SYMPOSIUM: SELECTED TOPICS IN MICROBIAL ECOLOGY[1]

I. Microbial Ecology of the Rumen

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Most natural habitats of microorganisms are difficult to analyze because of large and rapid changes in substrate, moisture, temperature, oxygen, and other factors of the physical environment. With the accompanying fluctuations in the microbiota the possible permutations and combinations of the environmental variables are so numerous that information obtained in one investigation is only questionably applicable in another. This situation is particularly evident in soil, the primary habitat of so many microbes.

Certain habitats such as the bodies of plants and animals are more nearly constant. The defense mechanisms of the host prevent massive invasions by unselected species and the number of successful parasitic types at any one time is small. This facilitates ecological analysis and, because parasitism is so important in the human economy, a great body of knowledge on the ecology of parasitic microorganisms which penetrate the tissues of plants and animals has accumulated. This knowledge is almost exclusively qualitative in nature and of little help in understanding interrelationships in open systems containing a wide variety of forms.

Physical Environmental Factors of the Rumen

The rumen is an exceptional habitat, first, in providing constant conditions of moisture, pH, temperature, anaerobiosis, and food, and, second, in being an open system in which no stringently restrictive factors such as humoral defense mechanisms limit to a few the number of kinds of organisms which can survive. Some restrictions are inevitable in any habitat maintaining fairly constant conditions; in the rumen the type of food and the lack of oxygen prevent the growth of many types found in other habitats. But the food of ruminants is complex in nature, approximating in composition the organic residues normally undergoing decomposition in soil, and the rumen contains many diverse microbes. Ecological analysis of the rumen may throw light on some of the interrelationships in other habitats more difficult to analyze because of variability, but accomplishing similar degradations of organic matter.

The anaerobic nature of the rumen is an important factor in its constancy. Oxygen is used avidly by many organisms and is so insoluble in water that steep gradients in dissolved oxygen concentration arise as soon as even a small population develops. Some oxygen is often found in the gas at the top of the rumen, but in the great mass of rumen ingesta, 5 kg in a sheep and 70 kg in a steer, the oxygen tension is negligible. The redox potential of rumen contents is approximately -0.35 volts. The methanogenic bacteria of the rumen cannot grow if the oxidation-reduction (redox) potential of the medium is much above -0.33 volts (Smith and Hungate, 1958) and many other rumen bacteria require a potential below that at which resorufin (the pink reduction product of resazurin) is reduced to the colorless state. The E_0' of the reaction, resorufin (pink) $\rightleftharpoons$ dihydroresorufin (colorless), at pH 6.867 is -0.042 volts (Twigg, 1945).

It has been noted by many investigators that bacteria and protozoa in rumen contents exposed to air live for some time. Also, masses of protozoa obtained from the rumen and washed free of bacteria may remain active in cultures from which oxygen is not excluded (Sugden and Oxford, 1952). These and other observations are sometimes interpreted as evidence that the rumen organisms are not anaerobic. It is questionable in these cases whether there is a high oxygen tension at the surface of the microbial cells. There may be enough facultative (the author prefers the more descriptive term, euryoxic) bacteria to use the dissolved oxygen in their metabolism, or the large masses of anaerobic organisms may absorb enough oxygen to prevent accumulation to

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toxic levels, even though in excess oxygen they cannot use it in their metabolism.

A few bacteria ingested into the rumen are aerobes and a greater number, both ingested and growing in the rumen, are euryoxic, but these two types are usually much less abundant than the anaerobes. The reason may be that the genetic systems concerned with aerobic metabolism are so rarely useful in the rumen that the cost of forming them is greater than their contribution to growth. Types handicapped by carrying the genes necessary for synthesis of both anaerobic and unused aerobic enzymes may not grow as fast in the rumen as their less encumbered anaerobic competitors.

What Constitutes a Complete Ecological Analysis?

The following questions arise in a complete ecological analysis of a natural habitat:

1. What kinds and numbers of organisms are present? This involves identification, classification, and enumeration.
2. What are their activities? Food and metabolic products must be identified, and habits of growth, reproduction, and death known. A complete determination of activities necessitates a complete knowledge of the environment.
3. To what extent are their activities performed? This involves quantitative measurement of the entire complex as well as its individual components.

