EFFECT OF ROUGHAGE LEVEL IN THE RATION ON PRODUCTION OF SHORT CHAIN ACIDS AND SULFUR AMINO ACIDS IN RUMEN FERMENTATION

by

Roy S. Emery

A THESIS

Submitted to the School of Graduate Studies of
Michigan State University of Agriculture and Applied Science
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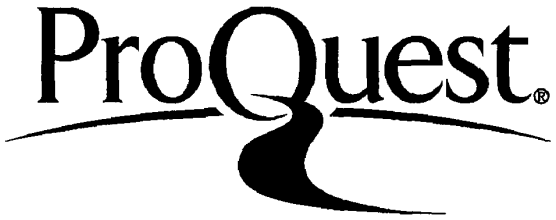

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EFFECT OF ROUGHAGE LEVEL IN THE RATION ON PRODUCTION OF SHORT CHAIN ACIDS AND SULFUR AMINO ACIDS IN RUMEN FERMENTATION

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Rumen liquid from 5 bovines (receiving hay alone or 75% of the total digestible nutrients as concentrate) was incubated with substrate and S^{35}-labeled inorganic sulfate. Two trials used the ration of the donor as substrate and two used a substrate of mixed alfalfa meal and concentrate for both hay inocula and grain inocula. In each trial, a separate fermentation flask was incubated for each time of 0.5, 1, 1.5, 2, 2.5, and 3 hours. Each ferment was fractionated into protein sulfur, free organic sulfur and inorganic sulfate. The protein and free organic sulfur fractions were further separated by chromatography. Glutathione accounted for 2 to 4% of the total activity. The mean partition of incorporated activity in a total of 44 flasks for all times on both treatments was 62% cyst(e)ine and 38% methionine plus an unidentified fraction. Exponential equations fitted to the total incorporations for each combination of substrate and inoculum are presented.

The concentration of acetic, propionic, and butyric acids in 3 bovine rumens was determined at various times after feeding either an all hay ration or one consisting largely of concentrates. Two trials were conducted on each ration. The concentration of each acid first increased and then decreased with time after feeding. The values obtained were fitted to turnover curves. Acetic acid disappeared from the rumen at the rate of 3.3%/hour and butyric acid at the rate of 2.7%/hour. The standard error was 0.3%. The disappearance rate of propionic acid on the high concentrate ration appeared to

be 4.6%/hour versus 3.2%/hour on the hay ration. The rates for conversion of feed to short chain acids ranged from 35 to 84%/hour.

Pooling data from the 4 trials, the amounts of acids per 100 gm. of rumen liquid which were produced from one pound of total digestible nutrients were, 0.2 to 0.67 acetic, 0.02 to 0.26 propionic, and 0.04 to 0.21 butyric. These are the 95% confidence level ranges. The average rumen in these trials was estimated to contain 70 kilograms of liquid. Using these values, it was computed that the cow obtains 3 to 13% of its energy from short chain acids.

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INTRODUCTION

Ruminants have long been known for their ability to utilize roughage. With the advent of intensive animal production and increased concentrate feeding, anomalies have been noted in rumen fermentation. These include changes in the production of short chain acids which are a major source of energy for the cow as well as an end product of the fermentation. Thus, information concerning amounts and types of these acids is imperative to a better understanding of feed utilization.

Large amounts of protein are synthesized in the rumen. Since methionine appears to be a limiting factor in this synthesis, a study of the formation of sulfur amino acids might well lead to increased production by ruminants.

The purpose of this study is to determine the quantity of short chain acids and sulfur amino acids made available to the ruminant as affected by the ratio of hay to concentrate in the ration.

REVIEW OF LITERATURE

The recent reviews on ruminant nutrition by Huffman (1953) and Owen (1954) establish the importance of microbial digestion for economical roughage utilization and for animal production. Doetsch and Robinson (1953) reviewed the bacteriology of the rumen while Jarrige (1953) gave particular attention to the degradation of carbohydrates and the resulting products. The literature discussed here pertains to the dynamics of rumen fermentation as influenced by the ration and portrayed by the production of short chain acids and the synthesis of protein.

Influence of Concentrate on Rumen Digestion and Passage

The fundamental problem of ruminant nutrition is to enhance the native ability of cattle to utilize coarse feed (Huffman, 1953). According to Phillipson (1953), carbohydrates can either enhance or depress roughage utilization depending on their quantity and quality. Jarrige (1953) lists research over fifty years dealing with the influence of soluble carbohydrate on roughage utilization.

Swift et al. (1947) reported a 5 to 10% depression of crude fiber digestibility when corn starch or sugar formed some 8 to 15% of a ration based on equal parts of mixed hay and corn. This could not be confirmed by Dowe et al. (1955)

who fed over 80% corn in the ration but no pure starch. However, Burroughs et al. (1949) obtained a 35% depression of organic matter digestibility with a ration of corncobs, skim milk and 30% starch but only a 4% depression when the starch level was reduced to 18%. Similar data were obtained with a ration of starch, alfalfa hay, and corncobs. According to Louw and Van der Wath (1943), supplementation of a hay ration with corn at the 8 to 33% level depressed cellulose digestibility.

A wood meal, studied by Koistinen (1948), which was 50% digestible when fed with hay or limited concentrate, became virtually indigestible when fed with silage. Balch and Johnson (1950) found that cotton thread suspended in the rumen was attacked several times faster on an all hay ration than on one containing over 25% concentrate.

Foreman and Herman (1953) increased cellulose digestion somewhat by adding 1 to 2 pounds of molasses to a basal ration of about 4 parts hay to 1 concentrate but this digestibility was usually depressed when the molasses level was such that the ration contained 30 to 40% total concentrate. The results varied with the type of hay fed. It should be noted that the water soluble carbohydrate of roughages can range from 4 to 30% of the dry matter depending on species, method of curing, stage of growth and other factors as reported by Waite and Boyd (1953).

Hale et al. (1940, 1947), utilizing the lignin ratio technique to compare rumen and fecal digestion, showed that the rumen accounts for the greater part of digestion in bovines fed alfalfa hay. This has been confirmed and extended to other feeds as reviewed by Jarrige (1953). The abomasal fistula technique for measuring forestomach digestion was used by Heald (1953), who found that about 40% of the ingested xylan was digested in the forestomach, and by Weller and Gray (1954), who found that nearly all of the starch in chopped potatoes was digested in the forestomach.

Balch et. al. (1951) state that the fermentation and digestion of feed in ruminants is a function of its rate of passage through the reticulo-rumen, its rate of dissimilation by the microbiota and finally, the rate of absorption or passage of the fermentation products.

Some 2 to 5% of the stained hay particles present in the rumen at a given time disappeared in the next hour according to the data of Balch (1950). Finer particles, such as cottonseed hulls or ground hay passed somewhat faster (5 to 10%/hour), but only if long hay or other roughage was also present. Moreover, by reclaiming the stained particles in the feces and expressing recovery as percentage of the total, he found that about 20 hours were required for passage from the rumen to the feces. Stained particle recovery appeared to be exponential approaching an asymptote; the rate of the exponential was again some 2 to 5%/hour. Earlier, Moore and Winter (1934)

found that ferric oxide passed from rumen to feces in 9 to 13 hours and small rubber rings in 10 to 20 hours. After the initial appearance, recovery was again roughly exponential approaching an asymptote at 2 to 5%/hour. The work of Balch et al. (1950, 1952b, 1953) showed that such factors as thyroxine administration, rations, restricted water intake and others had little effect on this rate. The rations studied included all hay, equal parts of hay and concentrate, hay plus mangolds, and finely ground hay.

The data of Agrawala et al. (1953) and of Chance et al. (1953a, b) for dry matter passage from the rumen measured between 6 and 24 hours also indicates a rate of 2 to 5%/hour. The disappearance was much faster between 0 and 6 hours after feeding due to digestion as explained by Hale et al. (1947), who showed that rumen digestion is virtually completed in 12 hours after feeding and over half completed in 6 hours. Balch and Johnson (1950) found cotton thread dissipating in the ventral portion of the rumen at as much as 50%/hour after a 20 hour lag phase. According to Balch et al. (1951), passage through the reticulo-omasal orifice is largely restricted to fine particles in a 90 to 95% aqueous suspension.

The duodenal or abomasal fistula presents another way of measuring passage from the forestomachs. In this way, Phillipson (1948, 1952a) measured a passage of 467 to 1864 ml./hour in sheep consuming 900 to 1700 gm. of dry matter/day; the larger volume was associated with heavier feeding. Considering

the total washed feed residue in 24 hour feces collections and in duodenal contents from a sheep maintained on hay, it was calculated that 10.7 liters of material passed through the duodenum daily. Masson and Phillipson (1952) calculated that two-thirds of this material was gastric juice and one-third omasal contents. Their calculations were based on the chloride content of the gastric juice, omasal contents, and abomasal contents. Passage of omasal contents was stimulated somewhat by concentrate but was largely independent of feeding. This suggests that passage of omasal contents corresponds to about 2 to 5%/hour of the mass of the total rumen ingesta which is in agreement with the passage of markers from the rumen.

The increased concentration of inorganic ions in omasal liquid over rumen liquid found by Garton (1951) indicates absorption of water from the omasum. Gray et al. (1954) confirmed this and calculated from the lignin ratios in rumen and omasal contents that 33 to 64% of the water and 40 to 69% of the volatile fatty acid in the rumen digesta entering the omasum is absorbed there. Using the same technique, they were able to show that little or no rumen digesta passed directly to the abomasum via the omasal orifice and that water was not preferentially passed from the omasum to the abomasum.

Influence of Concentrate on the Rumen Microbiota

Since forestomach digestion seems to occur largely through action of the rumen microbiota, as pointed out by Hastings

(1944), we might expect a qualitative or quantitative change in this microbiota with changes in digestion. Hastings (1944) notes changes in the quantity of various types of protozoa with marked changes in feed, but considers the popular idea that biology of the digestive tract varies widely with the normal changes in ration as unfounded. Baker (1943) concluded from his extensive microscopic observations that the rumen microbiota is persistant and stable with broad qualitative features which are independent of the nature of the diet.

However, Louw and Van der Wath (1943) increased the rumen bacterial count by providing 8 to 22% corn in a ration of low quality hay and meat meal. Cellulose digestion was not augmented by the increased population and greater concentrations of corn depressed bacterial numbers. Bortree et al. (1946) obtained a 100% increase in the iodophilic microbial population of the bovine rumen when 3 pounds of glucose were administered in addition to the usual feeding of hay. Starch administered under similar circumstances produced a negligible effect; quality of roughage or pasture influenced counts somewhat. Walter (1952) found no appreciable change in direct microscopic counts on rumen ingesta when barley supplemented the basal of alfalfa hay for sheep.

Some qualitative differences among certain indicator organisms were observed by Pounden and Hibbs (1948) when they examined the rumen microbiota of calves fed various proportions of hay and grain. The broad appearance of the rumen microbiota

and the importance of the indicator organisms in rumen fermentation were not reported. Utilizing both cultural and morphological techniques, Gall (1949) found greater numbers of fast growing organisms with increasing amounts of grain in the ration but few qualitative differences were noted.

Foreman and Herman (1953) conducted direct microscopic counts on the rumen ingesta of 12 cows in 3 groups fed different roughages. Four levels of cane molasses were fed within each group on a rotation basis. The basal ration contained about 4 parts of hay and 1 part concentrate. The total counts, which were grouped into 10 morphological classes, showed a persistant increase from about 67 x 10^9 to 106 x 10^9 organisms per gm. of moist ingesta as molasses supplementation increased from 0 to 4 pounds per day. The numbers of protozoa were consistantly 200 to 300 thousand per ml. of rumen fluid. The single cocci constituted about 70% of the population. The short rods which constituted about 20 to 25% of the population tended to increase slightly with 1 or 2 pounds of molasses, but were depressed by 4 pounds. Long rods and diplococci were distinctly depressed by molasses. However, the qualitative and quantitative changes in the population were as great with changes in roughage as with the molasses supplementation.

Williams et al. (1953) found that 1 part of starch added to 1 or 2 parts of oaten chaff containing 5% crude protein decreased the numbers of free rumen bacteria by 50% or more, but if the protein source was corn gluten meal, this depression

largely disappeared. The high starch, low protein ration produced a pleomorphic rod in long chains clustered on starch granules. This rod largely disappeared as protein increased and was absent on low starch rations. An increase in the proportion of gram-positive organisms was also noted on the high starch, low protein ration.

The rumen microflora from cows fed alfalfa hay and/or concentrate mixture or wheat straw was studied by Bryant and Burkey (1953a, 1953b) using both morphological and cultural methods. Qualitatively, the microflora were largely the same with numbers increasing on the concentrate ration; however, they estimated that only 10% of the total population are cultured by the present *in vitro* techniques and call attention to the pleomorphic nature of many of the ruminal microorganisms. The flora was more complex on the alfalfa hay containing rations than on the rations of concentrate or wheat straw. Cellobiose fermenting organisms were depressed by the concentrate ration and starch fermenters were augmented; hydrogen sulfide producing organisms were augmented somewhat by the straw ration. Variability was also noted in the flora of different animals on the same ration.

Rumen Production of Short Chain Acids

The early literature on this subject, reviewed by Phillipson (1947), dates back to 1884 when Tappeiner found large quantities of volatile acid formed in the bovine rumen. However,

the composition of their mixture was not known until Elsden et al. (1946) developed a method for their separation on silica gel. These workers found an average of 329 gm. of volatile acid in the reticulo-rumen of 2 oxen and an average of 64 gm. in that of 4 sheep. These acids consisted of about 67% acetic, 19% propionic and 14% butyric. This is at variance with the data of Marston (1948) who found about equal portions of acetic and propionic acids produced from fermentation of cellulose by rumen microbiota in vitro.

Rumen ingesta from several cows examined by Stone (1949) through a 9 hour period following eating, contained 133 to 162 milligram equivalents for several hours after eating. The residual feed in 100 ml. of ingesta removed 1 hour after feeding hay and grain and incubated at 39°C. was converted to volatile acid at the rate of about 60%/hour with a total production of 20 to 25 milligram equivalents of volatile acid. This amounts to some 12 to 15 gm./liter rumen ingesta.

Carroll and Hungate (1954) pointed out that production of volatile acid can not be determined directly in the rumen because absorption decreases the acid concentration simultaneously with its increase through fermentation. Moreover, rumen microbiota dissociated from the absorptive and secretory activities of the host may soon develop abnormal conditions. These workers also noted a rise in the volatile acidity of rumen ingesta during the first six hours after feeding. The average amount of acid produced in 1 hour of in vitro incubation

with rumen contents from hay fed steers was 1.37 milligram equivalents per 100 gm. The corresponding amounts with a grain diet and a pasture diet were 2.01 and 0.91 respectively. The average proportions of acetic, propionic and butyric acids in the mixture formed during 6 trials with hay fed animals were 61%, 20%, and 19% respectively. It was estimated that 66% of the total digestible energy in the ration is presented to the ruminant as volatile acids. In hay fed steers, acetic acid contributed 42% of this energy; butyric acid, 32% and propionic acid, 26%.

These estimates of Carroll and Hungate (1954) were based on the experimentally determined rates of acid production and estimates of total rumen mass. The feed was converted to volatile acids at the rate of about 40%/hour. They considered this rate as a minimum and pointed out that the best way of ascertaining rate of production of a product in the rumen would be to construct time-concentration curves and calculate the initial rate. They pointed out that a very large volume of data would be required.

Gray et al. (1951b) incubated wheaten and alfalfa hay with liquid rumen contents in vitro. The yield of volatile acids was about one-fourth of the substrate or 50% of the digestible matter. The acids produced from wheaten hay were 46% acetic, 41% propionic and 13% butyric while those from alfalfa were 59%, 27%, and 14% respectively.

Dealing with sheep fed wheaten hay, Gray and Pilgrim (1951) found volatile acid concentrations in the rumen liquid of 8.7 milligram equivalents/100 ml. at the time of feeding which increased to 20.6 six hours after feeding and reverting to the initial concentration in about 24 hours. In sheep fed alfalfa hay, the corresponding initial value was 9.3 increasing to 25.5 four hours after feeding. Moreover, the propionic acid concentration increased faster than the acetic acid concentration and thus, it was concluded that propionic acid was produced in greater quantities than acetic acid and was also absorbed faster since the concentration reverted to the pre-feeding level. This hypothesis was tested by Gray and Pilgrim (1952a) by *in vitro* incubation of rumen microbiota with wheaten hay chaff. Volatile acid production was largely completed within 7½ hours and although the production of propionic acid exceeded that of acetic acid at first, the proportion of the acids in the final mixture was about 50% acetic, 40% propionic and 10% butyric.

The volatile acids of rumen fluid from hay fed sheep were more completely analyzed by Gray *et al.* (1951a) who found 2 to 3% valeric acid, 0.3 to 0.6% isobutyric acid, and 0.5 to 1.0% caproic acid in addition to the usual acetic, propionic and butyric acids. Using C^{14}-labeled acetic and propionic acids, they found that the carboxyl carbon of acetic acid occurred to an appreciable extent in butyric and valeric acids and also to some extent in propionic acid. The carboxyl carbon

of propionate appeared in valeric but not in butyric acid. The ability of Clostridium to reversibly interconvert acetic, propionic and butyric acids was more fully explained by Wood et al. (1945) and Stadtman and Barker (1949).

