The bacteriology of the porcupine caecum

by John LeRoy Johnson

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY in Bacteriology
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Abstract:
The porcupine caecum appears to be similar to the rumen in that it provides a suitable environment for the growth of large numbers of bacteria which rapidly ferment portions of the ingested food.

The principle fermentation products were identified as the short chain volatile fatty acids acetic, propionic and butyric. The acids accumulating in the caecal ingesta consisted of 73 percent acetic, 12 percent propionic and 14 percent butyric. - The average rates at which the acids were produced in the caecal ingesta were 0.110, 0.016 and 0.014 meg, per g,dry weight per hour for acetic, propionic and butyric respectively. It was estimated that a 10 Kg porcupine could obtain from 6 to 22 percent of its maintenance energy requirement from the absorption and utilization of the volatile acids.

Lactic' and. succinic acids appeared not to be important intermediate products in the caecal fermentation.

Approximately twice as much carbon dioxide as methane was produced during in vitro incubation of caecal content.

Naturally occurring nitrogen and carbon sources for the caecal bacteria were sought in the ingesta of the caecum and from the distal end of the small intestine. Substrate amounts, of ammonia were found in the ingesta from both organs. Several amino acids were detected but in less than substrate quantities. Soluble sugars and sugar acids were not detected in the soluble fraction of the ingesta, but arabinose, xylose, glucose and galactose were detected in an acid- hydrolysate of the particulate fraction from the small intestine.

Three hundred twenty-eight strains of caecal bacteria were isolated by randomly picking discrete colonies from 0.5 or 1.0 x 10-8 dilution roll tube cultures. The strains were placed into 43 groups based on morphological and physiological characteristics. Some of the groups were similar to but most could not be identified with the predominant bacterial groups in the bovine rumen.

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ABSTRACT

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INTRODUCTION

Herbivorous animals are characterized by having an expanded portion or portions in their alimentary tract, which in carnivorous animals are expanded to a lesser degree if at all. The herbivorous animals show wide variations in the anatomy of their digestive system. In ruminants, the distal end of the esophagus is expanded into three compartments: the rumen, which is the largest, the reticulum, and the omasum. These are followed by a relatively small true stomach, the abomasum. Some rodents, for example the hamster and field vole, have a gastric diverticulum and a large caecum. The elephant and the horse have a large caecum and colon. The rabbit and porcupine have only a large caecum.

The expanded portions of the digestive tract provide suitable environments for the growth of anaerobic microorganisms which may aid the host in the digestion of food material. In general the environments are semi-fluid, 60 to 90 percent water, have a constant temperature of 38 to 40 C, and have low oxidation-reduction potentials. In addition, the environment within a particular digestive organ may be influenced by glandular secretions such as saliva, mucin and bile.

Food materials degraded by microorganisms in the digestive tract include mono-, di- and polysaccharides, sugar acids and organic nitrogen compounds. The major products formed during ruminal, caecal, or large intestinal fermentations are the short chain volatile fatty acids, acetic, propionic and butyric, and the gases carbon dioxide and methane. Two common bacterial fermentation acids, lactic and succinic, normally do not accumulate in the ingesta but may be important intermediates in the fermentation of the food material. In addition to the digestion, there is also

synthesis of such materials as vitamins, proteins, and carbohydrates. Some of these are incorporated into the bodies of the microorganisms and may become available to the host only after digestion.

The role of the microflora has been recognized in the degradation of plant material ever since Tappeiner (1884) observed that cellulose disappeared in the rumen of cattle. Baker and Martin (1937a) also credited the microflora of the caecum with the disintegration of pectic substances, hemicelluloses and cellulose in the rabbit. In addition to these early observations, there have been many more on the decomposition of plant material in the rumen, caecum and colon, but only a few of these will be mentioned. The in vitro fermentation of cellulose by rumen bacteria was studied by Woodman and Evans (1938). According to Van der Wath (1948), the only diastases present in the sheep rumen are those secreted by the rumen bacteria. Hale et al. (1940) found 85 percent of the digestible cellulose ingested by a bovine to be digested in the rumen within 14 hours. They found that lignin and true fat were not digested in the rumen. Elsden (1945) observed in vitro fermentation of cellulose, glucose and lactic acid by sheep rumen content. Gray (1947b) obtained data indicating that 70 percent of the digestible cellulose of fodder was degraded in the rumen, 17 percent in the caecum, and 13 percent in the colon. No cellulose digestion occurred in the abomasum or in the small intestine. Baker et al. (1950) studied the breakdown of structural starches in the digestive tracts of ruminant and non-ruminant animals. They demonstrated the bacterial degradation of starch in the rumen, diverticulum, and caecum in ruminants, rodents and swine. Heald (1952) obtained in vitro fermentation of pentoses and uronic acids with

suspensions of sheep rumen bacteria. Doetsch et al. (1953) incubated suspensions of bovine rumen bacteria with cellobiose, maltose, glucose, xylose, pyruvate, lactate, oxalacetate, succinate, fumarate, malate, formate, acetate, propionate, butyrate and β-hydroxy-butyrate. All of these were attacked by rumen bacteria. McBee (1953) found that the addition of cellulose to the hay diet of a sheep increased the rate of cellulose and hemicellulose digestion by the rumen fluid; however, the addition of hemicellulose increased the fermentation rate of only the hemicellulose. Howard (1955) observed a rapid fermentation of purified wheat flour pentosan which consisted of chains of β-1:4-D-xylose units with single L-arabinose units attached along the chain.