Most ecological analyses of microbial habitats have been restricted to partial answers to the first question, with inferences about question two which may be correct but in many cases are not supported by adequate evidence. The quantitative measurement of microbial habitats is usually restricted to a culture count, an occasional direct count, and too often to isolations in which the number of organisms is not determined. In very few cases has the activity of microorganisms in a particular habitat been measured, yet in ecology, as in other disciplines, an analysis which falls short of quantitative formulation can hardly be considered complete.

The present paper is an attempt to review the extent to which rumen studies have contributed answers to the above three questions. A few comments on the rationale of techniques for analyzing a microbial habitat are also included. Many publications which illuminate detailed aspects of rumen microbiology have been omitted from the references since the intent of the discussion is to emphasize major ecological considerations rather than to review all pertinent knowledge.

The Rumen Bacteria

Identification by Direct Microscopic Examination

Distinctive bacteria identifiable in rumen contents by their appearance include: *Sarcina bakeri*, a gram-negative form with tetrads of cells 3 to 4 μ in diameter; *Borrelia* with characteristic spirochete morphology; *Lampropedia*, cocci dividing alternately in two planes with the daughter cells remaining attached to form rectangular sheets of as many as 1,024 cells (lampropediae may be ingested and not grow in the rumen); *Oscillospira guilliermondii*, large, motile trichomes quite similar in appearance to *Caryophanon*; *Selenomonas*, large crescentic cells with a tuft of flagella on one side; and *Peptostreptococcus elsdenii*, long chains of large cocci. Although these genera of bacteria as well as some others can be seen easily in rumen contents and several of them have been cultured, they have not been counted.

Cultivation in Habitat-Simulating Medium

Since most of the rumen bacteria cannot be identified by microscopic examination alone, they must be isolated and their physiological characteristics must be studied. In selecting a medium for isolating the rumen bacteria, ecological considerations play an important part. The maximal number and kinds will be isolated if the culture medium supplies all the needed factors operative in the rumen and introduces no inhibitors. Since the feed consumed by the ruminant constitutes the primary substrate in the rumen, a preparation of ruminant feed appears most suited to cultivation of the maximal number of rumen bacteria. In practice, the insoluble fraction of ruminant feed poses a problem in preparing a medium with the transparency required for colony counts and identification, and an extract is usually employed; or, glucose and cellobiose are supplied. Since carbohydrate is probably the primary energy-yielding substrate in natural anaerobic habitats (von Brand, 1952; Hungate, 1955, p. 195), a mixture of all soluble sugars in hay or forage, or occurring as digestion products of forage, should in theory satisfy the energy requirements of the rumen bacteria as satisfactorily as would the total feed components.

Other cultural features designed to simulate the rumen habitat include the use of balanced inorganic salts, in which sodium bicarbonate and carbon dioxide serve as the important buffer, as in the rumen. The carbon dioxide is also useful in displacing and excluding air. Various devices are used to obtain low redox potentials in which the rumen organisms can grow, including reduction by chemicals such as cysteine, thioglycolate, dithionite, and sodium sulfide, or by respiration, *e.g.*, by *Escherichia coli* (Smith and Hungate, 1958).

Rumen fluid is included in the medium to supply nutrients characteristic of the habitat, especially those nutrients arising through the action of the rumen microorganisms. In some experiments the medium has been based on 100 per cent rumen fluid, which might seem theoretically to duplicate most closely actual rumen conditions. But rumen fluid includes not only nutrients, it contains also the waste products of bacterial growth. By diluting rumen contents, the concentration of inhibitory products is decreased yet the concentration of nutrients is still adequate. In practice, one-third rumen fluid in the medium gives counts as high as or higher than those found with other concentrations.

Most tests of the relative effectiveness of rumen fluid media and artificial media containing peptones and sugars have shown a superiority of rumen fluid (King and Smith, 1955). Many of the bacteria isolated from the rumen fail to grow in common laboratory media lacking rumen fluid. This indicates a specific adaptation and supports the validity of habitat simulation in the design of media. In growing the bacteria from ruminants fed rations high in concentrates, rumen fluid appears not to be as important as in the case of animals on a hay ration (Maki and Foster, 1957), but even with concentrate feeds some of the isolates cannot grow without rumen fluid or its essential constituents (Bryant *et al.*, 1958*b*).

For comparisons of the microflora in different ruminants, a standard habitat-simulating medium with defined ingredients providing the same total nutrients and with no inhibitory factors would be even better than one employing rumen fluid, since the latter collected at different times from different animals varies in composition. But for growing the rumen microbes from any particular animal, rumen content from that individual is theoretically best and in practice (Hungate *et al.*, 1959) has proved nutritionally superior.