El-Shazly (1952) simultaneously obtained similar results by using a new and very sensitive gas partition method of chromatography. He found somewhat higher proportions of isobutyric acid (1.5 to 2.8 molar% of the volatile acid mixture) and showed that most of the valeric acid is branched chain. He also showed that these branched chain acids are formed largely by microbial decomposition of protein. This work was continued by Annison (1954) who identified 2-methyl-butyric acid among the branched chain acids in the rumen. Coon et al. (1952a, 1952b) showed that this acid could arise from isoleucine in the rat liver and could yield a 2-carbon ketogenic compound and a 3-carbon glucogenic compound with propionic acid as an intermediate.

According to Annison (1954), the total volatile acid concentration in the rumen declined exponentially at the rate of 3%/hour in fasted sheep, but the branched chain acids remained almost constant at 1.5 to 3.5 milligram equivalents/ 100 ml. rumen ingesta. The branched chain acids increased as the nitrogen content of the ration increased. A ration of 2.5 parts ground corn and 1 part hay was converted to volatile acid at a rate between 30 and 80%/hour.

Phillipson (1952b) studied production of volatile acids on a similar ration with similar results. The molar composition of the volatile acids was about 60% acetic, 30% propionic, 7% butyric and 4% higher acids 8 hours after feeding. The relative amount of propionic acid increased after feeding and hence, was either produced faster or absorbed slower than acetic acid. Lactic acid concentrations as high as 630 milligrams/100 ml. rumen liquid were found in lambs fed a corn ration. Waldo and Schultz (1955) obtained values of 0.3 to 41 milligram% lactic acid in rumen liquid from hay fed steers. These values increased to as much as 58 milligram% when 7.5 pounds of grain was included in the ration. Maximum concentration in the rumen occurred 1 hour after feeding hay or one-half hour after feeding grain. Disappearance was equally rapid declining to one-half of peak concentration in less than 2 hours. However, when Phillipson (1952b) drenched 2 lambs with lactate, disappearance of lactate from the rumen of one lamb was much slower than in the other. Waldo and Schultz (1955) found that 1 pound of glucose increased the lactic acid concentration in the rumen much more than 1 pound of starch, cellulose, or pyruvic acid. Both groups of investigators propose that lactic acid is rapidly converted primarily to propionic acid in the rumen. This would explain the very high fermentable carbohydrate concentration which is apparently required in the rumen to produce a detectable concentration of lactic acid. Johns (1952) and Gutierrez (1953)

isolated bacteria from the rumen which dissimilate lactate with production of propionic and acetic acids.

Washed suspensions of rumen bacteria prepared by Robinson et al. (1955) produced lactic acid from maltose and cellobiose and to a smaller extent from glucose, pyruvate, or malate, but not from xylose or arabinose. When the smaller organisms were eliminated by using lower centrifugal force, lactic acid was produced at pH 5.0 or 8.0 but not at pH 6.9.

The molar composition of short chain acids was about 65% acetic, 20% propionic, and 15% butyric in a number of cows fed a control ration of hay, silage, and concentrate (Tyznik, 1951). The molar ratios of acetate and propionate were usually reversed 2 to 3 weeks after restricting roughage and liberally feeding concentrate or finely ground hay. It was hypothesized that the production of acetate was suppressed in favor of propionate production.

Card and Schultz (1953) studied production of volatile fatty acids in 28 cows on widely different rations and found a significant difference in the molar percentage of each acid due to ration. The molar % composition of the total volatile fatty acids for acetic, propionic, and butyric acids respectively were as follows: mixed hay - 60.0, 21.0, 19.0; hay plus grain - 57.7, 18.8, 23.5; pasture - 55.6, 17.9, 26.5; pasture plus grain - 53.0, 20.3, 26.7 and; grain alone - 47.3, 23.2, 29.5. Roughage from a later date of cutting tended to increase the proportion of acetic and decrease the proportion

of butyric acid which could be explained by decreased protein (El-Shazly, 1952). The total volatile acid concentration was highest on the all concentrate ration and lowest on the pasture. Schultz (1954) found the total volatile acid concentration in 8 bovine rumens was depressed about 25% by a 16 hour fast and the relative proportion of propionic acid was also depressed. This is in agreement with the data of Robertson and Thin (1953).

Johns (1953) found that glucose in the rumen of sheep quantitatively yielded a mixture of acetic, propionic and butyric acids within an hour. Both in vitro and in vivo, acetate production was much greater than propionate or butyrate production. Under the same conditions, glycerol was attacked more slowly yielding almost entirely propionic acid. According to Sijpesteijn and Elsden (1952), succinate also quantitatively yields propionate in the rumen.

The products of in vitro fermentation reported by Mc Naught (1951) were different for rumen microbiota from cows on pasture than for microbiota from hay and grain fed cows. The products also varied with substrate. Pasture microbiota acting on maltose or arabinose produced acids with a molar composition of about 46% acetate, 46% propionate, and 8% butyrate while the hay-concentrate microbiota produced a corresponding composition of 53%, 32%, and 15%. Xylose yielded more total acid than maltose, but the composition was the same. A carbon balance study showed that 24 to 25%

of the maltose, arabinose, or xylose carbon was converted to volatile acid. Lactic acid accounted for 6 to 10% of the maltose carbon while only a trace or no lactic acid was produced from arabinose or xylose. This was confirmed by Heald (1952), except for larger proportions of acetate from xylose, and extended to glucuronolactone with similar results. Heald (1953) estimated that pentosans form 20 to 25% of the dry matter in common ruminant rations.

Suspensions of rumen bacteria prepared by Doetsch et al. (1953) fermented cellobiose or pyruvate yielding a mixture of acids with a molar composition of about 64% acetic, 21% propionic and 15% higher acids; glucose and maltose yielding 36% acetic, 40% propionic and 24% higher acids; xylose yielded 40% acetic, 30% propionic and 30% higher acids and succinate yielded only propionic acid. Lactate and oxalacetate were attacked slowly with only a trace of acid production. Propionate yielded a trace of acetate; acetate was not attacked; butyrate was converted quantitatively to acetate. Beta-hydroxybutyrate yielded some acetate while malate was converted to acetate and propionate. Many of these results were confirmed by Sirotnak et al. (1954) and were extended, using the same technique, to alanine which was not attacked, fumarate which was dissimilated without acid production, and to L-aspartate which appeared to be deaminized to succinate and then decarboxylated to propionate.

Gray and Pilgrim (1952b) examined the fatty acids produced *in vitro* from the cellulose and hemi-cellulose of wheaten hay and found approximately equal parts of acetic and propionic acids and a small amount of butyric acid produced from either or both substances. Butyric acid seemed to come only from protein sources which also yielded rather large amounts of acetic acid. This pattern is found again in the work of Belasco (1954) where only acetate and propionate with a trace of butyrate were formed by the action of rumen microbiota *in vitro* on cellulose and starch. Protein nitrogen was replaced by non-protein nitrogen in this work and as the concentration of the latter increased, more valeric and butyric acids were formed.

Tyznik *et al.* (1954) tested the effect of various substances on short chain acid production when replacing cellulose on an equal carbon basis. Maltose or starch increased total volatile acid production with a 100% increase in the proportion of butyric acid. Glucose alone or with cellulose gave the same proportions of volatile acids as cellulose alone but significantly increased the total acid production. Lactic acid increased the proportion of propionic acid and increased total acid production but depressed cellulose fermentation above the 25% carbon replacement level. Replacing 25% of the carbon with starch, lactic acid, maltose, or sodium pyruvate yielded varying amounts of valeric acid amounting to from 1 to 7.5% of the total volatile acid. Addition of acetic,

propionic and butyric acids to replace 25% of the carbon had no effect on total volatile acid production.

Absorption and Utilization of Short Chain Acids

The literature dealing with absorption of volatile acids was reviewed by Phillipson (1947) who, citing earlier work done by his group, concludes that more than 5 gm. of volatile acid per hour are absorbed from the bovine rumen. Pfander and Phillipson (1953) point out that, although numerous experiments have been conducted on absorption rates of short chain acids, none of them sufficiently simulate normal conditions to allow deductions concerning quantities of these acids normally absorbed.

McClymont (1951) reported a maximum arterial volatile acid level of 1.2 to 2.3 milligram equivalents/liter 2 to 5 hours after feeding. This declined to 0.3 to 0.9 milligram equivalents/ liter 16 hours after feeding with a molar composition of over 90% acetic acid. Similar values were found by Reid (1950) in sheep.

The peripheral blood and venous blood draining the rumen in 14 sheep on pasture was examined by Kiddle et al. (1951). Peripheral blood contained 0.4 to 1.7 milligram equivalents volatile acid/ liter consisting largely of acetic while venous blood draining the rumen contained 1.7 to 8.6 milligram equivalents volatile acid/liter with a molar composition of 57 to 95% acetic, 10 to 33% propionic and 3 to 16% butyric acid.

Craine and Hansen (1952) obtained similar values in venous blood draining the rumens of adult goats. Even after correction for the acetic acid in peripheral blood, the proportion of acetic acid in blood draining the rumen was always higher than in rumen ingesta. Lymph collected from the thoracic duct in 4 animals contained the same concentration of volatile acids as arterial blood. However, when Masson and Phillipson (1951) continued this work by measuring the disappearance of individual acids from emptied, washed rumens ligated at the oesophagus and omasal-abomasal orifice, they found that all acids disappeared at about the same rate. The rumen tended to buffer at pH 7.5 to 7.8 by influx of chlorides and CO_2 from blood. Administration of butyrate and propionate to normal animals had little effect on the arterial volatile acid concentration while acetate caused a three-fold increase.

These anomalous results were partially clarified when Pennington (1952, 1954) found rumen epithelium metabolizing butyrate much more readily than propionate or acetate. Ketones were produced from butyrate and acetate, but not propionate. Propionate metabolism was increased by increasing CO_2-tension up to 20%. Malonate inhibited utilization of each acid and caused production of succinate from propionate. The ammonium ion depressed metabolism of propionate, but not butyrate, possibly by blocking the tricarboxylic acid cycle. This depression occurred at ammonium levels which often occur in the rumen after feeding. Robertson and Thin (1953) confirmed the extra-ruminal nature of ketone body production.

Schambye (1951) conducted similar studies using rations of chopped or unchopped hay, crushed oats, and linseed meal. The prefeeding volatile acid concentration in portal blood was double that of arterial blood. After feeding, the portal level rose about 50% paralleling the increased concentration of volatile acids in the rumen while the arterial level rose only slightly. Injection of acetic acid into the rumen increased the concentration in portal blood but not in venous blood draining the liver. Thus, the volatile acids were largely removed from portal blood by the liver. The portal glycemia showed little change after feeding and the portal-arterial differences were nearly zero. Oral administration of 1 to 2 gm. of glucose/kilogram body weight resulted in a small and transitory hyperglycemia. The very large molar ratio of absorbed volatile acid to absorbed glucose leaves little doubt that volatile acids constitute the greater source of calories passing from the digestive tract to the liver. Moreover, glucose is absorbed more rapidly from the abomasum of sheep than from the abomasum of non-ruminants (Reid; 1950, 1952). Weller and Gray (1954) observed only a 10-fold increase in starch passage through the abomasum when starch ingestion was increased 100-fold. The starch was not attacked in the abomasum.

Johnson (1951) infused neutralized volatile acid mixtures into the rumens of fasted goats to demonstrate the rates of absorption without exceeding physiological concentrations.

He concluded that when rumen volatile acid analysis shows 65 molar % acetic acid, 20% propionic acid and 15% butyric acid, the amounts actually absorbed are probably about 60%, 20% and 20% respectively. This was confirmed and extended by Pfander and Phillipson (1953) showing that acetic acid disappears from the rumen in the greatest quantity due to its high concentration, but propionic and particularly butyric acids disappear at faster rates and contribute equal or greater amounts of energy to the ruminant. This agrees with the conclusions of Carroll and Hungate (1954), Gray et al. (1951b), Gray and Pilgrim (1951, 1952a, 1952b), and McNaught (1951).

A rumen liquid tonicity of 0.165 molar was reported by Parthasarathy and Phillipson (1953). Absorption of chloride and acetate but not sodium and potassium was inhibited by 0.002 molar mercuric chloride. Acetate was absorbed faster at a lower pH (confirming results of Danielli et al., 1945) but could not be absorbed against a concentration gradient.

An arterio-venous difference in sheep head tissue of 2 to 3 milligram % for acetate compared to less than 2 milligram % for glucose was reported by Reid (1950) who concluded that acetate might be utilized in preference to glucose by ruminant tissue. Jarrett and Potter (1950) showed that 27 to 40% of the acetate injected intravenously into phlorizinized sheep could give rise to sugar. This sugar was excreted in the urine since phlorizin blocked resorption. Formation of ketones was also extensive under these conditions. Jarrett et al.

(1952), following the time-concentration curves for blood volatile acids injected intravenously, found propionate disappearing more rapidly than acetate or butyrate. Acetate caused no significant glycemia while propionate was markedly glucogenic. Acetate and butyrate alone were markedly ketogenic, but simultaneous injection of propionate prevented their ketonic effect.

Pennington (1952, 1954) found that ruminant liver and kidney *in vitro* could utilize acetate and butyrate and to a lesser extent, propionate. Kidney was particularly active while heart muscle weakly utilized acetate and butyrate, but not propionate.

Less than 5% of the injected acetate (500 milligrams/kilogram body weight) appeared in the urine of 16 normal ewes studied by Pugh and Scarisbrick (1952). The half-time for disappearance of blood acetate in normal sheep was 25 minutes versus 60 minutes in ketonic sheep. Allen *et al.* (1955) point out that both hepatic and extra-hepatic cells, including even brain, can oxidize both sugars and fatty acids. They demonstrated a basal fatty acid catabolism occurring generally in tissues which was not suppressed even by abundant presence of carbohydrate. Bentley *et al.* (1954a) found 1 pound of lactic, acetic, or propionic acid equivalent energetically to 2.75, 1.6, or 1.4 pounds of corn when fed to sheep.

Indirect evidence for the essentiality of acetate in formation of milk fat started accumulating when Powell (1941)

found that a ration of severely restricted or very finely ground roughage depressed the fat content of milk. Further study of this phenomenon by Allen and co-workers was reviewed by Tyznik (1951). The depressed fat content caused by restricted roughage-high concentrate rations or by finely ground roughage could be reversed by oral administration of sodium acetate. Although Tyznik (1951) found a lowered rumen concentration of acetic acid on these rations, both the rumen fermentation and the butterfat test of most cows spontaneously reverted to normal within a few weeks.

Balch et al. (1952a, 1954a, 1954b) studied the depression of the fat content of milk associated with changing cows from dry feed to pasture. They considered this an effect of the lack of coarse material in young pasture. According to these workers, the solids not fat content is not affected by a restricted roughage ration. High concentrate rations depressed the fat content regardless of their protein content; however, the effect became less severe later in lactation. Hay consumption had to be less than 12 pounds per day per cow to produce the effect, but depression was not furthered by decreasing hay ingestion below 8 pounds.

Milk fat production was stimulated 3.2% in cows fed 350 gm. of acetate daily by Zelter (1952). Pyruvate feeding also caused some stimulation over the controls while no significant increase in production could be attributed to butyrate. Van Soest et al. (1954) confirmed these results using cows whose

milk fat production had been depressed by a restricted rough-age-high concentrate ration and found that the increased production accounted for all of the carbon administered as acetate. However, Miller and Allen (1955) could not find significant differences in either the fat test or the amount of milk secreted between control cows fed hay plus corn silage and 16 pounds of concentrate daily and experimental cows fed the same ration plus one pound of sodium acetate. The experiment could detect differences of 0.1% in fat test or 0.8 pounds of milk/day.

This indirect evidence for acetate utilization in milk fat synthesis is augmented by mammary gland studies reviewed by Popjak (1952). Folley and French (1948) reported that ruminant liver slices do not utilize acetate better than rat liver slices, but ruminant kidney and mammary tissues do have a greater acetate utilizing ability than equivalent non-ruminant tissues and even show a greater utilization of acetate than glucose. Ruminant mammary tissue utilizes acetate but not glucose with an R.Q. greater than 1. Non-ruminant mammary tissue uses only glucose with an R.Q. greater than 1. When Popjak *et al.* (1951a, 1951b) injected lactating goats with carboxy-C^{14}-acetate, the maximum specific activity of the plasma fat acid occurred in 24 hours whereas specific activity of milk fat was maximum in 3 to 4 hours and was several times greater than the plasma fat maximum. During the first 12 hours after injection, activity of the 8 to 12 carbon acids in milk

exceeded that for the 14 to 16 carbon acids. Half-time for the disappearance of blood acetate was 50 minutes versus 4 hours for the volatile acids of milk. Only the odd-numbered carbons of acetate, butyrate, and caproate were active and the carboxyl-carbon of caproate was more active than carbons 3 or 5. About 40% of the milk butyrate came from acetate via beta condensation and 60% from some non-isotopic 4-carbon compound. Higher acids were formed exclusively by a stepwise condensation of acetate with caproate. Fifty percent of the non-oxidized, labeled acetate was used for milk fat synthesis.

Cowie et al. (1951b) circulated acetate through an isolated bovine udder and recovered 40% of it in the milk fat along with traces in milk cholesterol and lactose. Since butyric acid specific activity corresponded with that of the other acids, the 4-carbon precursor of milk butyrate noted above came from blood. Carbon dioxide incorporation into milk fat and lactose was negligible. Shaw and Petersen (1943) showed that the bovine mammary gland readily utilizes beta-hydroxybutyric acid and Kumar and Lakshmanan (1954) showed that this acid is probably the 4-carbon precursor of milk butyrate hypothesized by Popjak (1952).

Glucose was more effective than acetate as a precursor for milk fat in the rabbit mammary tested by Popjak et al. (1953) but the synthesis was still via pyruvate and some 2-carbon unit. Glycerol was also formed much more slowly from acetate than glucose.