The conversion of organic nitrogen compounds in the digestive tract has also been studied but such studies have primarily been limited to the rumen. The degradation of urea has received much attention because of its economic importance as a substitute for protein nitrogen in livestock feed. Pearson and Smith (1943a) observed high levels of urease activity in bovine rumen ingesta. Pearson and Smith (1943b) and Smith and Baker (1944) obtained evidence which indicated that urea may be used as a nitrogen source by rumen microorganisms. Sirotnak et al. (1953) incubated 22 amino acids with suspensions of bovine rumen bacteria. Aspartic acid, glutamic acid, serine, arginine, cysteine, and cystine were found to be dissimilated. MacLeod and Murray (1956) found that a combination of valine, leucine and isoleucine stimulated cellulose digestion. The major products formed when an acid hydrolysate of casein was incubated with sheep rumen ingesta were ammonia, carbon dioxide and volatile fatty acids (El-Shazly, 1952). Feeding

sheep on a diet in which zein contributed 94 percent of the total nitrogen resulted in an incorporation of approximately 40 percent of the zein into microorganism protein (McDonald, 1954). By in vitro experiments, Belasco (1954) compared urea with feed proteins and found urea to be a superior nitrogen source in promoting cellulose digestion by rumen microorganisms. Moore and King (1958) followed the changes in the concentrations of 7 different fractions of nitrogenous materials in the bovine rumen. The maximum changes in concentrations occurred within 1 hour after feeding, regardless of the ration tested, and returned to the prefeeding levels within about 5 hours. Blackburn and Hobson (1960a) studied the proteolytic activity of whole and fractionated sheep rumen contents and found it to be independent of the animals diet. Both the protozoan and bacterial fractions showed proteolytic activity. They also found (1960b) that the proteolytic activity of rumen microorganisms was relatively constant and comparatively high at all times, even before feeding. The metabolic fates of the nitrogenous compounds seem to be dependent upon the general properties of the microorganisms and are not greatly influenced by the diet.

The principal products of carbohydrate fermentation in different parts of the digestive tract have been shown to be remarkably similar, not only in the kinds of acids but in their relative concentrations. Phillipson (1947) credits Tappeiner (1884) for being the first to detect volatile fatty acids as products of cellulose fermentation. Acetic, propionic and butyric acids have been shown to be the main volatile fatty acids in the rumen of sheep (Elsden, 1945). Acetic acid accounted for 55 to 75 percent of the total. Elsden et al. (1946) determined the volatile fatty acids in the

digesta of the sheep, red deer, horse, rabbit, rat and pig. Mixtures of acetic, propionic, and butyric acids were found to be the characteristic fermentation products in the alimentary tracts of all of these species and their average compositions consisted of 67 percent acetic, 19 percent propionic and 14 percent butyric. The in vitro fermentation of cellulose and glucose by rumen contents yielded acetic, propionic and butyric acids (Elsden, 1945). Propionic acid was the major fatty acid in both cases. Very little, if any, butyric acid was formed during the fermentation of cellulose. Gray and Pilgrim (1952) found that when cellulose or hemicellulose was fermented, equal amounts of acetic and propionic acids were produced, with only a small amount of butyric acid. When cellulose was fermented in the presence of casein, a high level of butyric acid was produced. Carroll and Hungate (1954) found that the volatile acids produced during in vitro fermentation rate studies consisted of approximately 62 percent acetic, 22 percent propionic and 16 percent butyric regardless of diet. Balch and Rowland (1957) studied the diurnal variations in the concentrations of volatile fatty acids in the bovine rumen and found that the level of propionic acid was directly proportional to the amounts of readily fermentable carbohydrates in the ration and that the levels of butyric and higher acids were directly proportional to the levels of protein. These results were confirmed by Eusebio et al. (1959), who found that increasing the availability of readily fermentable carbohydrates resulted in an increase of propionic acid at the expense of acetic acid. When linseed-oil meal was included in the diet, the level of butyric acid increased at the expense of the propionic. The metabolic fate of uniformly

C^{14}-labeled glucose introduced into a bovine rumen has been investigated (Otagaki et al., 1963). The specific activity of C^{14} reached a maximum in respired CO_2 at 45 minutes, in the blood volatile fatty acids at 15 minutes and in the plasma glucose at 2 hours after administration of the labeled glucose. Their results indicate that there is little, if any, direct absorption of glucose from the rumen, but that the plasma glucose is synthesized from the absorbed volatile acids.