Counts. The importance of determining numbers of individuals of each kind of organism in a natural microbial habitat can hardly be overemphasized. A habitat-simulating medium, if completely successful, theoretically enables the investigator, through the use of serial dilution techniques, to detect and count all kinds of viable bacteria in the habitat. But in practice there are so many cells of abundant types in the highest dilutions containing the rare types that the latter escape detection. In many cases the rare bacteria are quantitatively unimportant, performing in small measure the activities accomplished chiefly by the abundant types. But there is a chance that numerically insignificant species may be unique in performing an essential function. Recognition of such a role for poorly represented species requires special evidence of importance. The abundant types are important simply because their metabolism must constitute a significant fraction of the total.

Application of Selective Enrichment Techniques

In a complete ecological analysis, even the rare types should be known. For their cultivation and detection the selective-enrichment rather than the habitat-simulating medium is indicated. A common selective enrichment technique consists in inoculating a large quantity of the habitat into a liquid medium, serially repeating this procedure by inoculating a fraction of the previous enrichment into a subculture, and attempting isolation in solid medium when the desired type preponderates. This is highly successful for obtaining microbial strains capable of accomplishing many selected metabolic activities and is adequate if it is desired merely to obtain bacterial strains for laboratory study. But unless accompanied by a dilution procedure which permits an estimate of numbers, the ecological importance of the selected organism in the investigated habitat is difficult to assess.

It is sometimes assumed that if selective enrichment yields an organism which performs in pure culture a particular action occurring also in the habitat, the action in the habitat can be ascribed to the isolate. This would follow if the enrichment method *exactly* simulated the natural condition. But exact simulation should yield the kinds and proportions of bacteria characteris-

tic of the natural habitat. It would not select or enrich. An enrichment must provide conditions *different* from those of the habitat in order to be selective. It may not select for the most abundant type performing the action in question and, unless the selective enrichment is performed with various dilutions of the natural material, it may give a misleading impression of the nature of the responsible organisms. The more abundant bacteria performing the action in the natural habitat may have peculiar and unsuspected culture requirements not supplied at an optimal level by the enrichment method and may be overgrown by less abundant competitors for which the medium happens to be more favorable. When the rarer competitors are absent, as in higher dilutions of the habitat material, the most numerous types can develop even though conditions are not optimal.

Use of solid medium. Enrichment cultures have in the past used liquid media almost exclusively. The best selective medium would theoretically be that which supported abundant growth of the desired bacterium but no others. This goal can rarely be achieved, particularly as regards the complete repression of unwanted forms. However, many media are sufficiently selective that a single cell of the desired type can develop even in the presence of many cells of other species. If the medium is actually this selective it can be used in the solidified state to obtain quantitative counts of the viable cells of the selected type in the natural habitat. The selective factors support more rapid development of cells of the type sought, making them identifiable as larger colonies. This assumes that the inoculum can be ensconced in the agar without damage by heat or other factors during the inoculation procedure. The separation of cells by a solid medium may protect sensitive types against vicinal competitors and permit less abundant and slower growing forms to obtain a share of nutrients greater than they could command in a liquid medium.

Kinds of Bacteria

By diluting rumen contents into rumen-simulating agar medium or into selective modifications of it, numerous previously undescribed bacteria have been isolated, identified, and counted. New genera abundantly represented include *Ruminococcus* (Sijpesteijn, 1948), *Butyrivibrio* (Bryant and Small, 1956*a*), *Succinivibrio* (Bryant and Small 1956*b*), *Succinimonas* (Bryant *et al.*, 1958*a*), and *Lachnospira* (Bryant and Small, 1956*b*). In addition, new species of *Bacteroides*, *Peptostreptococcus*, *Eubacterium*, *Clostridium*, *Selenomonas*, *Methanobacterium*, *Sarcina*, and *Lactobacillus* are at times common. Familiar types, some common and others rare, are *Streptococcus bovis*, *Corynebacterium acnes*, *Veillonella alcalescens* (*V. gazogenes*), *Desulfovibrio desulfuricans*, and *Lactobacillus*, *Fusobacterium*, and *Borrelia* species. It seems possible that most of the more abundant rumen bacteria have been encountered and given an initial characterization, although as selective methods are developed many additional less abundant types will undoubtedly be found.