McClymont (1951) found 40 to 80% of the acetate removed from blood circulating through the intact, lactating bovine mammary. Arterial acetate levels were not affected by the high concentrate-restricted roughage ration although the milk fat test was depressed. Hyperinsulinism or delayed milking also failed to affect acetate utilization. A high correlation was found between CO_2 production and acetate utilization by the mammary tissue even when excess acetate was administered. It was concluded that the volatile acid of milk is independent of blood volatile fatty acid and that acetate is used in the mammary gland for either synthesis or useful oxidative metabolism.

Bryant and Doetsch (1954, 1955) and Bentley et al. (1954b) have found that a mixture of the branched-chain volatile acids and straight-chain 5 to 8 carbon acids normally present in the rumen is required for growth of some rumen microorganisms and for optimum cellulose digestion and nitrogen utilization.

Rumen Synthesis of Carbohydrate

Several rumen microorganisms, examined by Baker (1942), synthesized iodine staining polysaccharide. These organisms were not subject to peptic or tryptic digestion, but were digested in large quantities by the ciliate protozoa. Baker and Harriss (1947) reviewed the subject of synthesis by rumen microbiota. A rumen protozoan, cultured by Hungate (1943), produced a reserve iodine staining polysaccharide 2 to 7 hours

after exposure to cellulose. The polysaccharide disappeared within 12 hours after removing the cellulose. Van der Wath (1948) confirmed the ability of rumen microbiota to synthesize iodophilic polysaccharide from starch.

Heald (1951) prepared relatively pure batches of rumen microorganisms from hay fed sheep and found that they contained 4 to 5% glucose-yielding-carbohydrate on the dry basis. A maximum of 5 gm. of glucose per day passed from the abomasum of hay fed sheep assuming that all abomasal passage was rumen ingesta. Masson and Phillipson (1952) showed that two-thirds of this material was gastric juice which would revise the estimate to about 2 gm. This amounts to a negligible portion of the total feed energy.

Weller and Gray (1954) used the lignin ratio technique with rumen and abomasal fistulae to show that starch was largely destroyed in the omasum along with the protozoa. Starch passing through the abomasum increased from 1 gm. per day when 3 gm. were fed to only 8 gm. when 148 gm. were fed. Destruction of starch in the rumen and omasum was almost complete. Heald (1951) found the fermentable, glucose-containing carbohydrate content of the rumen microbiota increased from a prefeeding level of 2 to 7% to 7 to 21% of the dry matter 2 hours after feeding. This returned to the prefeeding level within 6 to 8 hours. Centrifugation showed that this synthesis was largely a function of the intermediate and large sized organisms.

Holotrich ciliates from the rumen of hay fed sheep deposit a polysaccharide which Oxford (1951) and Masson and Oxford (1951) found to be starch. This starch, which could be prepared with a 1% yield from the dry matter of strained rumen liquid from sheep grazed on starch-free spring grass, was synthesized *in vitro* from glucose, fructose, sucrose, inulin and bacterial levan with 15 to 21% yields and from cellobiose with a 6% yield, but not from maltose.

McNaught (1951) recovered 15 to 39% of the carbon of maltose, arabinose, or xylose as bacterial polysaccharide when they were fermented *in vitro* by rumen microbiota. Mc Naught *et al.* (1954) found polysaccharide accounting for 32% of the rumen bacterial dry matter and 62% of the protozoal dry matter. Sugden (1953) found that certain oligotrich protozoa could not form polysaccharides from simple sugars or cellulose when their symbiotic bacteria were eliminated by streptomycin.

Robinson *et al.* (1955) concluded from their work with washed suspensions of rumen microbiota that polysaccharide synthesis is probably a mechanism which enables the microbiota to store a reserve energy supply. They failed to obtain this synthesis with maltose substrate which confirms the work of Masson and Oxford (1951).

Rumen Synthesis of Protein

The literature concerning urea as a protein replacement for ruminants has been reviewed by Reid (1953). According to this review, Zuntz recognized the value of urea as a partial protein replacement for ruminants in 1891. Hart et al. (1939) confirmed the value of simple nitrogenous compounds as protein substitutes for ruminants and considered the rumen microbiota responsible for their utilization. The literature reviewed here pertains to the role of rumen microbiota in this protein synthesis.

Pearson and Smith (1943b) concluded that protein synthesis during rumen fermentation, in vivo or in vitro, is accompanied by protein catabolism and either process may predominate. The amount of synthesis during the first two hours of incubation of centrifuged rumen liquid with 0.3% starch and urea added was equivalent to 8 milligrams N/100 gm. rumen liquid which would amount to some 450 gm. of protein in the intact bovine rumen. Synthesis was stopped by less than 0.1% sodium fluoride indicating its microbial nature. Starch had a relatively high stimulatory effect on synthesis compared to other compounds studied while 1% gelatin markedly inhibited synthesis. Amino acids had relatively little effect. Burroughs et al. (1951) examined the effect of various proteins on urea utilization during rumen fermentation in vitro and concluded that the nitrogen requirements of rumen microbiota are restricted to ammonia.

About 50% of the total rumen nitrogen is microbial according to Gray et al. (1953). Moreover, the ratio of total nitrogen to lignin is nearly the same in the rumen as in the fodder at all times after feeding so net loss of nitrogen from the rumen occurs only with lignin passage to the lower alimentary tract. McDonald (1954) pointed out that the insoluble protein, zein, does not alter the ammonia concentration in the rumen while the soluble proteins, casein and gelatin, cause marked increases. When sheep were fed a partially purified ration to which zein contributed 94% of the total nitrogen, approximately 40% of the zein was converted to microbial protein.

Using steers fed natural and purified rations, Agrawala et al. (1953) and Duncan et al. (1953) studied protein synthesis by the rumen passage technique of Hale et al. (1940, 1947). Minimum values for protein synthesis varied from 33 to 109 gm. while considerable amino acid synthesis was demonstrated on the ration containing only non-protein nitrogen. Smith (1953) confirmed the synthesis of lysine utilizing the same technique with natural rations, but could not demonstrate an accumulation of methionine in the rumen.

McNaught (1951) found that cooked starch provided a better source of energy for protein synthesis than raw starch; arabinose, xylose, fructose, and cellobiose were particularly good energy sources with maltose and glucose ranking as intermediate; glucuronic, acetic, propionic, butyric, citric, malic, tartaric, fumaric, succinic, lactic, and beta-hydroxybutyric acids and

mannose or sorbose were either poor or ineffective sources. Bacterial protein accounted for 7 to 18% of the carbon of maltose, arabinose, or xylose fermented in vitro by rumen microbiota. Arias et al. (1951) noted that small amounts of soluble carbohydrates (0.1 to 0.2 gm./100 ml. volume in the fermentation flask) aided cellulose digestion which in turn yielded energy for protein synthesis. Salsbury (1955) confirmed this stimulatory effect of 0.1 to 1% soluble carbohydrates.

A low rate of sugar and cellulose fermentation by ruminal contents from sheep fasted 48 hours was reported by Quin et al. (1951). The fermentation ability reverted to normal faster with a low protein ration than with a high protein ration. Bell et al. (1953) studied retention of urea nitrogen by steers fed different carbohydrates concluding that urea nitrogen was utilized with equal efficiency in rations of different cereal grains but with less efficiency in molasses containing rations.

Walter (1952) studied the effect of a number of carbohydrates on the magnitude of rumen fermentation in vitro and found that activity with sucrose substrate exceeded slightly that with glucose which was several times greater than activity with xylose, hemicellulose, and starch substrates. When barley supplemented the alfalfa ration of the ovine source of rumen microbiota, fermentation on all substrates was intensified. According to Reid (1953), efficient protein synthesis from

non-protein nitrogen depends upon an optimal balance between ammonia liberation and carbohydrate fermentation.

Reed et al. (1949) found that the digestibility and biological value of rumen bacterial protein for rats are 62 to 65% and 79 to 80% compared to 101% and 80% for casein. Crude protein constituted 48 to 51% of the dry matter of the bacterial preparation. McNaught (1954) confirmed these values and found a digestibility of 91% for the protein of rumen protozoa.

The amino acid composition of rumen bacterial protein, studied by Holmes et al. (1953), varies little if any with the change from dry feed to pasture. The amino acid composition of rumen bacterial protein is similar to recorded values for a number of microorganisms excepting for a somewhat higher content of arginine, histidine, tryptophane and glutamic acid and lower content of isoleucine. The content of leucine, threonine, phenylalanine and particularly methionine and isoleucine was sub-optimal for maximum nutritional value as also noted by Reed et al. (1949). Duncan et al. (1953) confirmed the low methionine content of rumen bacterial protein but found somewhat higher proportions of isoleucine, leucine, phenylalanine and threonine with lower proportions of arginine, histidine and tryptophane. The methionine content of rumen dry matter was very low as reported by Chance et al. (1953b) and Smith (1953).

Investigations of the sulfur amino acid requirements of ruminants began when Huffman and Duncan (1940) failed to

stimulate milk production with cystine supplementation to a legume hay ration. However, Smuts et al. (1941) found that supplementation of alfalfa with cystine increased retention of the alfalfa nitrogen in lambs. Alfalfa contains 0.3% sulfur according to Davis et al. (1954) and Lewis (1954) cites values for sulfate-sulfur in forages ranging from 0.3 to 1.0% of the dry matter. All of these are high values compared to other feeds. Loosli and Harris (1945), Jones and Haag (1946), Starks et al. (1954) and Noble et al. (1955) have demonstrated increased nitrogen retention and/or growth or production when sulfur supplemented basal rations containing either less than 0.1% or unspecified amounts of total sulfur. Methionine sulfur is slightly more valuable than inorganic sulfur according to Loosli and Harris (1945). Utilization of field pea or alfalfa protein by sheep was stimulated by methionine supplementation as reported by Klosterman et al. (1954) who noted that the proteins of these feeds are deficient in methionine. Hunt et al. (1954) reported a greater in vitro activity of rumen microbiota when methionine or sodium sulfate supplied their sulfur than when cystine or elemental sulfur served this function.

Block and Stekol (1950), Block et al. (1951), and Block (1953) demonstrated conversion of ingested radioactive, inorganic sulfate to cystine and methionine in the rumen. The milk protein, which also contained radioactive cystine and methionine, attained a maximum activity in 2 to 3 days. The

ratios of cystine nitrogen-methionine nitrogen in rumen microbiota, serum albumin, and milk protein were about 1:1, 6:1 and 1:5 respectively and the ratios of cystine activity:methionine activity followed the same pattern. Methionine and cystine were apparently formed in equal amounts in the rumen. Since Lewis (1954) found that sulfate disappears from the rumen with a half-time of less than 2 hours accompanied by a simultaneous increase in rumen sulfide, sulfate utilization must proceed largely via sulfide. Rumen microbiota degrades cysteine to ammonia, sulfide, CO_2, and acetic acid according to Sirotnak et al. (1953) and Lewis (1954).

Rumen sulfate and sulfide concentrations are normally about 0.15 and 0.08 milligram equivalents/100 ml. as reported by Patel (1952) and Lewis (1954). Weir and Rendig (1954) report serum inorganic sulfur values of 0.02 milligram % increasing to 4 milligram % as sulfur ingestion was increased.

About 70% of the inorganic, radioactive sulfate fed with silage to cows by Keener et al. (1953) was recovered in the urine along with 1 to 2% in the milk and the remainder in the feces. Kulwich et al. (1954) obtained a similar sulfate partition in sheep fed soybean meal, but when urea provided the dietary nitrogen, only half of the activity appeared in urine indicating increased incorporation by rumen microbiota.

The reviews by Bersin (1950) and Fromageot (1953) reveal the central role of sulfur and sulfur amino acids in metabolism. This is particularly true for cysteinsulfinic acid which appears

to be a key intermediate among several metabolic acids and amino acids. These amino acids include hypotaurine and taurine which are urinary excretion products and are also synthesized by the chick embryo (Chatagner et al., 1955; Lowe and Roberts, 1955; Mačklin et al., 1955). Their characteristics are similar to the unidentified compounds synthesized by bacteria noted by Cowie et al. (1950, 1951a). Block et al. (1952) discuss the role of glutathione in transfer of amino acids to protein.

Horowitz (1947) isolated cystathionine and showed that Neurospora synthesizes methionine from inorganic sulfur via cysteine, cystathionine, and homocysteine consecutively. Cowie et al. (1950, 1951a, 1952), Bolton et al. (1952), Wijesundra and Woods (1953), and the work of others reviewed by them showed that the same general mechanism applies in other microorganisms. Hess (1952), Arnstein (1953) and their literature reviews show essentially the same mechanism in rat tissue only reversed; that is, methionine goes to homocysteine to cystathionine.

To whatever extent Escherichia coli metabolism parallels that of rumen microbiota, the following facts found by Cowie et al. (1950, 1951a, 1952, 1954), and Bolton et al. (1952) apply to this problem: Inorganic sulfate of media and cells is in instantaneous equilibrium while the organic, cell-bound sulfur amounts to 3.5 milligrams S/ml. centrifuged cells and is stable; inorganic sulfate is not bound by non-growing cells and sulfur incorporation by growing cells is directly correlated

with protein synthesis provided adequate sulfur is present; glutathione accounts for 25 to 30% of the organic, cell-bound sulfur and acts as reserve sulfur for cell growth in the absence of an external supply; cysteine and sulfite sulfur markedly suppress while methionine and homocysteine sulfur only slightly suppress incorporation of sulfate sulfur into cells; it is concluded that sulfate sulfur is incorporated as cysteine via sulfite while methionine is formed more slowly via cysteine.

PART I

INCORPORATION OF INORGANIC SULFATE BY RUMEN MICROBIOTA IN VITRO

Preliminary Experiments

Fermentation Technique. This experiment was initiated in 1953 with the object of measuring cell growth and proliferation during *in vitro* fermentations by rumen microbiota. This initial study utilized the fermentation method described by Salsbury (1955, pp. 73-76) with the mineral complex modified by substituting chloride salts for the equivalent sulfates. About 0.8 gm. of air dry alfalfa leaf meal was placed inside the dialyzing sacs with 25 ml. of cheese-cloth strained rumen liquid from fistula steer C741 receiving an alfalfa hay ration. Caution was used in this and the following experiments to avoid delay, temperature change, and air exposure in transferring inocula from the rumen to the fermentation vessel. These inocula were always collected about 2 hours after feeding. Following inoculation, the dialyzing sac was suspended in 175 ml. of mineral complex containing about 2.5 microcuries of S^{35}-labeled inorganic sulfate/ml. The carrier sulfate concentration was less than 4.5×10^{-9} moles/ml. One complete assembly was incubated at 40°C. for each time of 1, 2, 4, and 6 hours and two complete assemblies were refrigerated as controls.

After incubation, the residual inorganic sulfate activity was exchanged out by dialysis against dilute ammonium sulfate at 6°C. Thick smears of the dialyzed fermentation residue were shielded so a constant area was exposed and counted under an end-window, G-M detector. These counts are presented in Table 1, page 40.

The control flasks, designated as 0 and 0', were exposed to the radioactive mineral mix for 1 and 3 hours respectively before exchange against ammonium sulfate. The activity in the controls was apparently inorganic sulfate as demonstrated by the chromatographic results. This demonstrated incomplete exchange with non-labeled sulfate in spite of the fact the ammonium sulfate solutions were changed repeatedly until practically no exchange activity could be detected. For this and other technical reasons, the dialyzing sac was not used after this trial. A supplemental experiment demonstrated that inorganic sulfate equilibrates across these visking tubing dialyzing sacs at a rate of 2% per minute. Therefore, the apparent rate of sulfate incorporation is a function of both the rate of binding by the cells and the rate of sulfate diffusion into the fermentation sac.

The remaining fermentation residues were hydrolyzed with 6M HCl, the excess acid removed by evaporation, and the amino acids separated chromatographically on filter paper as described by Patton (1950) with the exception that larger paper and cylinders were used. The developed chromatograms were cut

TABLE 1

SULFUR ACTIVITY IN DIALYZED FERMENTATION RESIDUES

Incubation Time (Hours)	Radioactivity in the Cells		[3]Relative Activities	
	[1]Net	[2]Relative	Cystine	Methionine
0	11.13	----	----	----
0'	23.03	----	0	0
1	14.88	41.9	4.7	0
2	23.66	66.7	8.2	5.8
4	34.00	95.8	48.7	9.0
6	35.49	100.0	26.5	11.9

1. Counts per second corrected for natural background activity.
2. Each count as its percentage of the six hour count.
3. Area under curves obtained by graphing activity versus R_f value.

into strips and the strips counted as described by Winteringham et al. (1952). Methionine yielded an R_f of 0.85 under these conditions. While all of the strips from incubated flasks contained appreciable activity near R_f 0.8 and R_f 0.2, over 95% of the activity in the controls was below R_f 0.2. Although this conclusively demonstrated methionine synthesis, cystine was obliterated by inorganic sulfate. The chromatographing process was repeated using a solvent mixture of 80% methanol, 18% water, and 2% glacial acetic acid which gave an R_f of 0.7 for methionine and an R_f tailing from 0.5 for cystine. Inorganic sulfate did not move above R_f 0.2. The relative activities corresponding to methionine and cystine are presented in Table 1, page 40.