Pure cultures of numerous rumen bacteria produce lactic and succinic acids as fermentation products (Bryant, 1959). However, the concentrations of these acids in rumen content are usually low. A transitory rise in lactic acid concentration of sheep rumen content was observed when large amounts of glucose or ground grain were placed into the rumen of hay-fed sheep (Hungate et al., 1952). Soluble starch, cellulose, pyruvic acid and glucose were tested for lactic acid production by incubating them in vitro with rumen content (Waldo and Schultz, 1956). They found lactic acid to be produced only from glucose. Jayasuriya and Hungate (1959) did not consider lactate to be an important intermediate in the rumen fermentation of hay-fed steers, but believed that it may have been an intermediate for as much as one-sixth of the total substrate in grain-fed steers. Lactic acid accumulation appeared to be dependent upon the presence of high levels of readily fermentable carbohydrates. In isotope experiments with lactate-2-C^{14}, most of the labeled carbon was recovered in acetic acid. Bruno and Moore (1962) incubated commercial alfalfa meal, heated and unheated alfalfa, glucose and heated starch in vitro with ruminal ingesta and observed that a transitory accumulation of lactic acid occurred with glucose, heated alfalfa and heated

starch. Their results also suggest that the accumulation of lactic acid is dependent upon readily fermentable carbohydrates which either have to be added or else made available by heating. The authors suggested that the lactic acid accumulation might reflect the resistance of the lactic acid fermentation to low pH values. They did not believe that the experiment indicated the importance of lactic acid as an intermediate in the rumen fermentation, since the absence of lactic acid may have reflected either a rapid metabolism or a decreased rate of production.

Low concentrations of succinic acid have been found in sheep rumen content; only a slight increase in concentration occurred after feeding (Sijpesteijn and Elsden, 1952). Suspensions of washed rumen bacteria rapidly converted succinic acid to propionic acid and carbon dioxide. Blackburn and Hungate (1963) calculated velocity constants for the conversion of succinic acid to propionic acid in bovine rumen content. By determining the size of the succinic acid pool in the rumen content and by the use of the conversion velocity constant, they concluded that succinic acid was the major precursor of the propionic acid found in the rumen.

The principal fermentation gases detected from the digestive tract content are carbon dioxide and methane. Pure cultures of carbohydrate-fermenting rumen bacteria, however, have been found to produce CO_2 or CO_2 and H_2 (Bryant, 1959). Smith and Hungate (1958) cite Reiset (1863) for first demonstrating methane formation in the rumen. Tappeiner (1884) detected methane and carbon dioxide as products of cellulose fermentation by rumen bacteria. Lugg (1938) measured the combustible metabolic gases of a sheep and found that about 18 liters of methane were produced per day.

Olson (1940) found CO_2:CH_4 ratios of 3.3-4:1 from cows feeding on different kinds of pasture. Only trace amounts of hydrogen were detected. Marstron (1948) obtained CO_2:CH_4 ratios of 3.0-3.8:1 during cellulose fermentation with suspensions of washed rumen bacteria. When CO_2:CH_4 ratios were measured with respect to time after feeding, they varied from 1:1 following a period of fasting to 3:1 two hours after feeding (Kingwill et al., 1959). McNeill and Jacobson (1955) presented evidence to suggest that their failure to find more than trace amounts of hydrogen in rumen contents was due to extremely active hydrogenase systems. In vitro experiments with a hydrogen gas atmosphere resulted in no carbon dioxide production. The authors suggested that hydrogen gas might be the limiting factor for methane production in a normal rumen. Their observations, however, are not supported by the work of Kingwill et al. (1959) who found that continuous sparging with H_2 showed no effect on the CO_2:CH_4 ratios. Carroll and Hungate (1955) incubated rumen content in vitro with C^{14} formic acid. The labeled carbon was recovered primarily as CO_2 indicating hydrogenlyase activity. An estimation of the maximum rate at which formic acid could be produced in the rumen indicated that methanogenic substrates in addition to formic acid were involved in the synthesis of methane. Beijer (1952) incubated various substrates with rumen fluid. Formic acid was converted to methane quantitatively and succinic acid gave a notable rise in methane production. Acetic, propionic and butyric acids did not appear to be fermented. Oppermann et al. (1957) stabilized rumen fluid enrichment cultures which produced methane from formic and acetic acid. Cultures utilizing butyric acid could be established only at 45 C, but cultures utilizing propionic acid were not obtained.

Nelson et al. (1958) obtained enrichment cultures of rumen bacteria which fermented butyric and valeric acids. Butyric acid was completely degraded but propionic accumulated when valeric was fermented.

Methane production was found to be lower in the caecum and large intestine than in the rumen of several African ruminants (Hungate et al., 1959).