Reasons for feeling that a large proportion of important rumen bacteria are known are as follows: (a) Many distinctive morphological types identifiable in the rumen contents by direct microscopic examination have been grown in axenic culture. (b) The prevalent gram-negative reaction and the morphology of the predominant organisms in stained smears of rumen contents are consistent with the morphology of the pure cultures and with the gram-negative nature of the majority of them. (c) Culture conditions simulate the rumen so closely that most rumen types should find them suitable for growth. (d) All colonies which appear in initial dilution series grow in agar subcultures and can be maintained in pure culture. (e) Metabolic processes such as fiber digestion, starch hydrolysis, protein degradation, hydrolysis of urea; production of acetic, propionic, butyric, lactic, formic, and succinic acids, methane, hydrogen, and carbon dioxide; and conversion of formate, lactate, succinate, carbon dioxide and hydrogen, all known to occur in the rumen, have been demonstrated in at least one of the pure bacterial cultures. Acceptance of these criteria leads to the tentative conclusion that the most abundant rumen bacteria have been cultured, although their characterization is still incomplete.

Numbers of Bacteria

The direct microscopic count is the chief method for ascertaining the total number of bacteria in the rumen. It is subject to a fairly high percentage error but in experienced hands can give consistent and reproducible values. The direct counts reported in the literature range

roughly from 1×10^{10} to 1×10^{11} cells per gram of rumen contents. In general, the more nutritious the ration the higher the direct count.

Solidified habitat-simulating and selective media, inoculated with appropriate dilutions of the natural habitat, give reliable culture counts if the restriction customary in plate counts is followed, namely, if the count is based on dilutions showing 30 to 300 colonies per tube. In the past many culture counts of rumen bacteria, including those in the author's laboratory, have been based on the highest dilution showing growth rather than on the most probable number derived from replicate tubes of several dilutions (Halvorson and Ziegler, 1933) or on tubes showing 30 to 300 colonies.

Most of the culture counts in habitat-simulating agar media, using glucose-cellobiose or an alfalfa-hay extract as fermentable substrate, are of the order of 1 to 2×10^9 per g when the animal is on a good hay ration (Bryant and Burkey, 1953). Animals on high grain rations show rumen culture counts up to 1×10^{11}. However, these are based on the highest dilution showing growth rather than on more statistically reliable methods. The great variations in counts are probably due to variations in sampling procedures, counting method, time of sampling after feeding, culture medium, exposure to oxygen during dilution, and other factors. When standardized methods are consistently employed, culture counts show a considerable degree of reproducibility (Bryant and Burkey, 1953; Huhtanen *et al.*, 1952).

The reason for the large discrepancy between the direct and culture count for rumen contents from animals on a hay ration has not been discovered. Perhaps the great majority of bacteria are dead. This fits the hypothesis that the total number of cells is near the maximal level achievable in a batch culture, yet if a large percentage of the bacteria in the rumen of cattle on a forage ration are dead, a similar proportion of dead bacteria would be expected in the rumen of cattle on high grain or concentrate. But in these animals the culture count is occasionally a high percentage of the direct count (Maki and Foster, 1957). It is possible that bacteria in cattle on a hay ration die more readily than those in animals fed much concentrate, or that they are sequestered in the fibrous materials in a fashion not easily disrupted by mixing. Until the discrepancy between the direct and culture counts is explained, the possibility that numerous rumen bacteria have still eluded cultivation *in vitro* cannot be excluded.

It is of interest that in a particular animal the total number of rumen bacteria, both dead and alive, is an inverse function of the rate of fermentation. This was first demonstrated by Walter (1952) in sheep sampled at various times after feeding. After a meal of the host had been largely fermented, the microbial population was maximal but the rate of fermentation was low due to exhaustion of substrate. Freshly ingested food served as substrate and increased the fermentation even though the microbial population was diluted. This emphasizes the fact that the amount of substrate normally limits the rate of the rumen fermentation.

The axenic cultivation, identification, and enumeration of bacteria in a natural habitat such as the rumen is extremely time-consuming. It is little wonder that most ecological investigations are restricted to a few components of the total flora. Development of methods permitting rapid and accurate identification would tremendously stimulate ecological studies. The fluorescent antibody technique holds promise for rapid identification and enumeration (Hobson and Mann, 1957) but has not yet been developed to the point of usefulness in the solution of rumen ecological problems.