Hydrogen Sulfide Production. This experiment was conducted to measure any loss of radioactive sulfur as free hydrogen sulfide. A 25 ml. portion of cheese-cloth strained rumen liquid from hay fed rumen fistula cow A55 was added to each of two 40 ml. centrifuge tubes containing 1 gm. of alfalfa leaf meal and 750 microcuries of S^{35} as 1×10^{-7} moles of inorganic sulfate. The tubes were closed with stoppers containing capillary tubes joined to a manifold. Air was drawn through the manifold at a slightly reduced pressure and washed through cupric acetate. After 3 hours incubation at 40°C., the fermentation was stopped by cooling and the sulfide oxidized to sulfate with hydrogen peroxide. The cupric oxide-cupric sulfate residue was then dissolved in hydrochloric

acid. This solution contained less than 0.4 microcuries of S^{35}.

Glutathione Production. This experiment was concerned with the form in which the radioactive cystine and cysteine occurred in the cells. Twenty-five ml. portions of cheese-cloth strained rumen liquid from rumen fistula steer C707 (receiving a restricted roughage-high concentrate ration) were added to 50 ml. Erlenmeyer flasks containing 0.73 gm. of concentrate mixture. Sufficient S^{35}-labeled sulfate was added to give an activity of 7.4 microcuries/ml. One flask was then incubated at 40°C. for each of the following times; 0, 0.5, 1, 1.5, 2, 3, 4 and 6 hours.

The fermentation was stopped by pouring the ferment into sufficient ethanol to yield a final concentration of 80% ethanol. After addition of 0.002 moles of sulfuric acid and 4 to 6 milligrams of cysteine hydrochloride as carrier, the protein was removed by filtration. Sulfhydral compounds were precipitated from the filtrate as their cadmium salts (Waelch and Rittenberg, 1941) and the sulfhydral compounds redissolved by precipitating cadmium as its sulfide. The resulting solution was assayed for radioactivity. The protein was hydrolyzed and assayed for radioactivity along with the combined filtrates. The non-protein sulfhydral compounds, recovered in this manner, accounted for 2.6 to 3.7% of the total activity regardless of incubation time. The activity of the protein hydrolysates after removal of inorganic sulfate ranged from 2.4% of the

total activity after 0.5 hour incubation to 19.4% after 4 hours incubation.

The sulfhydral compounds were separated chromatographically on paper by the method of Miller and Rockland (1952). Strong ninhydrin reactions were obtained at the positions occupied by cystine and glutathione with a fainter reaction at the cysteine location. S^{35}-activity was largely concentrated at the glutathione location, but activity as inorganic sulfate coinciding with the cysteine location made a quantitative interpretation impossible.

Microradioautographs. This experiment was undertaken to visually demonstrate incorporation of sulfate into the cells of rumen microbiota and to distinguish the morphological types responsible for this incorporation.

A 25 ml. portion of cheese-cloth strained rumen liquid from rumen fistula steer C707 (receiving a restricted roughage-high concentrate ration) was added to a 40 ml. centrifuge tube containing 0.73 gm. of the concentrate mix and 2×10^{-7} moles of S^{35}-labeled inorganic sulfate containing 0.6 millicuries of radioactivity. This tube was then incubated for 3.5 hours at 40°C. and the fermentation stopped by adding 10 ml. of M/10 $(NH_4)_2SO_4$ in 0.5M aqueous NaF. The tube was cooled in ice water, centrifuged for 30 minutes at 1,240 x G.; the residue was resuspended in 30 ml. of the sulfate-fluoride solution, and centrifuged for 30 minutes at 1,240 x G. A small amount of the white, cell-containing layer on top of

the residue was removed with a transfer loop and spread on a microscope slide. A control tube containing S^{35} was processed in exactly the same way except that the fluoride-sulfate solution was added immediately after inoculation and then this tube was refrigerated while the experimental tube was incubated.

The smears of incubated and control cells were flame fixed and then the slides were suspended in M/100 H_2SO_4 in 80% ethanol for 15 minutes followed by rinsing with distilled water. Before the washing procedure, both smears yielded over 10,000 counts per minute with a defined counting procedure, while after washing no activity could be detected in the control smear and the smear of incubated cells still yielded over 10,000 counts per minute.

These smears were then stained by the method of Gram and cross-hatched by cutting strips about 1 millimeter wide out of the smear at 2 millimeter intervals. This left a series of small rectangles with 4 to 20 oil immersion fields per rectangle which made it possible to accurately locate a given field. These smears were then clipped in apposition to nuclear track plates (NTB)*, exposed for 1 week and the NTB-plates developed as described by Fitzgerald et al. (1953). He states that this fine grained photographic emulsion produces a latent image portraying the radiation source on the smear with reasonable fidelity on the cytological level. However, in this work

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*NTB-5 microns; Emulsion No. 497, 943-52; Eastman Kodak Company; Rochester, New York.

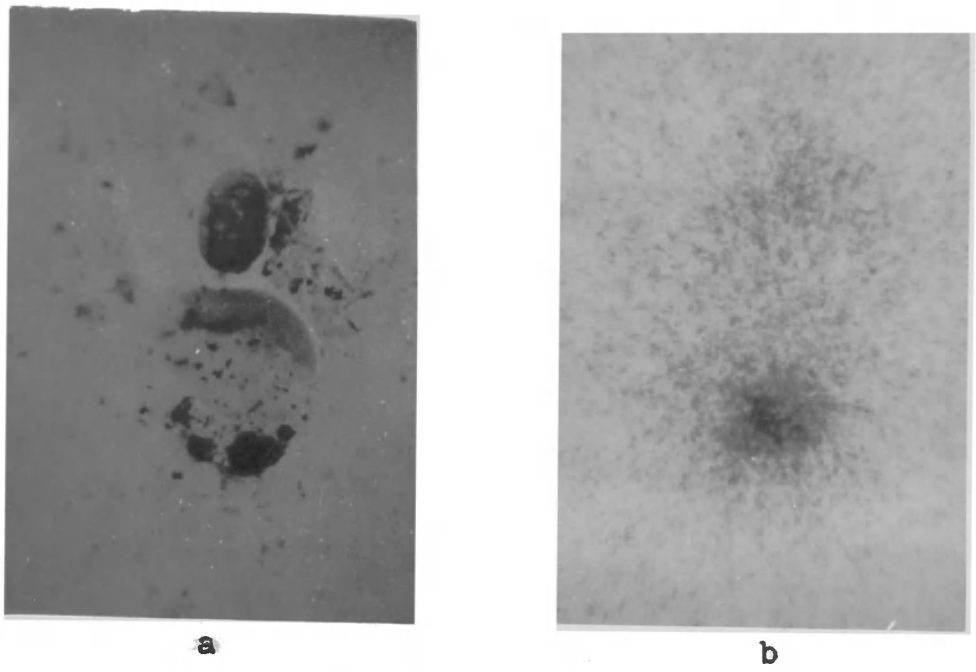
the edges of the image are blurred since a 5 micron thickness cannot be sharply focused under the oil immersion objective.

Figure 1, page 46, portrays a field from the microbial smear, the image this field produced on an NTB-plate and a representative field from the NTB-plate which was exposed to the control smear. It can be concluded that many of the rumen microorganisms incorporated radioactive S^{35} as a result of the in vitro fermentation and that the larger bacteria and protozoa either did not incorporate the S^{35} or the incorporation was localized yielding an image appearing much like small bacteria. Resolution was not adequate to distinguish bacterial morphology and many of the smaller rumen microorganisms do not stain or photograph well.

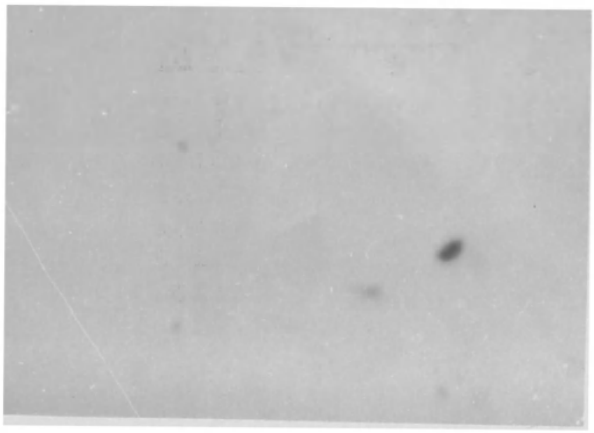
On the basis of these preliminary findings, an investigation of the effect of roughage level in the ration on incorporation of inorganic sulfate by rumen microbiota was undertaken.

Principal Experiment

Procedure. Inocula for use in these studies were obtained from 3 mature steers (C707, C741, and CS126) and 2 mature twin cows (T1 and T2) which were fitted with the plastic fistula plug described by Hentschl et al. (1954). C707, CS126, and T1 received a restricted roughage-high concentrate ration wherein 3 to 6 pounds of alfalfa hay represented the roughage and the concentrate was represented by 10 to 22 pounds of a mixture containing 77% ground corn, 20% soybean oil meal, 1% Ca_2HPO_4,



a b



c

Figure 1. Microradioautographs

a. A field from the smear of incubated microorganisms, Gram stain. (x470)
b. Latent image produced by the field in a on an NTB-plate. (x470)
c. Latent image produced by a representative field on the smear of control microorganisms. (x470)

1% NaCl, 1% $CaCO_3$ plus vitamin A and irradiated yeast. C741 and T2 received an all hay ration consisting of 30 to 34 pounds of alfalfa. Inocula were obtained by straining contents from the ventral portion of the rumen through cheese-cloth into flasks previously flushed with CO_2, warmed to 40 to 43°C, within an insulated carrying case.

In the first 2 trials, 25 ml. portions of inocula from the all hay or high concentrate fed donors were transferred with a graduated cylinder to a series of 50 ml. Erlenmeyer flasks containing 1 gm. of alfalfa leaf meal as substrate for the all hay inocula, or 0.73 gm. of the concentrate mixture for the high concentrate inocula. These fermentation flasks had previously been flushed with CO_2, warmed to 40°C. and about 0.1 ml. of S^{35}-labeled inorganic sulfate in about 0.05M HCl added, giving an activity of about 10 microcuries/ml. The carrier sulfate amounted to less than 1×10^{-7} moles per flask.

Following inoculation, the flasks were stoppered, rotated to mix, and placed in a 40°C. water bath. At 15 minute intervals, the contents of the flasks were rotated to mix and pressure relieved by briefly loosening the stoppers. Approximately 3 minutes were required to inoculate the entire series of flasks. The order of inoculation and the appropriate zero time for incubation was noted. One flask for the hay series and one for the high concentrate series was removed at each of the following times: 1, 1.5, 2, 2.5, and 3 hours. After incubation, the contents from these flasks were immediately

poured into 80 ml. of alcohol and then processed as described in Table 2, page 49.

In the second 2 trials, 25 ml. portions of inocula from the all hay (T1), or high concentrate (T2) fed donors were handled in the same manner except that all flasks contained 0.87 gm. of a mixture of the concentrate and alfalfa leaf meal in proportions of 0.73/1. Also, 0.5% of tympanol* was added to the inocula to facilitate handling. The radioactivity as S^{35}-labeled inorganic sulfate in these trials was about 27 to 40 microcuries per ml. An 0.5 hour incubation time was also included in the second 2 trials. Assays for the inorganic, water-soluble sulfate content of the inocula and substrates by the method of Lewis (1954) showed that the rumen liquid from T1 contained 5.4×10^{-8} moles/ml., that from T2 contained 11.0×10^{-8} moles/ml., the alfalfa leaf meal contained 2.9×10^{-5} moles/gm., and the concentrate mixture contained 7.9×10^{-7} moles/gm.

The chemical procedures are described in Table 2, page 49. Selection of 80% ethanol as the protein precipitant was based on the findings of Pearson and Smith (1943a).

Assays for radioactivity were conducted by placing 3 ml. of the solution of known volume in a 5 ml. beaker under a G-M detector with a window thickness of less than 2 milligrams per square centimeter. The same geometry and tube was used

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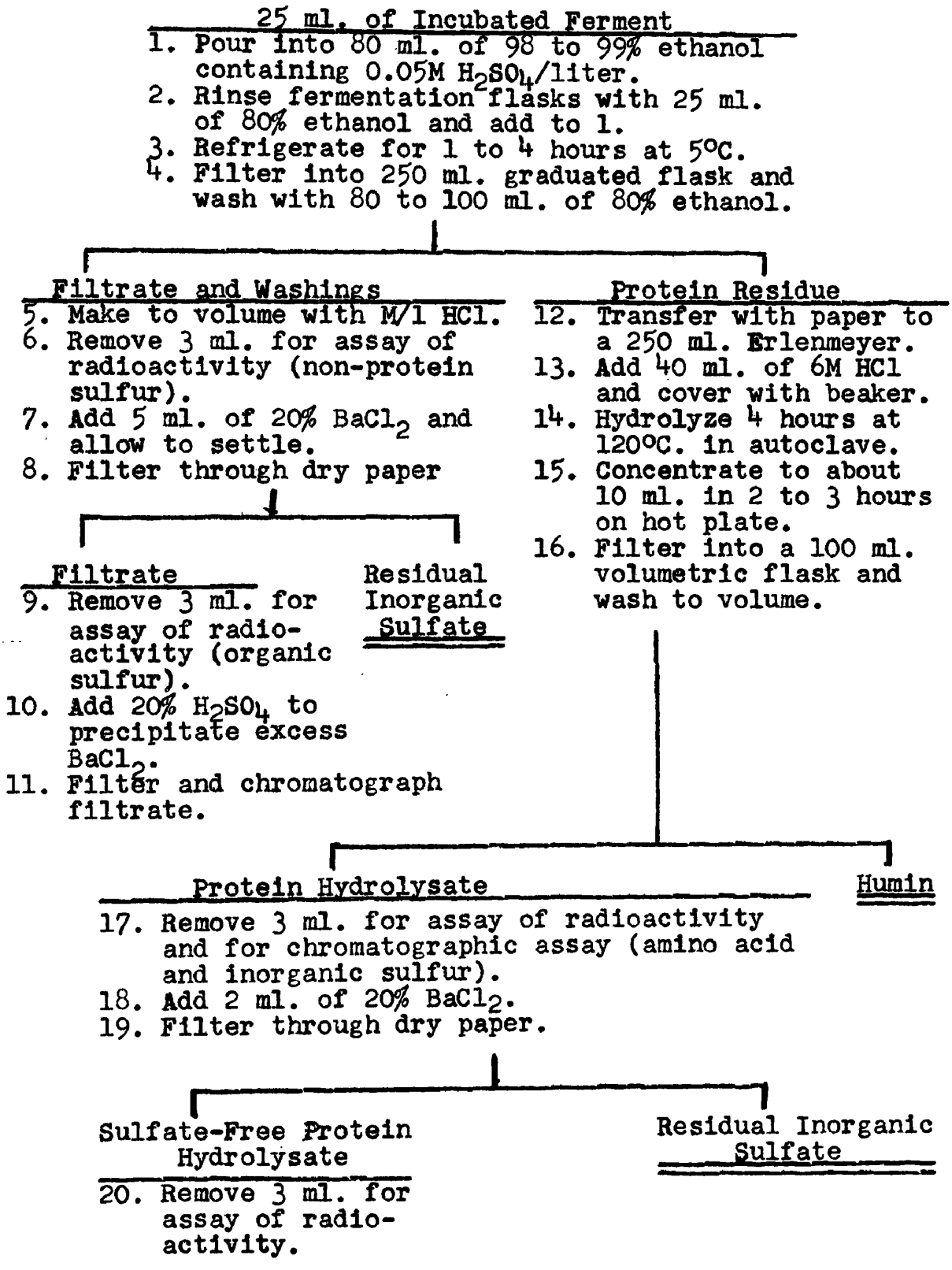
*Silicone anti-foam preparation; Jen-Sal Laboratories; Kansas City, Missouri.

TABLE 2

FLOW DIAGRAM FOR PARTITION OF S^{35}-ACTIVITY



throughout each trial. Corrections for background activity were determined at 1 to 2 hour intervals and subtracted from the raw counts to obtain the net counts. The net counts per second (C.P.S.) obtained in this manner were proportional to the concentration of S^{35} in microcuries/ml. No differences in self-absorption could be detected between the solutions used in these experiments. Net C.P.S. times the ml. of solution in a given fraction was then recorded as the relative activity in that fraction after application of appropiate dilution factors. The combined activity from steps 6 and 17 of Table 2, page 49, constitutes the total recovered activity of which the other fractions were expressed as a percentage.

Chromatographic separation of the amino acids in steps 11 and 17 of Table 2 was carried out on filter paper by the method of Patton (1950). The activity on the chromatograms was assayed as described by Winteringham (1952) and the activity of the liquid fractions partitioned according to the distribution of activity on the chromatograms.

Results. The total incorporation of labeled-sulfate into the organic sulfur fractions is presented in Table 3, page 51. The exponential equations fitted to these values graphically and the statistics applied to these equations as described by Stevens (1951) are presented in Table 4, page 52. Figure 2, page 53, visually presents the same equations. Table 5, page 54, shows the partition of incorporated activity of the chromatographic fractions. These fractions were not chromatographically

TABLE 3

TOTAL INCORPORATION OF LABELED-INORGANIC SULFATE

Incubation Time (t hours)	$\frac{\text{[1]Organic Activity}}{\text{Total Recovered Activity}}$ (100) = A_t	
	Hay Inoculum	Grain Inoculum
Means of first 2 trials with individual substrates:		
1.0	2.6	4.3
1.5	3.0	12.6
2.0	8.1	12.2
2.5	7.8	11.4
3.0	6.2	15.4
Means of second 2 trials with mixed substrates:		
0.5	6.39	3.78
1.0	16.14	5.80
1.5	13.29	5.18
2.0	18.68	7.35
2.5	24.79	7.63
3.0	27.03	9.88

1. The combined activities from steps 9 and 20 of Table 2.

TABLE 4

ASYMPTOTIC REGRESSION EQUATIONS FOR INCORPORATION OF SULFATE

Equation Form: $A_t = A_1 - A_1 e^{-rt}$

A_t = % of total activity which is incorporated at time t

A_1 = % of the total activity which would be incorporated in an infinite time (the asymptote)

e = base of the natural system of logarithms (2.71828)

r = the rate of incorporation of inorganic sulfate into organic sulfur

t = hours of incubation

(100)r = %/hour conversion of inorganic sulfate remaining to be converted to organic sulfur at that time.