The digestive organs, being highly vascularized, enable the fermentation products to become available to the animal by being absorbed into the blood. Barcroft et al. (1944) found the blood draining the rumen of sheep to have higher concentrations of the volatile fatty acids than peripheral blood. Increased levels of acids were not detected in the blood draining the abomasum or the small intestine, but significant amounts were found in blood draining the caecum. Relatively high concentrations of volatile acids were found in the blood draining the caecum and/or colon of the horse, pig and rabbit. The rates at which the sodium salts of acetic, propionic and butyric acids were absorbed from the sheep rumen, appeared in the inverse order of their increasing molecular weights. Danielli et al. (1945) observed that the rate of fatty acid absorption from the rumen was dependent upon pH. At pH 7.5 the salts were lost in the order acetate>propionate> butyrate, whereas at pH 5.8 the order of the absorption rates were butyric > propionic>acetic. It was concluded that the free acids were absorbed at a greater rate than the anions and that different mechanisms were involved. Gray (1947a) studied the rate of acetate and propionate absorption from the rumen of a sheep fitted with a rumen fistula. When alkaline solutions of acetate and propionate were introduced into the rumen, the absorption rates

of the two acids were constant and the ratios remained the same. When the sheep ate during the experiment, propionate appeared to be absorbed at a higher rate. Propionate was also absorbed faster when phosphate buffer, at a concentration similar to that in the saliva, was included in the solution. Lowering the pH of the solution to pH 6.5 caused the propionic acid to be absorbed faster than the acetic. It was concluded that the ratios in which the volatile fatty acids are found in the rumen may not depict the rates of production.

The metabolism of the volatile fatty acids in animal tissue has been studied by many investigators. Phillipson (1947) and Elsden and Phillipson (1948) have reviewed the older literature. The later literature has been reviewed by Barnett and Reid (1961). In general it can be said that the volatile fatty acids are used for both energy and biosynthesis. The amount of energy made available through the oxidation of volatile fatty acids may be calculated from their heats of combustion. Armsby and Moulton (1925) (cited by Carroll and Hungate, 1954) estimated the maintenance energy requirement of a 1000 lb. bovine to be 7,300 Kcal per day. Carroll and Hungate (1954), assuming that the energy requirement of an animal is proportional to the three-fourths power of its weight, as is the basal metabolism rate (Kleiber, 1947), calculated the maintenance requirement of a 500 Kg bovine to be 7,850 Kcal per day. Using a method of zero time fermentation rates, they approximated the amount of volatile acids formed in the bovine rumen. From their calculations, for a 500 Kg cow containing 70 Kg of rumen content, they estimated that during a 24 hour period a hay-fed animal obtained 7,070 Kcal, a grain-fed animal 11,450 Kcal and a grass-fed animal

4,810 Kcal through the oxidation of the volatile acids. The energy obtainable from the volatile acids accounted for 70 percent of the estimated total energy requirements. Emery et al. (1956) came to the conclusion that a cow obtains 3 to 13 percent of its energy from the short chain volatile fatty acids. Stewart et al. (1958) determined that 63 percent of the maintenance energy requirements for steers were supplied by the volatile fermentation acids. Hungate et al. (1959) measured the fermentation rates in the rumen, caecum, and large intestine of zebu, gazelle, eland, suni and camels. The highest fermentation rates occurred in the rumen. Due to the differences in the sizes of the organs, the rumen accounted for 95 to 98 percent of the total fermentation. Hungate et al. (1961) determined the zero time fermentation rates of the individual volatile fatty acids, acetic, propionic, and butyric in bovine rumen content. The microorganisms were credited for contributing 90 percent of the animals carbon requirements. As much as 50 percent of the maintenance energy of the deer may be provided by the rumen fermentation (Short, 1963).

Microorganisms important in the fermentation of ingested food materials have been studied both microscopically and in pure culture. Baker (1933) and Baker and Martin (1937b) studied microscopically the disintegration of vegetable remains in the caecum of the guinea pig. Numerous types of bacteria, thought to be of functional importance, were classified on the basis of their morphology. Protozoa were observed in the caecal content but were found in low numbers and were considered not to be of functional importance. The bacterial population was divided into the iodophilic and aniodophilic bacteria. The iodophilic bacteria contained starch-like

polysaccharides which stained with iodine. Baker and Martin (1937a) investigated the microflora of the rabbit caecum with regard to the disintegration of cell wall substances. Many of the same morphological types of bacteria were found as in the studies on the guinea pig; however, protozoa were not observed in the animals studied. Baker and Martin (1939) studied the caecal microbiology of the horse and found the protozoan population to be far more conspicuous than in either the guinea pig or the rabbit. Baker (1943) divided the rumen population of the ox into three major groups: (1) iodophilic microorganisms; (2) aniodophilic microorganisms; and (3) protozoa.

Few of the bacteria important for the degradation of plant materials in herbivorous digestion were grown in pure culture until Hungate (1947) developed cultural methods for growing cellulose-fermenting bacteria isolated from the rumen. The success of the method can be attributed to strict anaerobiosis and the incorporation of sterile rumen fluid as an enrichment. Bryant and Burkey (1953) and Bryant and Robinson (1961b) modified Hungate's medium so that it was less-selective. The so-called "non-selective" media have been used in studies on the composition of bacterial populations within the rumen.

Since 1947 many of the important rumen bacteria have been isolated and studied in pure culture. The literature has recently been reviewed by Bryant (1959, 1963).

The isolation and study of pure cultures of bacteria from the caeca of nonruminant herbivorous animals have been limited. Barker and Haas (1944) isolated a straight to slightly curved gram positive, anaerobic, rod shaped bacterium from the intestine of a rat. The bacterium produced chiefly

acetic and butyric acids from glucose. It was named Butyribacterium rettgeri. Alexander et al. (1952) isolated cocci similar to Streptococcus bovis and Veillonella gazogenes from the colon of a horse. Hall (1952) isolated a cellulose fermenting coccus, which resembled Ruminococcus flavefaciens, from the caecum of a rabbit.