The Rumen Protozoa

Identification

The protozoa have been identified by microscopic examination of fresh and stained preparations and classified according to the size of the cell and the type and location of the cilia, macro- and micro-nucleus, skeletal plates, reserve polysaccharides, and various spines and projections of the external cuticle. The protozoa are restricted to the rumen. Many kinds are common to several hosts but there are usually species differences which increase with the distance in the relationship of the hosts.

The most important genera of protozoa in the rumen and the ones which have received the most study are the holotrich ciliates *Isotricha* and *Dasytricha*, and oligotrichs of the genera *Endodinium*, *Diplodinium*, *Epidinium*, and *Ophryoscolex*. *Diplodinium* can be split into a number of genera (Kofoid and MacLennan, 1932; Dogiel, 1927).

Cultivation

Many of the protozoa have been cultivated *in vitro* under agnotobiotic conditions by following ecological considerations in the design of the culture medium and methods. Materials composing the food of the host, such as dried grass, cellulose, and ground cereal grains, have been used as food, with bicarbonate-carbon dioxide buffer plus appropriate balanced salts as the remaining ingredients. Rumen fluid or other materials produced in part by the ruminant are not required for the cultivation of the protozoa. Certain strains of *Diplodinium* have been grown as long as 22 months *in vitro* and species of *Endodinium* and *Epidinium* have been grown outside the host long enough to show conclusively that host secretions are not essential. The holotrichs have been cultured successfully for short periods but have not been maintained indefinitely *in vitro.*

Counts

The kinds and numbers of protozoa in the rumen are profoundly influenced by the diet of the host, and total counts may range from a few thousands to over a million per gram. Rumen contents from an animal on rations high in forages usually contain large numbers of holotrichs. They readily utilize sugars for synthesis of reserve starch and for growth (Sugden and Oxford, 1952), and soluble carbohydrates in the forage are assumed to supply their energy requirements in the rumen.

The genera of oligotrichs also occur in animals on a predominantly forage ration. *Diplodinium* ingests cellulose in large quantities, both in the rumen and in flask cultures, and can digest it. But cellulose digestion by the protozoa is probably not a large fraction of the total and exclusion of the protozoa does not seriously impede cellulose utilization by the total microbiota. Bacterial activity expands to replace that lost with the protozoa.

With host rations high in starch the oligotrichs develop to an extent which makes inescapable the conclusion that they are important in its dissimilation. All of them ingest starch very rapidly and with an excess of this substrate become so filled with grains that their internal structure is completely masked. The genera and species predominant in ruminants on concentrate rations vary with the ration but in nearly all cases one or several oligotrichs are prominent (Oxford, 1959; Gutierrez 1959).

Relation of the Protozoa to Rumen Bacteria

In recent years, through the investigations of Gutierrez (Gutierrez and Hungate, 1957; Gutierrez, 1958; Gutierrez and Davis, 1959) it has become evident that various rumen bacteria are used as food by the protozoa, presumably as a source of nitrogen. Studies indicate that proteinaceous materials of the feed may also be rapidly used by the protozoa (Williams, Gutierrez, and Doetsch, 1960). If particular bacterial types are selectively consumed, and Gutierrez' results indicate selection, the types ingested may be produced in large numbers and be responsible for a large turnover of substrate in the rumen, yet be present in numbers small as compared to less active bacteria not eaten by the protozoa.

IDENTIFICATION OF ACTIVITIES IN THE RUMEN

Nearly all the described pure cultures of bacteria isolated from the rumen have been subject to extensive study. The natural substrates utilized, fermentation products, and nutrient requirements are of ecological interest and have been collected in table 1. Inspection of the table shows that the substrates attacked include sugars, starch, cellulose, xylan, pectin, lactate, hydrogen, carbon dioxide, and certain volatile and nonvolatile acids. The important products are formic, acetic, propionic, butyric, and branched- and straight-chain acids from C_4 to C_6; lactic and succinic acids; carbon dioxide and hydrogen; ethanol; and methane. Figure 1 shows the probable interrelationships between the products formed in the pure cultures and those found as final products in the rumen.

The conversions of protein have not been considered thus far and will not be discussed in detail, in part because of lack of space but also because the individual reactions in the rumen are not known. The proteins in the food are converted rapidly to ammonia (McDonald, 1952) which is then reassimilated by the bacteria. Nitrogenous constituents such as amides in the feed are also rapidly converted to ammonia and are equally suitable as a source of nitrogen. The use of urea in ruminant rations reflects the capacity of the bacteria to convert nitrogenous materials to ammonia.