Ration of Inoculum Donor	Substrate	A_1	r	D.F.	Standard Error of Estimate	Standard Error A_1	Standard Error r
All Hay	Alfalfa Meal	10.5	0.4	3	1.79	1.4	0.7
All Hay	Alfalfa Meal and Concentrate	37.5	0.4	4	2.88	2.0	1.1
High Concentrate	Concentrate	16.5	0.6	3	2.74	2.2	1.1
High Concentrate	Alfalfa Meal and Concentrate	11.6	0.52	4	1.09	0.8	0.4
Mean of All Treatments		----	0.48	14	----	----	0.45

Figure 2. Asymptotic Curves For Incorporation of Sulfate

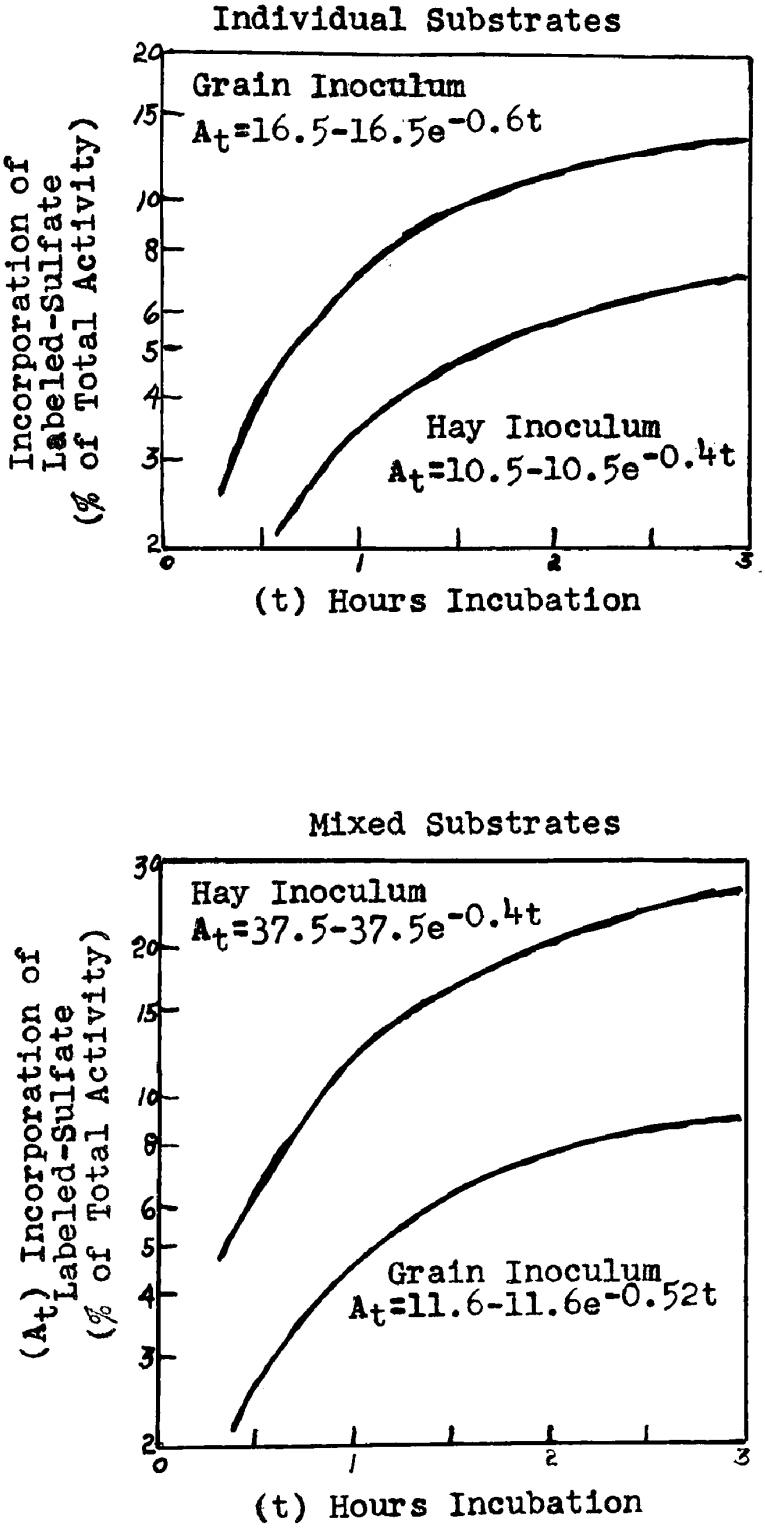


TABLE 5

ACTIVITY RECOVERED IN ORGANIC FRACTIONS AS PERCENT OF THE TOTAL RECOVERED ACTIVITY

Incubation Time (Hours)	Cysteine /Cystine	Methionine	[1]Unidentified
Grain Inoculum-Mixed Substrate:			
0.5	2.85	0.48	0.45
1.0	3.91	1.13	0.78
1.5	3.04	1.31	0.83
2.0	4.48	1.88	1.00
2.5	3.99	2.48	1.16
3.0	4.06	3.54	2.30
Hay Inoculum-Mixed Substrate:			
0.5	4.87	0.76	0.78
1.0	12.32	2.13	1.68
1.5	8.89	2.71	1.69
2.0	11.74	4.37	2.56
2.5	16.04	5.63	3.14
3.0	13.77	7.89	5.39
Total----------	90.0	34.3	21.8
Percent Distribution---	61.6%	23.5%	14.9%

Incubation Time (Hours)	Cysteine /Cystine	Methionine/ [1]Unidentified
Grain Inoculum-Individual Substrate:		
1.0	2.6	1.8
1.5	8.1	4.5
2.0	7.2	5.1
2.5	8.8	2.6
3.0	9.0	6.5
Hay Inoculum-Individual Substrate:		
1.0	1.5	1.2
1.5	2.4	0.6
2.0	5.3	2.8
2.5	3.1	4.7
3.0	4.3	2.0
Total----------	52.3	31.8
Percent Distribution------	62.2%	37.8%
Mean of All Trials--------	61.9%	38.1%

1. R_f is greater than 0.9 on paper chromatography with 80% Phenol.

pure and contained smaller amounts of activity as unidentified compounds.

PART II

PRODUCTION AND ABSORPTION OF ACETIC, PROPIONIC, AND BUTYRIC ACIDS IN THE BOVINE RUMEN

Procedure

Twin Holstein cows, T1 and T2, weighing 1050 and 1110 pounds and a Guernsey steer, C707, were used in this experiment. The steer weighed 1420 pounds when used for the restricted roughage trial and 1500 pounds for the all hay trial. All three animals were fitted with screw cap, plastic fistula plugs described by Hentschl et al. (1954) which facilitated sampling of the rumen contents. T1 was in the second month of lactation and T2 was dry.

The experimental rations were fed twice daily at 8 A.M. and 3 P.M. for at least three weeks before each trial. Alfalfa hay was used for the roughage and the concentrate mixture was the same as described in the procedures for Part I. For the restricted roughage trials, T1 received a ration of 28 pounds of concentrate plus 4 pounds of hay and C707 received 10 pounds of concentrate plus 4 pounds of hay. For the all hay trials, T2 received a ration of 30 pounds of hay and C707 received 24 pounds of hay.

On the days of the experiment, feeding was restricted to 2 hours for the all hay trials and 3/4 hour for the high concentrate-restricted roughage trials. Water was permitted at

will during the next 24 hours. During the feeding period, T1 ate 11 pounds of concentrate and 2 pounds of hay, T2 ate 8 pounds of hay, C707 ate 5 pounds of concentrate and 2 pounds of hay for the restricted roughage trial and 6 pounds of hay for the all hay trial.

The time halfway between initiation and termination of feeding was taken as 0 hour. The sample recorded as 0 hour was taken just prior to feeding. Samples of the liquid in the middle of the ventral sac of the rumen were taken at definite intervals after 0 hour and strained through cheesecloth into a volumetric flask containing 50% sulfuric acid equal to 3% of its volume. This volume of sulfuric acid lowered the pH to 1.5 to 2.0 and yielded a final sample containing about 91.3% rumen liquid on a dry matter free basis. Most of the suspended solids in the initial sample were precipitated by the sulfuric acid. These samples were stored at 6°C. until analyzed which was always less than a month.

Acetic, propionic, and butyric acids were determined by direct chromatography of rumen liquid on silica gel as described by Harper (1953) with modifications. Thirteen gm. of dry silicic acid (Mallinkrodt special chromatographic No. 2847, further graded by suspending 2 times in an equal volume of water, allowing to settle 30 minutes and discarding the supernatant) and 7.5 ml. of 1.6M potassium phosphate pH 6.3 buffer were thoroughly mixed in a mortar and then about 50 ml. of chloroform (U.S.P. washed 3 times with an equal volume of water and

filtered to remove suspended water) were used to form a slurry. This slurry was poured into a 38 millimeter diameter column with a fritted glass filter overlaid with filter paper to form the bottom. The column was tapped to remove air bubbles and then kept covered with chloroform added slowly from a separatory funnel. A filter paper disc 38 millimeters in diameter was placed on the buffer section. The sample section was not added until the chloroform level was about 1 millimeter above this paper.

The sulfuric acid section of Harper (1953) was omitted since it had no effect on the results in this experiment. The sample section was prepared by weighing 6 to 7 gm. of rumen liquid directly on to 10 gm. of dry silicic acid on a watch glass. The silicic acid and sample were then quantitatively transferred to a mortar and thoroughly mixed. Fifty ml. of chloroform were used to form a slurry and used as a vehicle to transfer this section to the column.

Eluate fractions of appropiate size as determined by preliminary trials were collected starting immediately after adding the sample section. The flow rate was about 150 ml./ hour. Four columns were run simultaneously. Further additions of solvent were made using a separatory funnel adjusted to keep 5 to 10 ml. of solvent covering the column. A 50 ml. addition of pure chloroform was followed by 50 ml. portions of 1%, 2.5%, 5%, 10% and 20% n-butanol in chloroform solutions. Additional 20% n-butanol in chloroform was used to give a total eluate

volume of 500 ml. Blank determinations were made with each batch of reagents and appropiate corrections made.

Results

The chromatographic procedure was tested with pure acids alone, and added to rumen liquid. Recovery in 4 trials with 0.5 to 3.8 millimoles of added acetic acid was 93% with a standard deviation of 8%. Recovery of 0.2 to 0.5 millimoles of butyric acid was similarly 96±6%. Threshold volumes for butyric, propionic, and acetic acids were about 70, 200, and 280 ml., but varied somewhat with changes in reagents and the amount of short chain acid applied to the column. The values reported as butyric acid could include traces of higher acids.

Figure 3, page 60, portrays the regression of rumen concentrations on time after feeding for acetic, propionic, and butyric acids. Table 6, page 61, lists the estimated amounts per 100 ml. of rumen liquid of acetic, propionic, and butyric acids produced from each pound of total digestible nutrients ingested as the all hay ration or the high concentrate ration on the day of the experiment. It was assumed that the hay contained 50% total digestible nutrients and the concentrate 75%. Table 7, page 61, gives the rates at which the acids disappeared from the rumen. The regression equations are summarized in Table 8 (Appendix) and Table 9 (Appendix) gives the concentrations of acetic, propionic, and butyric acids for each cow, ration, and sample time.

Figure 3. Concentration of Short Chain Acids in the Rumen After Feeding.

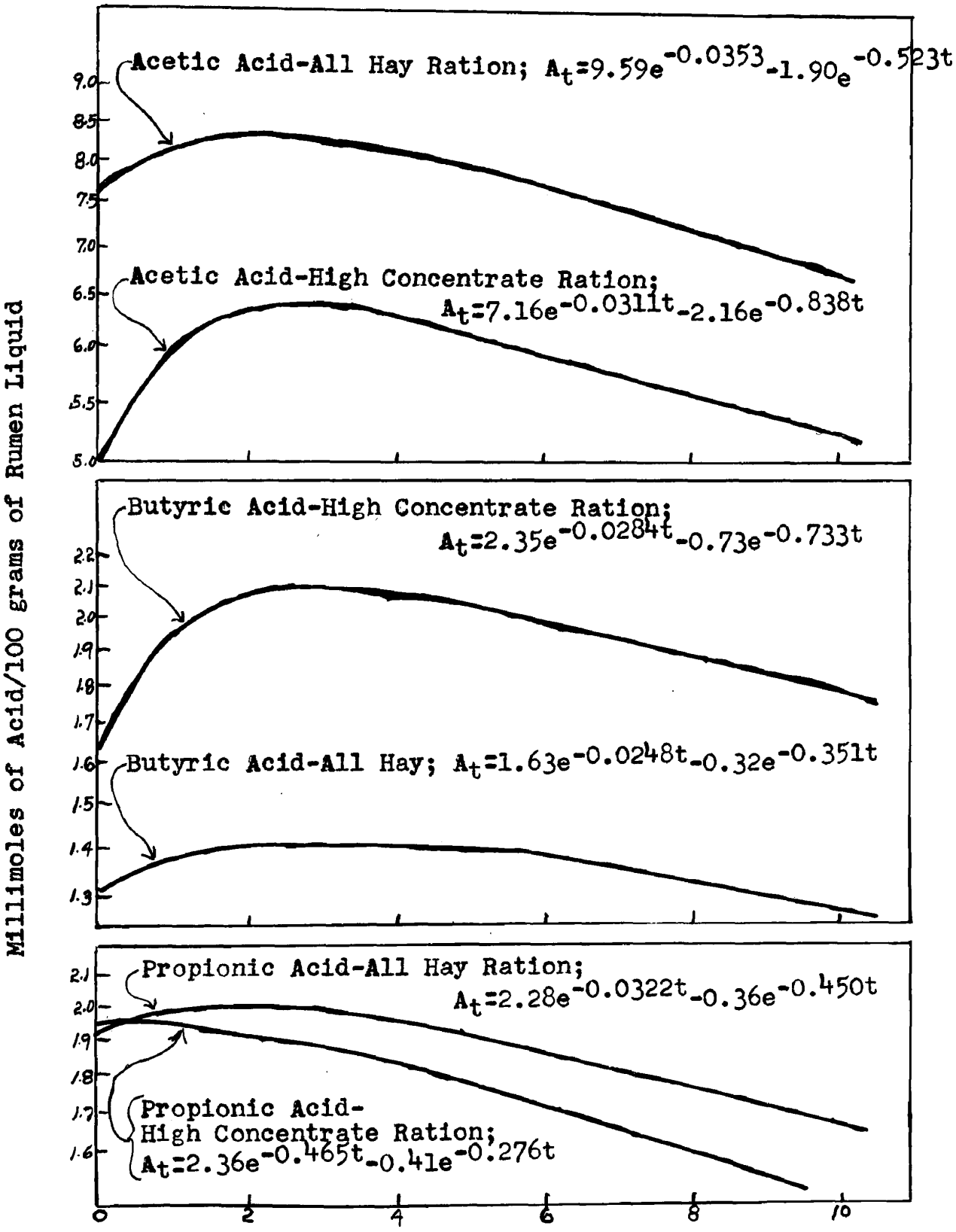


TABLE 6

THE AMOUNT OF ACETIC, PROPIONIC, AND BUTYRIC ACIDS PRODUCED FROM ONE POUND OF TOTAL DIGESTIBLE NUTRIENTS

Acid	All Hay Ration		Restricted Roughage -High Concentrate Ration		Pooled Data From Both Rations
	[1]mM/100g.	%	[1]mM/100g.	%	[2]mM/100g.
Acetic	0.52	68.4	0.30	65.2	.24-.67
Propionic	0.15	19.7	0.06	13.0	.02-.26
Butyric	0.09	11.8	0.10	21.7	.04-.21
Total	0.76	----	0.46	----	----

1. Millimoles of acid/100 grams of rumen liquid.
2. Range at 95% confidence level.

TABLE 7

THE DISAPPEARANCE RATES FOR ACETIC, PROPIONIC, AND BUTYRIC ACIDS FROM THE RUMEN

Acid	All Hay Ration		Restricted Roughage Ration		Pooled Data From Both Rations	
	%/Hr.	[1]S.E.	%/Hr.	[1]S.E.	%/Hr.	[1]S.E.
Acetic	3.5	0.4	3.1	0.5	3.3	0.3
Propionic	3.2	0.5	4.6	0.9	---	---
Butyric	2.5	0.5	2.8	0.5	2.7	0.3

1. Standard Error.

The type equation used to describe the data is:

$$A_t = A_o e^{-k_1 t} - A_p e^{-k_2 t}$$

where, A_t = the concentration of the acid in millimoles per 100 gm. of rumen liquid;

A_o = the disappearance regression extrapolated to 0 time; this could represent the amount of acid which would be present if the fermentation were completed without any disappearance of the acid;

A_p = $(A_o - A_t)$ when t is 0; this should represent the amount of acid produced from the current feeding in millimoles/100 gm. of rumen liquid;

e = base of the natural system of logarithms (2.71828);

k_1 = rate of disappearance of the acid from the rumen; $100k_1$ = %/hour disappearance of the acid present at any given time;

k_2 = rate of acid production approaching A_p which would represent the rate of conversion of fermentable feed into its acid product;

