substances. In all trials, total carbohydrates decreased from 0-12 hours of fermentation with a steady increase in VFA, with acetic acid predominating. The reducing sugar level generally reached a peak after 3 hours of fermentation and then tended to disappear during the remaining 9 hours, much faster than total carbohydrates. In trials when the experimental animals were either on a whole milk or a basal milk replacer diet, lactic acid was produced in extremely low concentrations. When pregelatinized starch was added to the whole milk diet of a donor, lactic acid was produced in large quantities from 0 to 6 hours fermentation and then decreased in concentration during the 6 to 12 hour fermentation period. This decrease in the level of lactic acid was associated with an increase in total VFA, especially acetic acid. The production of total VFA averaged 7.1 milliequivalents per gram of starch fermented. Either an increase in the age of the animal, a change of diet, or probably both, influenced the conversion of soluble starch to VFA.

In another experiment, *in vitro* digestion of raw, pregelatinized and soluble starches was studied, making use of two

donors in which the order of feeding raw and pregelatinized starches was reversed to study the effect of change of diet on intestinal microorganisms. With one animal as donor, *in vitro* digestion of soluble starch was greater on a raw starch diet than on a pregelatinized starch diet. With the second animal as donor the reverse was true. With raw starch included in the diet there was a marked difference in the *in vitro* digestion of soluble starch between the two donors; with pregelatinized starch in the diet the difference between donors was not significant. There was no difference between donors in the *in vivo* digestion of raw or pregelatinized starch.

The results of these experiments demonstrated that *in vitro* techniques can be successfully used to study various aspects of intestinal digestion in young calves.