The purpose of the present investigation was to obtain a better understanding of the caecum and its functional importance to the porcupine digestive system. The yellow haired porcupine (Erethizon dorsatum epixanthum) was selected as the experimental animal because it is a relatively large rodent, thus provides large quantities of caecal material, is readily available in this geographical area, can be collected without special permits, is relatively easy to catch and, after the quills are sheared, is easy to handle. The porcupine is also of interest because its diet consists not only of succulent plants but may also include bark and the cambium layer from conifers and deciduous trees. The nutrition of the porcupine is not complicated by coprophagy as it is in some rodents. The investigation was divided into three major parts: (1) the identification of the major fermentation products, the amounts of them produced and the nutritional value of those products utilized by the porcupine; (2) the identification of carbon and nitrogen sources available to the bacteria of the caecum; and (3) the study of populations of caecal bacteria and the major groups of bacteria in these populations. After some preliminary experiments early in the investigation the assumption was made that the caecal environment is similar to that of the rumen. The culture studies in this investigation were based on that assumption which proved to be incorrect. Therefore, the

studies on the carbon and nitrogen sources were commenced as a result of the culture studies and not prior to them.

MATERIALS AND METHODS

A. Animal Collection

The porcupines were caught by local ranchers, usually in grain fields or hay meadows. Those which have been designated as field animals were used for experiments within 24 hours after capture. Their ingesta consisted of the porcupines natural food in this area. The laboratory porcupines were kept in captivity for several weeks or months before being used in experiments. They were fed commercial rabbit feed containing no antibiotics, Purina Rabbit Chow, WO.

The majority of the experiments required sacrificing the animal. If blood was to be collected from the vessels of the caecal region, the animal was anesthetized with ether, opened with a midline incision and following blood collection the animal was killed by inhalation of chloroform. If it was not necessary to have the animal alive at the time of opening the abdominal cavity, it was first killed by an intramuscular injection of 2 ml of a 40 percent nicotine solution.

B. General Observations

The alimentary tract of each porcupine was removed. The stomach and caecum were weighed, and the lengths of the small and large intestines measured. In some cases the caecum was also weighed after the contents had been removed to determine the actual weight of the fermenting content.

Ingesta were removed from the stomach, caecum, and 3 sections of the large intestine. The sections of the large intestine were designated LI_1 (the first 25 cm of large intestine distal to the caecum), LI_2 (25 cm section

of large intestine distal to LI_1), and LI_3 the remainder of the large intestine containing the fecal pellets in varying states of dehydration.

The percent dry matter in the content of the different organs or parts of an organ was determined by drying samples in an 80 C oven to constant weight.

A glass electrode was used to measure the pH of the caecal content at the base and at the apex of the caecum.

The oxidation-reduction potentials of caecal content were determined with a platinum electrode on samples removed from nonspecific locations in the organ. They were determined at room temperature, in an air atmosphere, on content removed shortly after the animals death. The platinum electrode required about 15 minutes to come to equilibrium.

C. Fermentation Products

The importance of the caecal fermentation to the porcupine can be estimated if one knows what the fermentation products are, the rate at which they are produced, the total amounts produced during a given length of time, and the amounts of these products absorbed and utilized by the animal. The ingesta of the porcupine were quantitatively analyzed for acetic, propionic, butyric, lactic, and succinic acids. The arterial and the venous blood of the caecum was analyzed for total volatile fatty acids.

Portions of the content from the different organs or parts of an organ to be analyzed were mixed 1:1 w/v with 0.2 N H_2SO_4 to inactivate the biological processes. These samples were allowed to stand for at least 24 hours for the equilibration of the soluble components between the liquid

and particulate fractions and after equilibration, they were centrifuged for 20 minutes at 4,000 X g. The particulate fraction was discarded and the supernatant was examined for volatile fatty acids, lactic acid, and succinic acid.

Rates at which volatile fatty acids are produced in the caecum were determined in vitro by the zero time method (Carroll and Hungate, 1954). This procedure permits one to estimate the rate of production of the fermentation products in the animal by plotting their concentrations determined at several successive time intervals of incubation after the removal of the fermenting material from the animal. The slope of the tangent to this curve at zero time approximates the rate of production of the fermentation product in the living animal. Caecal contents were removed from each porcupine shortly after death and placed in a flask gassed with carbon dioxide. A slow stream of CO_2 was run into the flask through a capillary tube during the course of the experiment. The first sample was inactivated at the time the content was placed into the flask and the rest of the samples were removed at 1 hour intervals after the animal's death. The samples were prepared for analysis as described in the preceding paragraph.