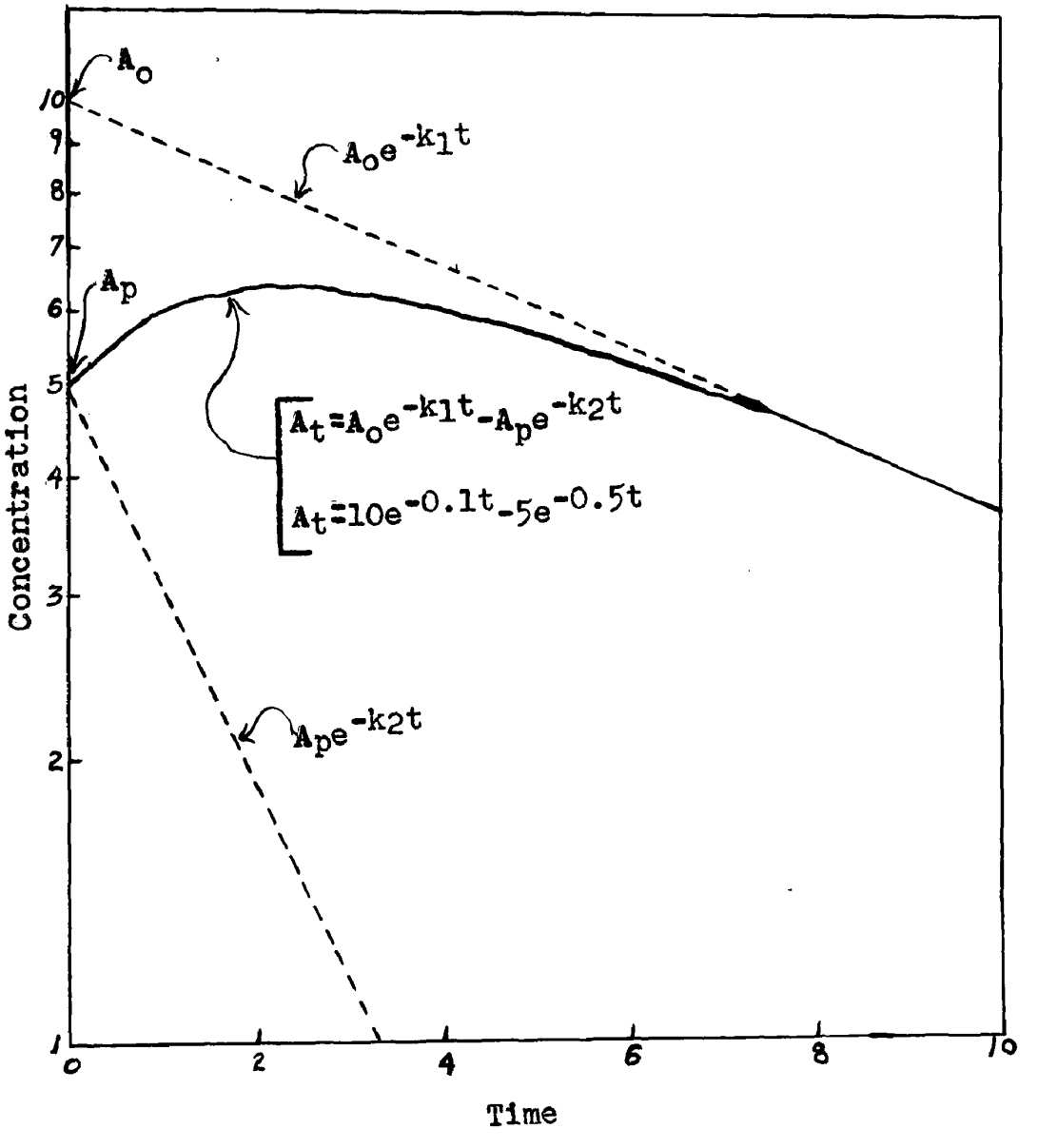
t = time after feeding in hours.

A graphic portrayal of this equation is given in Figure 4, page 63.

The natural logarithms of the acetic acid concentrations from 3 hours after feeding, and of the propionic and butyric acid concentrations from 3.5 hours after feeding, through the fasting period were fitted to a regression on time by the method of least squares as described by Snedecor (1946). The 0 hour extrapolation of this regression is the natural logarithm of A_o and the slope is k_1. The concentrations of acetic, propionic, and butyric acids determined between 0 and 3 or 3.5 hours after feeding were subtracted from the A_o value adjusted for time after feeding according to the disappearance regression.

Figure 4. A Graphic Portrayal of the Type Equation:

$$A_t = A_o e^{-k_1 t} - A_p e^{-k_2 t}$$



The natural logarithms of these differences were fitted to a regression against time as before. The slope of this second regression equation is k_2 and the 0 hour extrapolate is the natural logarithm of A_p. The tests for significance were carried out on the logarithms.

There were no significant differences at the 5% level between any of the disappearance rates although the pooled rate for acetic acid approached significance over that for butyrate. The disappearance rate for propionic acid with the restricted roughage ration approached significance over that with the all hay ration. The rates of production of the acids were not significantly different in any case and ranged from 27 to 83%/hour. The amounts of each acid produced from a pound of T.D.N. were not significant between the rations. However, acetic acid production from hay approached significance over that from the high concentrate ration.

DISCUSSION

The energy supplied to the cow by the short chain acids can be computed from the data in Table 6, page 61. The average animal in this experiment weighed 1270 pounds. Carroll and Hungate (1954) concluded that rumen contents constitute 14% of the bovine body weight. The average weight of the rumen contents in this experiment would then be 178 pounds which would contain about 70 kilograms of rumen liquid (dry matter free). Computing from the 95% confidence level ranges given in Table 6, a pound of total digestible nutrients yields 168 to 469 millimoles of acetic acid, 14 to 182 millimoles propionic acid, and 28 to 147 millimoles butyric acid. Converting the values to kilogram calories, a total energy of 55.0 to 232.3 Kcal./lb. of total digestible nutrients is obtained, 35.2 to 98.3 are represented by acetic acid, 5.1 to 66.9 by propionic acid, and 14.7 to 77.1 by butyric acid. If one pound of total digestible nutrient is equivalent to 1814 calories (Brody, 1945), the cow obtains 3 to 13% of its energy from short chain acids.

A constant rumen volume was assumed in computing these regression equations. Irregular fluctuations in volume would increase the standard error and hence, be corrected for by the range in 3 to 13% estimate. However, if rumen volume increases appreciably and regularly after eating due to salivation,

drinking, or influx of water from the tissues, these estimates would be too low. Similarly, if rumen volume decreases appreciably with fasting, as suggested by the data of Weller and Gray (1954), both the disappearance rates and the energy estimates are biased downwards.

The carbon balance studies of McNaught (1951) and of Heald (1952) indicate a 25 to 50% conversion of feed energy to short chain acid energy. Carroll and Hungate (1954) estimate that short chain acids account for 66% of the available feed energy while Pfander and Phillipson (1953) imply a similar value. These estimates were based on data obtained under extremely abnormal conditions. The 3 to 13% estimate in this experiment is considerabley lower than the values cited above and is probably biased downwards. However, this method utilized normal fistulated cattle under physiological conditions.

If a method for determination of rumen volume at frequent intervals were available, the accuracy of these estimates would be greatly enhanced. Perhaps this could be accomplished with dye dilution techniques similar to those used for blood volume determinations. Accuracy would be further enhanced by increasing feed ingestion during the limited feeding period and lowering the 0 hour concentration of rumen acids. Both of these objectives could be accomplished by once daily feeding. Error occasionally entered the chromatographic determinations through pipetting solids from the bottom of the flask along

with rumen liquid. This could have been avoided by a preliminary separation.

Acetate production from hay approached a significantly greater value than that from grain. The disappearance of acetate approached a significantly faster rate than that for butyrate which is in agreement with Pfander and Phillipson (1953). Although ration had no significant influence on the rates, propionic acid disappearance was faster with the high concentrate than with the hay ration. The molar ratio of acetic to propionic acid was reduced by the high concentrate ration but, the reduction was not as marked as that reported by Tyznik (1951).

Microscopic examination of rumen contents from animals on the all hay and the restricted roughage-high concentrate rations failed to reveal any appreciable or consistent differences. This does not preclude the possibility of less obvious morphological differences. Smears of the inocula and ferments from the in vitro experiments showed no readily detectable or consistent differences with incubation times under 3 hours. Longer incubation times often resulted in a dissolution of protozoa and an increase in gram-positive rods. Carroll and Hungate (1954), using a similar fermentation technique, found that certain physiological characteristics which changed after 2 to 4 hours incubation were quite constant before this time. They also found buffer solutions were unnecessary for short time in vitro fermentations. The values obtained between 2

and 3 hours in Experiment I were more erratic than values with shorter incubation times. More accurate estimates could probably have been obtained by using shorter incubation periods.

Robinson et al. (1955) found that 1,952 x G. for 10 minutes sedimented most rumen microbiota although more were sedimented by 5,782 x G. The 1,240 x G. used in the preparation of microradioautographs for Experiment I was probably somewhat low but the 30 minute centrifugation time used should partially offset the lower force.

The decomposition of cyst(e)ine and methionine noted by Olcott and Fraenkel-Conrat (1947) and Van Halteren (1951) complicated the amino acid partition data. Cyst(e)ine and its decomposition products moved as much as half-way to the solvent front with three areas of peak concentration. Methionine formed 2 or more peaks nearer the solvent front than cyst(e)ine. Another distinct radioactive area appeared very near the solvent front and was either in too low a concentration to visibly react with ninhydrin or was not an amino-sulfur compound. The activity could be divided into a cyst(e)ine fraction containing largely cyst(e)ine activity and a similar methionine fraction. Both fractions were probably contaminated with smaller amounts of unidentified compounds. More extensive separation was neither warranted nor practical for this study.

Lewis (1954) found that rumen microorganisms rapidly reduce sulfate to sulfide. Only a trace of hydrogen sulfide was found in this experiment but small amounts of dissolved

sulfide of high specific activity might have contaminated the cyst(e)ine and/or methionine fractions. The possibility of sulfate incorporation via sulfide needs further investigation.

The synthesis of methionine and cyst(e)ine found in this experiment agrees with the results of Block and Stekol (1950), Block et al. (1951, 1953), and Duncan et al. (1953). Cyst(e)ine appeared to be formed at a faster rate than methionine, but these data were entirely inconclusive. Of the labeled-sulfate incorporated during the first 3 hours of incubation, about two-thirds was found in the cyst(e)ine and one-third in the methionine fraction.

Reed et al. (1949) and Block et al. (1951) found equal amounts of cyst(e)ine and methionine sulfur in the rumen microbiota. The data in this experiment do not conflict with the earlier work since methionine could well be forming at a slower rate than the cyst(e)ine and still eventually attain an equal status provided cyst(e)ine formation ceases before that of methionine. Also, as much as 30% of microbial sulfur may occur as glutathione (Cowie et al., 1954) which may have been lost in the fractionation procedures of Reed et al. (1949) and Block et al. (1951). The data presented here support the hypothesis that inorganic sulfate is first incorporated into cyst(e)ine which is then converted to methionine (Cowie et al., 1954; Block, 1953).

The over-all rate of sulfate incorporation was about 48% /hour. The rate for the concentrate microbiota was slightly

but not significantly faster than that for the hay microbiota. The concentrate microbiota acting on a concentrate substrate incorporated more activity than the hay microbiota acting on a hay substrate. This can be at least partially explained by a greater dilution of the labeled-sulfate by the sulfate already present in the hay substrate. When the substrates were a mixture of hay plus concentrate, the hay microbiota incorporated more activity than the concentrate microbiota. Since Cowie et al. (1954) found that sulfate incorporation by Escherichia coli is directly proportional to cell proliferation, and since the sulfate content of the mixed substrates was equal, the higher incorporation of activity by inocula from a hay fed cow would seem to represent greater cell proliferation. However, this could represent either a greater number of cells used as inocula or a shorter generation time.

Where sulfate concentration of substrates is not equal, incorporation of activity is a function of both cell proliferation and the specific activities of the inorganic sulfate in the ferments. Control of inocula size by use of centrifuged cell suspensions and control of specific activity by use of sulfate free substrates would provide more accurate data. Although, rumen microbiota from cows fed all hay may differ somewhat from the rumen microbiota of high concentrate fed cows in their ability to utilize sulfate, the rate and the pattern of sulfate utilization is predominatly similar in both cases.

SUMMARY

Rumen liquid from 5 bovines (receiving either hay alone or 75% of the total digestible nutrients as concentrate) was incubated with substrate and S^{35}-labeled inorganic sulfate. Two trials used the ration of the donor as substrate and two used a substrate of mixed alfalfa meal and concentrate for both hay inocula and grain inocula. In each trial, a separate fermentation flask was incubated for each time of 0.5, 1, 1.5, 2, 2.5, and 3 hours. Each ferment was fractionated into protein sulfur, free organic sulfur and inorganic sulfate. The protein and free organic sulfur fractions were further separated by chromatography. Glutathione accounted for 2 to 4% of the total activity. The mean partition of incorporated activity in a total of 44 flasks for all times on both treatments was 62% cyst(e)ine and 38% methionine plus an unidentified fraction. Exponential equations fitted to the total incorporations for each combination of substrate and inoculum are presented.

The concentration of acetic, propionic, and butyric acids in 3 bovine rumens was determined at various times after feeding either an all hay ration or one consisting largely of concentrates. Two trials were conducted on each ration. The concentration of each acid first increased and then decreased with time after feeding. The values obtained were fitted to

turnover curves. Acetic acid disappeared from the rumen at the rate of 3.3%/hour and butyric acid at the rate of 2.7%/hour. The standard error was 0.3%. The disappearance rate of propionic acid on the high concentrate ration appeared to be 4.6%/hour versus 3.2%/hour on the hay ration. The rates for conversion of feed to short chain acids ranged from 35 to 84%/hour.

Pooling data from the 4 trials, the amounts of acids per 100 gm. of rumen liquid which were produced from one pound of total digestible nutrients were, 0.24 to 0.67 acetic, 0.02 to 0.26 propionic, and 0.04 to 0.21 butyric. These are the 95% confidence level ranges. The average rumen in these trials was estimated to contain 70 kilograms of liquid. Using these values, it was computed that the cow obtains 3 to 13% of its energy from short chain acids.

LITERATURE CITED

Agrawala, I. P., C. W. Duncan and C. F. Huffman. A quantitative study of rumen synthesis in the bovine on natural and purified rations. I. Protein, dry matter and non-protein nitrogen. J. Nutr., 49: 29-39.
1953

Allen, A., B. Friedmann and S. Weinhouse. Tissue preference for fatty acid and glucose oxidation. J. Biol. Chem., 212: 921-933.
1955

Annison, E. F. Some observations on volatile fatty acids in the sheep's rumen. Biochem. J., 57: 400-405.
1954

Arias, C., W. Burroughs, P. Gerlaugh and R. M. Bethke. The influence of different amounts and sources of energy upon In Vitro urea utilization by rumen microorganisms. J. Animal Sci., 10: 683-692.
1951

Arnstein, H. R. V. and J. C. Crawhall. The metabolism of D L-(beta C^{14})- and DL-(S^{35}) -Cystine by the rat. Biochem. J., 55: 280-285.
1953

Baker, F. Microbial synthesis and autolysis in the digestive tract of herbivora. Nature, 149: 582.
1942

Baker, F. Direct microscopical observations upon the rumen population of the Ox. I. Qualitative characteristics of the rumen population. Ann. of Applied Biol., 30: 230-239.
1943

Baker, F. and S. T. Harriss. Microbial digestion in the rumen (and caecum), with special reference to the decomposition of structural cellulose. Nutr. Abs. and Revs., 17: 3-12.
1947

Balch, C. C. Factors affecting the utilization of food by dairy cows. I. The rate of passage of food through the digestive tract. Brit. J. Nutr., 4: 361-388.
1950

Balch, C. C., D. A. Balch, S. Bartlett, C. P. Cox and S. J. Rowland. Studies on the secretion of milk of low fat content by cows on diets low in hay and high in concentrates. 1. The effect of variations in the amount of hay. J. Dairy Res., 19: 39-50.
1952a

Balch, C. C., D. A. Balch, S. Bartlett, V. W. Johnson and S. J. Rowland. 1952b Factors affecting the utilization of food by dairy cows. 5. The digestibility and rate of passage of foods during L-thyroxine administration. Brit. J. Nutr., 6: 356-365.

Balch, C. C., D. A. Balch, S. Bartlett, C. P. Cox, S. J. Rowland and J. Turner. 1954a Studies of the secretion of milk of low fat content by cows on diets low in hay and high in concentrates. II. The effect of the protein content of the concentrates. J. Dairy Res., 21: 165-171.

Balch, C. C., D. A. Balch, S. Bartlett, Z. D. Hosking, V. W. Johnson, S. J. Rowland and J. Turner. 1954b Studies of the secretion of milk of low fat content by cows on diets low in hay and high in concentrates. III. The effect of variation in the amount and physical state of the hay and a comparison of the Shorthorn and Friesian breeds. J. Dairy Res., 21: 172-177.

Balch, C. C., D. A. Balch, V. W. Johnson and J. Turner. 1953 Factors affecting the utilization of food by dairy cows. 7. The effect of limited water intake on the digestibility and rate of passage of hay. Brit. J. Nutr., 7: 212-224.

Balch, C. C. and V. W. Johnson. 1950 Factors affecting the utilization of food by dairy cows. 2. Factors influencing the rate of breakdown of cellulose (cotton thread) in the rumen of the cow. Brit. J. Nutr., 4: 389-394.

Balch, C. C., A. Kelly and G. Heim. 1951 Factors affecting the utilization of food by dairy cows. 4. The action of the reticulo-omasal orifice. Brit. J. Nutr., 5: 207-216.

Belasco, I. J. 1954 Comparison of urea and protein meals as nitrogen sources for rumen microorganisms: The production of volatile fatty acids. J. Animal Sci., 13: 748-757.

Bell, N. C., W. D. Gallup and C. K. Whitehair. 1953 Value of urea nitrogen in rations containing different carbohydrate feeds. J. Animal Sci., 12: 787-797.

Bentley, O. G., T. U. Hershberger, L. E. Kunkle and D. S. Bell. 1954a The nutritive value of acetate, propionate, and lactate for lambs. J. Animal Sci., 13: 976-977.

Bentley, O. G., Lehmkuhl, R. R. Johnson, T. V. Hershberger and A. L. Moxon. The "Cellulolytic Factor" activity of certain short chained fatty acids. J. Amer. Chem. Soc., 76: 5000.
1954b

Bersin, T. Die Phytochemie des Schwefels. Advances in Enzymology, 10: 223-311.
1950

Block, R. J. Inorganic sulfur in the synthesis of protein in the rumen from non-protein sources with a note on the synthesis of cystine and methionine in Blatella Germanica. pp. 213-224, in C. L. Comar and S. L. Hood, ed. The Role of Atomic Energy in Agricultural Research. U. S. Atomic Energy Commission, TID 5115. Office of Technical Services, Dept. of Commerce, Washington, D. C., 483 pp.
1953

Block, K., J. E. Snoke and S. Yanari. "Enzymatic synthesis of glutathione." pp. 82-94 in W. D. McElroy and B. Glass Ed. A Symposium on Phosphorus Metabolism, Vol. II. John Hopkins Press, Baltimore.
1952

Block, R. J. and J. A. Stekol. Synthesis of sulfur amino acids from inorganic sulfate by ruminants. Proc. Soc. Exptl. Biol. Med., 73: 391-394.
1950

Block, R. J., J. A. Stekol and J. K. Loosli. Synthesis of sulfur amino acids from inorganic sulfate by ruminants. II. Snythesis of cystine and methionine from sodium sulfate by the goat and by microorganisms of the rumen of the ewe. Arch. Biochem., 33: 353-363.
1951

Bolton, E. T., D. B. Cowie and M. K. Sands. Sulfur metabolism in Escherichia coli. III. The metabolic fate of sulfate sulfur. J. Bact., 63: 309-318.
1952

Bortree, A. L., K. M. Dunn, R. E. Ely and C. F. Huffman. A preliminary report on the study of factors influencing rumen microflora. J. Dairy Sci., 29: 542-543.
1946

Brody, S. Bioenergetics and Growth. Reinhold Publishing Corporation, New York, 1023 pp.
1945

Bryant, M. P. and L. A. Burkey. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. J. Dairy Sci., 36: 205-217.
1953a

Bryant, M. P. and L. A. Burkey. Numbers and some predominant groups of bacteria in the rumen of cows fed different rations. J. Dairy Sci., 36: 218-224. 1953b

Bryant, M. P. and R. N. Doetsch. Factors necessary for the growth of Bacteroides succinogenes in the volatile acid fraction of rumen fluid. Science, 120: 944-945. 1954

Bryant, M. P. and R. N. Doetsch. Factors necessary for the growth of Bacteroides succinogenes in the volatile acid fraction of rumen fluid. J. Dairy Sci., 38: 340-350. 1955

Burroughs, W., C. Arias, P. DePaul, P. Gerlaugh and R. M. Bethke. In Vitro observations upon the nature of protein influences upon urea utilization by rumen microorganisms. J. Animal Sci., 10: 672-682. 1951

Burroughs, W., P. Gerlaugh, B. H. Edington and R. M. Bethke. The influence of corn starch upon roughage digestion in cattle. J. Animal Sci., 8: 271-278. 1949

Card, C. S. and L. H. Schultz. Effect of the ration on volatile fatty acid production in the rumen. J. Dairy Sci., 36: 599. 1953

Carroll, E. J. and R. E. Hungate. The magnitude of the microbial fermentation in the bovine rumen. Applied Microbiology, 2: 205-214. 1954

Chance, C. M., C. F. Huffman and C. W. Duncan. Antibiotics in rumen digestion and synthesis. I. The effect of aureomycin on rumen digestion and the passage of nutrients from the rumen during specific intervals after feeding. J. Dairy Sci., 36: 253-259. 1953a

Chance, C. M., C. W. Duncan, C. F. Huffman and R. W. Luecke. Antibiotics in rumen digestion and synthesis. II. The effect of aureomycin on the concentration of some amino acids and b-vitamins in the rumen. J. Dairy Sci., 36: 495-503. 1953b

Chatagner, F., H. Tabechian and B. Bergeret. Repercussion d'une carence en vitamine b_6 sur le metabolisme de l'acide L-cysteinesulfinique, In Vitro et In Vivo, chez le rat. Biochim. et Biophys. Acta, 13: 313-318. 1955

Coon, M. J. and N. S. B. Abrahamsen. The relationship of
1952a alpha-methylbutyrate to isoleucine metabolism. I. Acetoacetate formation. J. Biol. Chem., 195: 805-812.

Coon, M. J., N. S. B. Abrahamsen and G. S. Greene. The rela-
1952b tion of alpha-methylbutyrate to isoleucine metabolism. II. Propionate formation. J. Biol. Chem., 199: 75-84.

Cowie, D. B., E. T. Bolton and M. K. Sands. Sulfur metabolism
1950 in Escherichia coli. I. Sulfate metabolism of normal and mutant cells. J. Bact., 60: 233-248.

Cowie, D. B., E. T. Bolton and M. K. Sands. Sulfur metabolism
1951a in Escherichia coli. II. Competitive utilization of labeled and non-labeled sulfur compounds. J. Bact., 62: 63-74.

Cowie, D. B. and E. T. Bolton. Sulfur metabolism in Escheri-
1952 chia coli. IV. Influence of non-methionine sulfur compounds upon the methionine requiring mutant. J. Bact., 64: 87-96.

Cowie, A. T., W. G. Duncombe, S. J. Folley, T. H. French, R.
1951b F. Glascock, L. Massart, G. J. Peeters and G. Popjak. Snythesis of milk fat from acetic acid ($CH_3\ ^{14}COOH$) by the perfused isolated bovine udder. Biochem. J., 49: 610-615.

Cowie, D. B., R. B. Roberts and E. T. Bolton. Utilization of
1954 internal sulfur resvoirs by Escherichia coli. Science, 119: 579.

Craine, E. M. and R. G. Hansen. The short-chain fatty acids
1952 of the peripheral blood of goats. J. Dairy Sci., 35: 631-636.

Danielli, T. F., M. W. S. Hitchcock, R. A. Marshall and A. T.
1945 Phillipson. The mechanism of absorption from the rumen as exemplified by the behavior of acetic, propionic, and butyric acids. J. Exp. Biol., 22: 75-84.

Davis, R. F., C. Williams and J. K. Loosli. Studies on sulfur
1954 to nitrogen ratios in feeds for dairy cows. J. Dairy Sci., 37: 813-818.

Doetsch, R. N. and R. Q. Robinson. The bacteriology of the
1953 bovine rumen: A review. J. Dairy Sci., 36: 115-142.

Doetsch, R. N., R. Q. Robinson, R. E. Brown and J. C. Shaw. 1953 Catabolic reactions of mixed suspensions of bovine rumen bacteria. J. Dairy Sci., 36: 825-831.

Dowe, T. W., J. Matsushima and V. Arthaud. The effects of 1955 the corn-alfalfa hay ratio on the digestibility of the different nutrients by cattle. J. Animal Sci., 14: 340-349.

Duncan, C. W., I. P. Agrawala, C. F. Huffman and R. W. Luecke. 1953 A quantitative study of rumen synthesis in the bovine on natural and purified rations. II. Amino acid content of mixed rumen proteins. J. Nutr., 49: 41-49.

Elsden, S. R., M. W. S. Hitchcock, R. A. Marshall and A. T. 1946 Phillipson. Volatile acid in the digesta of ruminants and other animals. J. Exp. Biol., 22: 191-202.

El-Shazly, K. Degradation of protein in the rumen of the 1952 sheep. Biochem. J., 51: 640-653.

Fitzgerald, P. J., E. Simmel, J. Weinstein and C. Martin. 1953 Radioautography: theory, technic, and applications. Laboratory Investigation, 2: 181-222.

Folley, S. J. and T. H. French. Utilization of acetate by 1948 tissues of the ruminant. Biochem. J., 43: lv.

Foreman, C. F. and H. A. Herman. Effects of carbohydrate 1953 feeding levels on roughage digestion in cattle. Missouri Agr. Expt. Sta. Research Bull. 535. Columbia, Mo., 55 pp.

Fromageot, C. The metabolism of sulfur and its relations to 1953 general metabolism. pp. 1-36, in J. D. Hardy, ed. The Harvey Lectures, Vol. 49. Academic Press, N. Y. City., 299 pp.

Gall, L. S. Effect of ration upon rumen flora of cattle and 1949 sheep. J. Animal Sci., 8: 619.

Garton, A. G. Observations on the distribution of inorganic 1951 phosphorus, soluble calcium and soluble magnesium in the stomach of the sheep. J. Exp. Biol., 28: 358-368.

Gray, F. V. and A. F. Pilgrim. Fermentation in the rumen of 1951 the sheep. II. The production and absorption of volatile fatty acids during the fermentation of wheaten hay and lucerne hay in the rumen. J. Exp. Biol., 28: 83-90.

Gray, F. V. and A. F. Pilgrim. Fermentation in the rumen of the sheep. III. Intermediate stages in the fermentation of wheaten hay In Vitro by microorganisms from the rumen. J. Exp. Biol., 29: 54-56. 1952a

Gray, F. V. and A. F. Pilgrim. Origins of the volatile fatty acids in the rumen. Nature, 170: 375. 1952b

Gray, F. V., A. F. Pilgrim, H. J. Rodda and R. A. Weller. Volatile acids in the rumen of the sheep. Nature, 167: 954. 1951a

Gray, F. V., A. F. Pilgrim and R. A. Weller. Fermentation in the rumen of the sheep. I. The production of volatile acids and methane during the fermentation of wheaten hay and lucerne hay In Vitro by microorganisms from the rumen. J. Exp. Biol., 28: 74-82. 1951b

Gray, F. V., A. F. Pilgrim and R. A. Weller. Conversion of plant nitrogen to microbial nitrogen in the rumen of the sheep. Nature, 172: 347. 1953

Gray, F. V., A. F. Pilgrim and R. A. Weller. Functions of the omasum in the stomach of the sheep. J. Exp. Biol., 31: 49-55. 1954

Gutierrez, J. Numbers and characteristics of lactate utilizing organisms in the rumen of cattle. J. Bact., 66: 123-128. 1953

Hale, E. B., C. W. Duncan and C. F. Huffman. Rumen digestion in the bovine with some observations on the digestibility of alfalfa hay. J. Dairy Sci., 23: 953-967. 1940

Hale, E. B., C. W. Duncan and C. F. Huffman. Rumen digestion studies. II. Studies in the chemistry of rumen digestion. J. Nutr., 34: 747-758. 1947

Harper, W. J. Direct chromatographic determination of acetic, propionic and butyric acids in cheese. J. Dairy Sci., 36: 808-816. 1953

Hart, E. B., G. Bohstedt, H. J. Deabald and M. I. Wegner. The utilization of simple nitrogenous compounds such as urea and ammonium bicarbonate by growing calves. J. Dairy Sci., 22: 785-798. 1939

Hastings, E. G. The significance of the bacteria and the protozoa of the rumen of the bovine. Bact. Revs., 8: 235-254. 1944

Heald, P. J. 1951 The assessment of glucose-containing substances in rumen micro-organisms during a digestion cycle in sheep. Brit. J. Nutr., 5: 84-93.

Heald, P. J. 1952 The fermentation of pentosans and uronic acids by bacteria from the rumen contents of sheep. Biochem. J., 50: 503-508.

Heald, P. J. 1953 The fermentation of xylans in the rumen of the sheep. Brit. J. Nutr., 7: 214-130.

Hentschl, A. F., R. N. Berry and C. F. Huffman. 1954 A plastic plug for use in the bovine rumen fistula. M. S. C. Veterinarian, 14: 77-81.

Hess, W. C. 1952 Cystathionine in the liver following feeding of methionine and serine. Arch. Biochem., 40: 127-129.

Holmes, P., R. J. Moit and E. J. Underwood. 1953 Ruminal flora studies in the sheep. V. The amino acid composition of rumen bacterial protein. Australian J. Biol. Sci., 6: 637-644.

Horowitz, N. H. 1947 Methionine synthesis in Neurospora: the isolation of cystathionine. J. Biol. Chem., 171: 255-264.

Huffman, C. F. 1953 Ruminant nutrition. Ann. Rev. Biochem., 22: 399-422.

Huffman, C. F. and C. W. Duncan. 1940 Cystine as a possible deficiency in a ration of alfalfa hay for milk production. J. Dairy Sci., 23: 559.

Hungate, R. E. 1943 Further experiments on cellulose digestion by the protozoa in the rumen of cattle. Biol. Bull., 84: 157-163.

Hunt, C. H., O. G. Bentley, T. V. Hershberger and J. H. Cline. 1954 The effect of carbohydrates and sulfur on b-vitamin synthesis, cellulose digestion, and urea utilization by rumen microorganisms In Vitro. J. Animal Sci., 13: 570-580.

Jarrett, I. G. and B. J. Potter. 1950 The metabolism of acetate in sheep after injection of phlorizin. Australian J. Exp. Biol., 28: 595-602.

Jarrett, I. G., B. J. Potter and O. H. Filsell. 1952 Lower fatty acids in the intermediary metabolism of sheep. Australian J. of Exp. Biol., 30: 197-206.

Jarrige, R. 1953 L'utilisation des glucides alimentaires par les ruminants. Ann. Nutrit. Aliment., 7: 245-302, 339-406.

Johns, A. T. 1952 Mechanism of propionic acid formation by Clostridium propionicum. J. Gen. Microbiol., 6: 123-127.

Johns, A. T. 1953 Fermentation of glycerol in the rumen of sheep. New Zealand J. of Science and Technology, Sec. A., 35: 262-269.

Johnson, R. B. 1951 The relative rates of absorption of the volatile acids from the rumen and their relationship to ketosis. Cornell Vet., 41: 115-121.

Jones, I. R. and J. R. Haag. 1946 Utilization of non-protein nitrogen by dairy heifers. J. Dairy Sci., 29: 535-536.

Keener, H. A., A. E. Teeri, R. V. Harrington and R. R. Baldwin 1953 Metabolic fate of S^{35} in the lactating cow when fed $S^{35}O_2$ preserved silage. J. Dairy Sci., 36: 1205-1211.

Kiddle, P., R. A. Marshall and A. T. Phillipson. 1951 A comparison of the mixtures of acetic, propionic and butyric acids in the rumen and in the blood leaving the rumen. J. Physiol., 113: 207-217.

Klosterman, E. W., D. W. Bolin, E. L. Lasley and W. E. Dinusson. 1954 The effect of methionine supplementation upon the utilization of pea and alfalfa proteins by sheep. J. Animal Sci., 10: 439-446.

Koistinen, O. A. 1948 Ueber die Garung der nativen Kohlenhydrate des Holzes durch Einwirkung von mesophilen Bakterien aus dem Verdauungskanal des Schafes. Soumalaisen Tiedekatamian Tormituhsia Annales Academie Scientiarum Fennicae, A, II, Chemica, 31. Helsinki, 73 pp.

Kulwich, R., L. Struglia, H. C. Bourne and P. B. Pearson. 1954 Uptake of radiosulfur by sheep given repeated doses of labeled sodium sulfate. J. Animal Sci., 13: 990-991.

Kumar, S. and S. Lakshmanan. 1954 Beta-hydroxybutyric acid metabolism of the mammary gland. J. Dairy Sci., 37: 669.

Lewis, D. 1954 The reduction of sulfate in the rumen of the sheep. Biochem. J., 56: 391-399.

Loosli, J. K. and L. E. Harris. 1945 Methionine increases the value of urea for lambs. J. Animal Sci., 4: 435-437.

Louw, J. G. and J. G. Van der Wath. 1943 The influence of varying maize supplements on the digestibility of the cellulose of a poor veld hay in relation to the bacterial population of the rumen of sheep with a note on nitrogen metabolism. Onderstepoort J. Vet. Sci. Animal Ind., 18: 177-190.

Lowe, I. P. and E. Roberts. 1955 Incorporation of radioactive sulfate-sulfur into taurine and other substances in the chick embryo. J. Biol. Chem., 212: 477-483.

Macklin, L. J., P. B. Pearson and C. A. Denton. 1955 The utilization of sulfate sulfur for the synthesis of taurine in the developing chick embryo. J. Biol. Chem., 212: 469-475.

Marston, H. R. 1948 The fermentation of cellulose In Vitro by organisms from the rumen of sheep. Biochem. J., 42: 564-574.

Masson, F. M. and A. E. Oxford. 1951 The action of the ciliates of the sheep's rumen upon various water soluble carbohydrates including polysaccharides. J. Gen. Microbiol., 5: 664-672.