Total volatile fatty acids in the alimentary tract contents were determined by steam distillation in the Markham still (Markham, 1942). One to five ml aliquots of the prepared samples, 0.2 ml conc. H_2SO_4, and one drop of 0.1 percent thymol blue solution were placed into the still. More H_2SO_4 was added if the thymol blue was not in the red range (pH 1.2 to 2.8). Condensation of steam into the sample was prevented by heating

the sample prior to distillation by passing steam through the outer jacket of the still for 5 minutes. The amount of distillate collected equaled 15 times the volume of the sample. The distillate was titrated with 0.02 N NaOH to the brom thymol blue end point in the presence of CO_2 free air.

The individual volatile fatty acids were identified and determined quantitatively by gas-liquid chromatography. The acids from 10-20 ml of the acidified and centrifuged caecal content were collected by steam distillation and neutralized with NaOH beyond the thymol blue end point (pH 8.0-9.6) and evaporated to dryness on a steam bath. They were adjusted to the high pH to prevent loss of the volatile acids during drying. The salts of the volatile acids were quantitatively transferred to a 20 ml beaker by rinsing the evaporating dish with 5-7 ml of distilled water added in small portions. The salts were again evaporated to dryness in a near boiling water bath. They were redissolved in 0.2 ml of hot distilled water. The acids were extracted from the aqueous phase with dichloromethane. The beaker was placed into an ice bath and 5 ml of dichloromethane were added; the mixture was allowed to cool for 5 minutes. It was then stirred rapidly with an electric stirrer and conc. H_2SO_4 was added until the thymol blue turned red (pH 1.2-2.8), usually 2 or 3 drops. Two grams of anhydrous Na_2SO_4 were added to take up the water and the stirring was continued for 30 to 45 seconds longer. The stirring blade was then rinsed with 2 ml of dichloromethane, the washing going into the beaker. The beaker was removed from the ice bath and the contents filtered through a medium porosity sintered glass filter into a 10 ml volumetric flask. The beaker

was rinsed several times with small amounts of solvent. The volumetric flask was filled to volume just prior to removing a 30 μl sample for analysis by gas chromatography. This method was found to extract the volatile fatty acids quantitatively from the water solution.

A Beckman GC 2A gas chromatograph was used with a stainless steel column 6 feet long and 1/4 inch in diameter. The column packing material was a modification of that used by Erwin et al. (1961). One hundred ml of Gas Chrom P (60-80 mesh) was first coated with 0.74 g of H_3PO_4 dissolved in 65 ml of dichloromethane. The solvent was evaporated and the H_3PO_4 coated Gas Chrom P was coated with 7.4 g of Tween 80 dissolved in 65 ml of the solvent. The solvent was again evaporated. The packed column was operated at 130 C, with a helium flow of 110 ml per minute. The thermal detector current was set at 300 milliamperes, and the output, attenuated 1, 2, or 5 times, was recorded on a Texas Instruments Servo-Riter integrating recorder.

Standards of reagent grade acetic, propionic and butyric acids were prepared in dichloromethane. The units of area on the chromatographs were linear between the concentrations of 0.003 and 0.100 milliequivalents of acid per milliliter when using a 30 μl sample. An equation for the standard curve of each acid was calculated by the method of least squares.

Lactic acid was determined on diluted digesta using the colorometric Barker and Summerson method as described by Umbreit et al. (1957).

Succinic acid was extracted from digesta with ethyl ether for 16 hours with a Kutscher-Steudel extractor and determined manometrically with pig heart succinic dehydrogenase (Umbreit et al., 1957).

Fermentation gases were collected from caecal content which was removed from a porcupine immediately after death and placed into a flask connected by rubber tubing to a mercury filled gas burette. The flask was incubated in a 39 C water bath and the caecal gases collected for 4 to 6 hours. The gases were separated and quantitatively measured by gas-solid chromatography using a stainless steel column 4 feet long and 1/4 inch in diameter packed with 100-200 mesh silica gel. The column was operated at 40 C, the thermal detector at 150 milliamperes, and the helium flow was set at 55 ml per minute. Each gas sample was introduced into an evacuated 1 cc gas sample loop of the chromatograph through the gas sampling valve. The amount of sample under standard conditions was calculated from the temperature and the pressure at which it was introduced into the gas loop; usually about 100 mm mercury.

Standard curves of carbon dioxide, methane and air were obtained by introducing known samples at various pressures. Cylinder grade CO_2 was used. Methane was obtained in a purified state by distilling condensed natural gas from a vessel immersed in freezing isopropanol. The column gave good separation of H_2, CH_4, air, and CO_2. Nitrogen and oxygen were not separated. The sensitivity of the instrument to H_2 was low and to have been able to have measured it quantitatively one would have had to measure it by difference, but no evidence of H_2 was obtained.

Absorption of volatile fatty acids from the caecum was demonstrated by measuring the differences in their concentrations in blood removed from a large caecal artery and vein. Five ml blood samples were drawn with a syringe and transferred to tubes containing potassium oxalate.

These were refrigerated until analyzed, usually 1 or 2 days.

Volatile fatty acid analyses were made on blood deproteinized with $ZnSO_4$ (McClendon, 1944). Seven ml of the deproteinized blood were placed in a Markham steam still, together with 0.5 ml of 10 N H_2SO_4 and 1 drop of 0.1 percent solution of thymol blue. Seventy ml of the distillate were collected and titrated to the brom thymol blue end point with 0.02 N NaOH while being flushed with CO_2 free air.

Blood samples were also analyzed for urea to determine whether urea was excreted into the caecum. Protein free filtrates of the blood (Folin and Wu, 1938) were incubated with urease and the resulting ammonia determined colorimetrically with Nessler's reagent (Karr, 1924).

D. In Vitro Reactions of Caecal Content

Urease activity in caecal content was determined by the production of ammonia from added urea. Caecal content was mixed 1:1 w/v with a 1 percent aqueous solution of urea. Immediately after mixing, a zero time sample was removed and inactivated by mixing 1:1 v/v with 0.2 N H_2SO_4. The reaction flask was flushed with CO_2, fitted with a bunsen valve and incubated in a 39 C water bath. Ten ml samples were removed at 10 to 20 minute intervals. The inactivated samples were then centrifuged at 4,000 X g for 20 minutes. The supernatants were analyzed for ammonia. One to five ml samples were placed in a Markham still together with one drop of thymol blue and sufficient 10 percent NaOH to turn the mixture alkaline. The distillate was collected with the tip of the condenser immersed in 5 ml of water containing 5 drops of methyl red-methylene blue indicator (McKenzie and

Wallace, 1954). Forty ml of distillate were collected and titrated with 0.02 N H_2SO_4.

Lactic acid dissimilation by caecal bacteria was determined in vitro. Fresh caecal content was placed in a flask and mineral salts dilution solution (Bryant and Robinson, 1961b), containing 1 percent lactic acid, was added 1:1 w/v. The contents of the flask were mixed and a 10 ml zero time sample removed and inactivated with an equal volume of 0.2 N H_2SO_4. These were centrifuged at 4,000 X g for 20 minutes and the supernatants were analyzed for residual lactic acid.

Succinic acid dissimilation by caecal bacteria was determined in a similar manner; the mineral salts solution contained 1 percent succinic acid in place of lactic acid. The inactivated and centrifuged samples were analyzed for residual succinic acid.

E. Nitrogen and Carbon Sources for Caecal Bacteria

Compounds, which might be the natural nitrogen and carbon sources for the caecal bacteria were searched for in content from the caecum and from the distal end of the small intestine. The samples were inactivated by mixing them 1:1 w/v with 0.2 N H_2SO_4, and centrifuged at 4,000 X g for 10 minutes. The supernatant from each sample was analyzed for soluble organic compounds. The particulate fraction of the small intestine content was washed by suspending it in distilled water and recentrifuging. It was then dried in an 80 C oven, ground in a mortar and analyzed for polysaccharides. The particulate fraction of the caecal samples was discarded.

The supernatant samples were analyzed for ammonia and amino acids.

Ammonia was determined by steam distillation and titration. Amino acids were removed from each 10 ml supernatant sample by passing it through a 16 x 30 mm column of Dowex 50 cation exchange resin charged with 4 percent HCl. Amino acids were eluted with 10 ml of 6 N HCl and dried at room temperature under a stream of air. The residue was extracted several times with 1-2 ml volumes of absolute ethanol. The ethanolic extract, a total volume of 5-7 ml, was filtered through sintered glass and dried at room temperature under a stream of air. This second residue was dissolved in 1 ml of water and 2-3 drops of conc. NH_4OH were added to convert the amino acids to the free acids. The samples were then evaporated to dryness on a steam bath. The residue was dissolved in 0.5 ml of distilled water and 5 μl amounts were spotted on thin layer chromatogram plates prepared with a 250 μ layer of plain silica gel H (Research Specialties Company, Richmond, Calif.). The chromatograms were developed in two dimensions with n-butanol-acetic acid-water (2:1:2) for the first direction and isopropanol-NH_4OH (67:33) for the second. The amino acids were detected with ninhydrin.

Supernatant samples from the distal end of the small intestine and from the caecum were analyzed for soluble sugars and sugar acids. Particulate samples from the small intestine were hydrolyzed and analyzed for polysaccharide monomers. The neutral fraction, containing the sugars, from each supernatant sample was obtained by passing the sample through a 16 x 30 mm column of Dowex 50 cation exchange resin and neutralizing the eluate with Dowex 2 anion exchange resin charged with 8 percent $NaHCO_3$. The neutral fraction was decanted from the resin, and the resin was washed

several times with small amounts of distilled water. The washings were added to the neutral fraction which was then dried at room temperature in a stream of air. The residue was dissolved in 0.5 ml distilled water and 5-20 μl amounts were spotted on Whatman No. 1 chromatography paper. The anionic fraction, containing the sugar acids, was eluted from the anion exchange resin with 10 ml of 6 N formic acid. The eluate was evaporated in a vacuum desiccator over NaOH and $CaSO_4$. The residue was dissolved in 0.5 ml of distilled water and 5-20 μl amounts were spotted on Whatman No. 1 paper chromatograms.

One gram amounts of the particulate samples were treated with H_2SO_4 (Block et al., 1958) to hydrolyze cellulose and other polysaccharides. The hydrolysate was neutralized with $BaCO_3$. Excess $BaCO_3$ and $BaSO_4$ were removed by centrifuging at 4,000 X g for 10 minutes. The supernatant was concentrated to 5 ml at room temperature under a stream of air. Five μl amounts were spotted on chromatogram paper.