Masson, M. J. and A. T. Phillipson. 1951 The absorption of acetate, propionate and butyrate from the rumen of sheep. J. Physiol., 113: 189-206.

Masson, M. J. and A. T. Phillipson. 1951 The composition of the digesta leaving the abomasum of sheep. J. Physiol., 116: 98-111.

McClymont, G. L. 1951 Volatile fatty acid metabolism of ruminants, with particular reference to the lactating bovine mammary gland and the composition of milk fat. Australian J. Agr. Res., 2: 158-180.

McDonald, I. W. 1954 The extent of conversion of food protein to microbial protein in the rumen of the sheep. Biochem. J., 56: 120-125.

McNaught, M. L. 1951 The utilization of non-protein nitrogen in the bovine rumen. 7. A qualitative and quantitative study of the breakdown of carbohydrate which accompanies protein formation in bovine rumen contents during In Vitro incubation. Biochem. J., 49: 325-332.

McNaught, M. L., E. C. Owen, K. M. Henry and S. K. Kon. 1954 The utilization of non-protein nitrogen in the bovine rumen. 8. The nutritive value of the proteins of preparations of dried rumen bacteria, rumen protozoa and brewers yeast for rats. Biochem. J., 56: 151-156.

Miller, W. J. and N. N. Allen. 1955 The effect of sodium acetate feeding on milk and fat yield, blood sugar, and blood ketones of dairy cows. J. Dairy Sci., 38: 310-312.

Miller, J. M. and L. B. Rockland. 1952 Determination of cysteine and glutathione in citrus juices by filter paper chromatography. Arch. Biochem., 40: 416-423.

Moore, L. A. and O. B. Winter. 1934 Rate of passage of inert materials through the digestive tract of the bovine. J. Dairy Sci., 17: 297-305.

Noble, R. L., L. S. Pope and W. D. Gallup. 1955 Urea and methionine in fattening rations for lambs. J. Animal Sci., 14: 132-136.

Olcott, H. S. and H. Fraenkel-Conrat. 1947 Formation and loss of cysteine during acid hydrolysis of proteins--role of tryptophane. J. Biol. Chem., 171: 583-594.

Owen, E. C. 1954 Reviews of the progress of dairy science. Section A. Physiology of dairy cattle. Part II. Physiology and biochemistry of rumination. J. Dairy Res., 21: 408-453.

Oxford, A. E. 1951 The conversion of certain soluble sugars to a glucosan by holotrich ciliates in the rumen of sheep. J. Gen. Microbiol., 5: 83-90.

Parthasarathy, D. and A. T. Phillipson. 1953 The movement of potassium, sodium, chloride and water across the rumen epithelium of sheep. J. Physiol., 121: 452-469.

Patel, B. R. 1952 The composition, physical properties and nutritional value of rumen contents of dairy cows. Thesis. Univ. Wis., Madison.

Patton, A. R. 1950 Introducing paper chromatography to biochemistry students. J. Chem. Ed., 27: 60-61.

Pearson, R. M. and J. A. B. Smith. 1943a The utilization of urea in the bovine rumen. 1. Methods of analysis of the rumen ingesta and preliminary experiments in vivo. Biochem. J., 37: 142-148.

Pearson, R. M. and J. A. B. Smith. 1943b The utilization of urea in the bovine rumen. 3. The synthesis and breakdown of protein in rumen ingesta. Biochem. J., 37: 153-164.

Pennington, R. J. 1952 The metabolism of short-chain fatty acids in the sheep. 1. Fatty acid utilization and ketone body production by rumen epithelium and other tissues. Biochem. J., 51: 251-258.

Pennington, R. J. 1954 The Metabolism of short-chain fatty acids in the sheep. 2. Further studies with rumen epithelium. Biochem. J., 56: 410-416.

Pfander, W. H. and A. T. Phillipson. 1953 The rates of absorption of acetic, propionic and n-butyric acids. J. Physiol., 122: 102-110.

Phillipson, A. T. 1947 The role of the microflora of the alimentary tract of herbivora with special reference to ruminants. 3. Fermentation in the alimentary tract and metabolism of the derived fatty acids. Nutr. Abs. and Revs., 17: 12-18.

Phillipson, A. T. 1948 A method of measuring the flow of digesta from the stomach of the sheep. J. Physiol., 107: 21-22.

Phillipson, A. T. 1952a The passage of digesta from the abomasum of sheep. J. Physiol., 116: 84-97.

Phillipson, A. T. 1952b The fatty acids present in the rumen of lambs fed on a flaked maize ration. Brit. J. Nutr., 6: 190-198.

Phillipson, A. T. 1953 Digestion of cellulose by the ruminant. Biochem. Soc. Symposia, 11: 63-70.

Popjak, G. 1952 The metabolism of fat in the mammary gland and foetal tissues with special reference to the application of isotopic tracers. Nutr. Abs. and Rev., 21: 535-553.

Popjak, G., 1951a T. H. French and S. J. Folley. Utilization of acetate for milk fat synthesis in the lactating goat. Biochem. J., 48: 411-416.

Popjak, G., 1951b T. H. French, G. D. Hunter and A. J. D. Martin. Mode of formation of milk fatty acids from acetate in the goat. Biochem. J., 48: 612-618.

Popjak, G., 1953 G. D. Hunter and T. H. French. Biosynthesis of milk-fat in the rabbit from acetate and glucose. The mode of conversion of carbohydrate to fat. Biochem. J., 54: 238-247.

Pounden, W. D. and J. W. Hibbs. The influence of the ratio 1948 of grain to hay in the ration of dairy calves on certain rumen microorganisms. J. Dairy Sci., 31: 1051-1054.

Powell, E. B. Relation of the ration to the composition of 1941 milk. J. Dairy Sci., 24: 504-505.

Pugh, P. S. and R. Scarisbrick. Acetate metabolism in ovine 1952 ketosis. Nature, 170: 978-979.

Quin, J. I., W. Oyaert and R. Clark. Studies on the alimen- 1951 tary tract of the merino sheep in South Africa. XVIII. The effect of fasting on the activity of the ruminal flora of sheep and cattle. Onderstepoort J. Vet. Res., 25: 51-58.

Reed, F. M., R. J. Moir and E. J. Underwood. Ruminal flora 1949 studies in sheep. I. The nutritive value of rumen bacterial protein. Australian J. Sci. Res., B2: 304-317.

Reid, J. T. Urea as a protein replacement for ruminants: A 1953 review. J. Dairy Sci., 36: 955-996.

Reid, R. L. Studies on the carbohydrate metabolism of sheep. 1950 II. The uptake by the tissues of glucose and acetic acid from the peripheral circulation. Australian J. Agr. Res., 1: 338-354.

Reid, R. L. Studies on the carbohydrate metabolism of sheep. 1952 V. The effect of hyperglycemia and of insulin on the rate of extra-hepatic glucose assimilation. Australian J. Agr. Res., 3: 160-167.

Robertson, A. and C. Thin. A study of starvation ketosis in 1953 the ruminant. Brit. J. Nutr., 7: 181-195.

Robinson, R. Q., R. N. Doetsch, F. M. Sirotnak and J. C. Shaw. Production of lactic acid and an iodine staining substance by bovine rumen bacteria. J. Dairy Sci., 38: 13-19. 1955

Salsbury, R. L. Studies on the In Vitro digestion of cellulose by rumen microorganisms. Thesis, Mich. State Coll., East Lansing, 141 pp. 1955

Schambye, P. Volatile acids and glucose in portal blood of sheep. Nord. Vet.-Med., 3: 555-574, 748-762, 1003-1014. 1951

Schultz, L. H. Volatile fatty acids in the rumen of cows with ketosis. J. Dairy Sci., 37: 664. 1954

Sijpesteijn, A. K. and S. R. Elsden. The metabolism of succinic acid in the rumen of the sheep. Biochem. J., 52: 41-45. 1952

Sirotnak, F. M., R. N. Doetsch, R. E. Brown and J. C. Shaw. Amino acid metabolism of bovine rumen bacteria. J. Dairy Sci., 36: 1117-1123. 1953

Sirotnak, F. M., R. N. Doetsch, R. Q. Robinson and J. C. Shaw. Aspartate dissimilation reactions of rumen bacteria. J. Dairy Sci., 37: 531-537. 1954

Shaw, J. C. and W. E. Pertersen. The utilization of beta-hydroxybutyric acid by the perfused lactating mammary gland. J. Biol. Chem., 147: 639-643. 1943

Smith, C. K. A study of the activities of rumen bacteria. Thesis, Mich. State Coll., East Lansing, 53 pp. 1953

Smuts, D. B., B. A. Du Toit and J. G. Van der Wath. A study on the possibility of cystine synthesis in the rumen of sheep together with the effect of cystine supplementation on the nitrogen utilization of lucerne in young stock. Onderstepoort J. Vet. Sci., 16: 181-190. 1941

Snedecor, G. W. Statistical methods. 4th edition. Iowa State College Press, Ames. 485 pp. 1946

Stadtman, E. R. and H. A. Barker. Fatty acid synthesis by enzyme preparation of Clostridium kluyveri. J. Biol. Chem., 180: 1085-1124, 1169-1186. 1949

Starks, P. B., W. H. Hale, U. S. Garrigus, R. M. Forbes and
1954 M. F. James. Response of lambs fed varied levels of elemental S, sulfate-sulfur and methionine. J. Animal Sci., 13: 249-257.

Stevens, W. L. Asymptotic regression. Biometrics, 6: 247-
1951 267.

Stone, E. C. Fermentation ability of ingesta from normal and
1949 and atonic bovine rumens. Amer. J. Vet. Res., 10: 26-29.

Sugden, B. The cultivation and metabolism of oligitrich
1953 protozoa from the sheep's rumen. J. Gen. Microbiol., 9: 44-53.

Swift, R. W., E. J. Thacker, A. Black, J. W. Bratzler and W.
1947 H. James. Digestibility of rations for ruminants as affected by proportion of nutrients. J. Animal Sci., 6: 432-444.

Tyznik, W. J. The effect of the amount and physical state
1951 of the roughage upon the rumen fatty acid and milk fat of dairy cows. Thesis, Univ. of Wis., Madison, 53 pp.

Tyznik, W. J., O. G. Bentley, H. H. Weiser, T. S. Sutton, A.
1954 L. Moxon and Assistants. The effect of various carbon substance on In Vitro activity of sheep rumen microorganisms. J. Animal Sci., 13: 1002.

Van der Wath, J. G. Studies on the alimentary tract of Merino
1948 sheep in South Africa. XI. Digestion and synthesis of starch by ruminal bacteria. Onderstepoort J. Vet. Sci., 23: 367-384.

Van Halteren, M. B. Artefacts in the chromatography of mix-
1951 tures of amino acids containing cysteine. Nature, 168: 1090-1091.

Van Soest, P. J., N. N. Allen and L. R. Maki. The effect of
1954 restricted roughage, high concentrate diet upon milk fat, blood glucose and blood ketones. J. Dairy Sci., 37: 660.

Waelch, H. and D. Rittenberg. Glutathione: I. The metabolism
1941 of glutathione studied with isotopic glycine. J. Biol. Chem., 139: 761-774.

Waite, R. and J. Boyd. The water soluble carbohydrates of
1953 grasses. J. Sci. Food and Agr., 4: 197-204, 257-261.

Waldo, D. R. and L. H. Schultz. Lactic acid production in
1955 the rumen. J. Dairy Sci., 38: 605.

Walter, W. G. A study of microbial activities in the ovine
1952 rumen. Thesis, Mich. State Coll., East Lansing,
127 pp.

Weir, W. C. and V. V. Rendig. Serum inorganic sulfur as a
1954 measure of the sulfur intake of sheep. J. Nutr.,
54: 87.

Weller, R. A. and F. V. Gray. The passage of starch through
1954 the stomach of the sheep. J. Exp. Biol., 31:
40-48.

Wijesundera, S. and D. D. Woods. Cystathionine in relation
1953 to methionine synthesis by Bacterium coli. J.
Gen. Microbiol., 9: iii-iv.

Williams, V. J., M. C. Nottle, R. J. Moir and E. J. Underwood.
1953 Ruminal flora studies in sheep. IV. The influence
of varying dietary levels of protein and starch
upon digestibility, nitrogen retention, and the
free microorganisms of the rumen. Australian
J. Biol. Sci., 6: 142-151.

Winteringham, F. P. W., A. Harrison and R. G. Bridges.
1952 Radioactive tracer techniques in paper chromato-
graphy. Nucleonics, 10: 52-57.

Wood, H. G., R. W. Brown and C. H. Werkman. Mechanism of the
1945 butyl alcohol fermentation with heavy carbon
acetic and butyric acids and acetone. Arch.
Biochem., 6: 243-260.

Zelter, Z. Participation of acetic and butyric acid in the
1952 lipogenesis of the milk cow. Comp. rend., 234:
567-569.

APPENDIX

TABLE 8

SUMMARY OF REGRESSIONS OF ACETIC, PROPIONIC, AND BUTYRIC ACID CONCENTRATIONS IN THE RUMEN ON TIME AFTER FEEDING

	All Hay Ration		
	Acetic Acid	Propionic Acid	Butyric Acid
$\text{Log}_e A_o$	2.2602	0.8256	0.4915
$\text{Log}_e A_p$	0.6441	-1.0103	-1.1545
k_1	0.0353	0.0322	0.0248
k_2	0.5226	0.4499	0.3511
	Standard Error of Estimate		
Regression of $\text{Log}_e A_o$ on t	0.1230	0.1342	0.1308
k_1	0.0041	0.0048	0.0046
$\text{Log}_e A_o$	0.0497	0.0607	0.0592
Degrees of Freedom	16	14	14
Regression of $\text{Log}_e A_p$ on t	0.9739	0.5712	0.4966
k_2	0.2938	0.2372	0.1498
$\text{Log}_e A_p$	0.3872	0.4004	0.3355
Degrees of Freedom	6	3	6

TABLE 8 (Continued)

SUMMARY OF REGRESSIONS OF ACETIC, PROPIONIC, AND BUTYRIC ACID CONCENTRATIONS IN THE RUMEN ON TIME AFTER FEEDING

Restricted Roughage-High Concentrate Ration		
Acetic Acid	**Propionic Acid**	**Butyric Acid**
1.9683	0.8593	0.8562
0.7710	-0.8915	-0.3201
0.0311	0.0465	0.0284
0.8379	0.2763	0.7335
Standard Error of Estimate		
0.1348	0.2197	0.1279
0.0050	0.0089	0.0052
0.0613	0.1164	0.0680
12	10	10
0.6509	1.4099	0.9568
0.2664	0.6753	0.3520
0.2551	0.9338	0.6393
6	5	8

TABLE 9

THE CONCENTRATION OF ACETIC, PROPIONIC, AND BUTYRIC ACIDS IN RUMEN LIQUID

(Millimoles Acid /100 gm. Rumen Liquid[1])

Hours After Feeding	All Hay Ration					
	Acetic Acid		Propionic Acid		Butyric Acid	
	C707	T2	C707	T2	C707	T2
0.0	5.87	8.32	1.66	2.07	1.04	1.47
2.0	8.76	7.69	2.04	1.90	1.32	1.41
2.5	8.50	7.35	2.22	1.73	1.47	1.38
3.0	8.44	----	2.22	----	1.41	----
3.25	----	7.78	----	1.77	----	1.40
3.5	8.71	8.35	2.36	1.76	1.50	1.66
4.0	8.19	7.97	2.13	1.79	1.38	1.44
5.0	8.06	8.33	2.03	2.01	1.74	1.41
6.0	8.08	8.27	1.80	1.95	1.52	1.48
10.25	----	6.57	----	1.59	----	1.24
10.5	6.90	----	1.71	----	1.15	----
12.5	----	6.18	----	1.42	----	1.24
14.5	5.41	----	1.37	----	1.00	----
18.0	----	6.38	----	1.56	----	1.03
19.5	4.21	----	1.09	----	0.96	----
23.5	----	5.36	----	1.37	----	1.19
24.0	3.07	----	0.82	----	0.68	----

1. Sample weighed on to column contained 91.3% rumen liquid on a dry matter free basis; the values reported here are corrected to 100% rumen liquid.

TABLE 9 (Continued)

THE CONCENTRATION OF ACETIC, PROPIONIC, AND BUTYRIC ACIDS IN RUMEN LIQUID

(Millimoles Acid /100 gm. Rumen Liquid[1])

Hours After Feeding	Restricted Roughage-High Concentrate Ration					
	Acetic Acid		Propionic Acid		Butyric Acid	
	C707	T1	C707	T1	C707	T1
0.0	4.9	5.40	1.31	2.25	1.8	2.15
1.0	6.2	----	1.34	----	1.73	----
1.5	6.4	5.42	1.43	2.15	1.80	1.69
2.0	6.47	5.62	1.49	2.07	1.77	2.17
2.5	6.75	6.20	1.71	2.34	1.87	2.12
3.0	6.77	6.26	1.58	2.25	1.89	2.34
3.75	----	5.93	----	2.18	----	2.18
4.0	6.5	5.69	1.63	2.13	1.70	2.15
5.0	----	7.17	-----	2.29	----	2.52
6.0	6.57	----	1.58	----	2.13	----
8.0	5.97	----	1.54	-----	1.72	----
10.75	----	4.88	----	1.65	----	1.83
12.0	5.64	----	1.21	----	1.71	----
13.0	----	3.36	----	1.05	----	1.38
18.25	----	3.96	----	1.23	----	1.45
24.0	3.38	3.81	0.53	1.09	1.07	1.36

1. Sample weighed on to column contained 91.3% rumen liquid on a dry matter free basis; the values reported here are corrected to 100% rumen liquid.