The sugars and sugar acids were separated by descending paper chromatography. Ethyl acetate-pyridine-water (8:2:1) was used for the separation of sugars and the upper phase of n-butanol-acetic acid-water (4:1:5) was used for the separation of the sugar acids. The spots were developed with $AgNO_3$ solution (Trevelyan, 1950).

F. Caecal Bacteria

Studies on the populations of rumen bacteria have shown that the number of bacteria per gram of rumen content will vary with respect to time after feeding and with location within the rumen (Bryant and Robinson,

1961b, Munch-Peterson and Boundy, 1963). The investigations of Masson (1950), Bauman and Foster (1956), and Hungate et al. (1952) have shown that the composition of the bacterial population of the rumen may vary with changes in the diet; however, Hungate (1957) found no correlation between different rations and differences in the microflora. Culture experiments on the caecal bacteria were designed to determine the diurnal variations in the total numbers and kinds of culturable bacteria, and to compare the compositions of the bacterial populations from different animals.

In order to obtain more than one sample of caecal content from an animal, caecal cannulas were placed in a number of laboratory animals. These were made of plexiglass or teflon and had an inside diameter of one half inch and were closed with a screw cap. They were held in the caecum by a purse-string suture and were brought out through the animals left flank.

The anaerobic culture techniques developed by Hungate (1950) were used for growing caecal bacteria. Anaerobic conditions were continuously maintained in the culture tubes and flasks by displacing the air with oxygen free carbon dioxide, nitrogen, or a mixture of 10 percent carbon dioxide and 90 percent nitrogen. Traces of oxygen were removed from the flushing gases by passing them through a tube of hot reduced copper filings maintained at 520 C in a combustion furnace. The gas or gas mixture was passed into the vessels by means of a Pasteur pipette attached by rubber tubing to the gas source. The reentrance of oxygen was prevented by closing the vessels with rubber stoppers as the pipette was removed. The culture media employed in the study were modifications of those used by

Bryant and Robinson (1961b). The compositions of the various solutions used in the media and the media are given in the Appendix.

Caecal material to be cultured was serially diluted in anaerobic dilution medium and inoculated into roll tubes containing a nonselective medium, which were incubated for one week at 39 C. The colonies were counted under a dissection microscope. Isolated colonies were randomly picked from roll tubes of 0.5 or 1 x 10^{-8} dilution and inoculated into slants of the same medium. Growth from these cultures was used to inoculate differential media designed to detect: (1) fermentation of the carbohydrates glucose, xylose, cellobiose, starch, xylan, and cellulose; (2) gas production from glucose; (3) hydrogen sulfide production; (4) oxygen relationships; (5) gelatin hydrolysis; (6) growth in the absence of rumen fluid; and (7) growth in the absence of carbon dioxide. A weakly buffered glucose medium (pH glucose) was used for determining the terminal pH of fermentation. The results obtained from these media along with cell morphology, gram reaction, and motility were used to group the cultures isolated.

Visible characteristics were recorded for each medium at 1, 3 and 7 days after inoculation, and chemical tests were made at 7 days. Acid production was detected with the use of brom thymol blue, cellulose utilization was detected by visual observation, xylan utilization was quantitatively measured by the orcinol method (Umbreit et al, 1957) and gelatin hydrolysis was determined by liquefaction. Motility was substantiated by diffuse growth in the semi-solid H_2S-Anaerobiosis-Motility medium and by microscopic observation of a wet mount. The H_2S-Anaerobiosis-

Motility medium was not gassed when stab inoculated, which resulted in the oxidation of the upper 8 to 10 mm of medium. The facultatively anaerobic bacteria grew in the oxidized zone as well as in the bottom of the tube.

EXPERIMENTAL

A. General Observations

Since the purpose of the present investigation was to determine the importance of the caecal fermentation to the nutrition of the porcupine, it was important to study normal porcupines, caught in their habitat and eating their natural food. Catching animals not influenced by man and his agricultural crops proved to be difficult. More than half of the porcupines studied were caught while feeding in alfalfa, wheat or barley fields. Porcupines, being nocturnal animals, were easiest and therefore usually caught while feeding between the hours of 7 and 10 P.M. Few collectors were sufficiently interested to deliver the animals within an hour or so of capture at that time of night. Since the time of year, diet and interval between capture and examination were factors which could affect the results, they are recorded in Table I. In some instances data are insufficient to be of any value in interpreting the results.

The determination of the rate at which ingesta leave the caecum was necessary for estimating the amounts of fermentation products absorbed from the ingesta in both the caecum and large intestine. It was assumed that the dry weight loss from the content while passing through the large intestine would be insignificant. The rate of content passage from the caecum through the large intestine was measured by weighing the excreted feces. The feces from 4 laboratory animals were collected twice daily for 3 days and dried to constant weight. The results (Table II) indicate that the amount of ingesta passing through an animal is proportional to its size, being about 10 g per kilogram of body weight per day under those laboratory conditions.