TABLE 1

A few characteristics of some important rumen bacteria

Organisms	Nutrients	Fermentation Products										
		CO_2	H_2	Formate	Acetate	Propionate	Butyrate	Valerate	Lactate	Succinate	Ethanol	CH_4
Cellulose decomposers:												
Bacteroides succinogenes	Rumen fluid, butyric, isovaleric, biotin, PAB*, CO_2			+	+					+		
Butyrivibrio fibrisolvens	Feed constituents (including starch) sugars	+	+	+	+		+		+		+	
Ruminococcus	Rumen fluid, volatile and higher acids	±	+	+	+				+	+	+	
Clostridium lochheadii	Feed constituents (including starch)	+	+	+	+		+		+		+	
Starch decomposers:												
Bacteroides amylophilus	Peptones			+	+					+	+	
Bacteroides ruminicola	Rumen fluid, xylan, CO_2			+	+					+		
Selenomonas ruminantium	Rumen fluid, lactate	+		+	+	+	+		+	+		
Succinimonas amylolytica	Peptones, CO_2				+					+		
Streptococcus bovis	Biotin								+			
Using other substrates:												
Bacteroides amylogenes	Peptones, sugars				+	+	+		+			
Lachnospira multiparus	Peptones, pectin	+	+	+	+				+	+	+	
Veillonella gazogenes	Peptones, lactate, succinate	+	+		+	+						
Peptostreptococcus elsdenii	Peptones, lactate	+			+	+	+	+				
Borrelia	Rumen fluid, sugars, CO_2	+		+	+				+	+	+	
Methanobacterium ruminantium	Rumen fluid, CO_2, H_2											+

* PAB = *para*-aminobenzoic acid.

The conversions of protein in the rumen are important in providing certain organic acids required as nutrients by several of the fiber-digesting species of bacteria (Allison *et al.*, 1958; Bryant and Doetsch, 1954). This phase of rumen ecology is receiving increased attention and, as the nutritional requirements and metabolic products of the rumen organisms are more exactly defined, many interrelationships between species will undoubtedly be uncovered. These may provide clues from which additional selective media can be formulated.

Measurement of Activities

The most difficult aspect of the ecological analysis of a habitat is to obtain quantitative information on the extent of the activities. A complete analysis should include not only a measurement of the total activity but also the activity of each component step. The degree of success and completeness in an ecological analysis can be measured by the degree to which the algebraic sum of the activities of the component parts equals the integrated activity of the whole. The measurement of total activity is thus essential

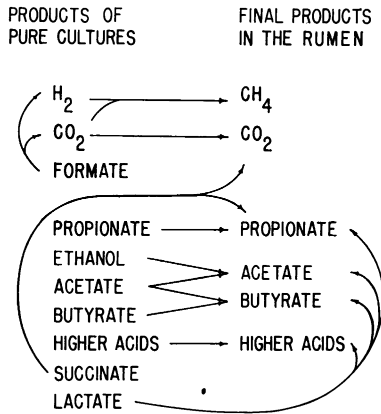


Figure 1. Probable interrelationships between fermentation products of pure cultures and final products in the rumen.

for a complete analysis. For many habitats this measurement is difficult, but for the rumen it is relatively easy.

Total activity. The volatile acid contents in the venous, arterial, and portal blood flow of the rumen, coupled with the rate of flow, were first used to estimate the total production of these fermentation products by the rumen microbiota (Barcroft, McAnally, and Phillipson, 1944). The values obtained were consistent with the assignment to the fermentation products of an important function in host energy metabolism. These findings initiated a large series of studies from which it has emerged that butyrate is metabolized to a considerable extent in the rumen wall (Pennington, 1952), thus decreasing the calculated production values below the true amount. Acetic acid is least susceptible to metabolic transformations in the rumen wall and is absorbed into the blood with the least change.

Another method for measurement of rumen microbial activity is the zero time rate method (Carroll and Hungate, 1954). This consists in (a) removing from the rumen a fairly large sample, as representative as possible, (b) immediately removing from the sample an aliquot which is treated instantly to stop all metabolic activity and in which the amount of the material of interest, whether substrate or product, is determined, (c) incubating the remaining sample at rumen temperature anaerobically, and (d) removing periodically additional aliquots in which the amount of the material is measured. A curve of concentration against time can be constructed; the slope at zero time represents the rate of change in concentration, *i.e.*, the rate at which the material is produced or used, at the instant of removal from the rumen. It is assumed to represent also the rate at which the material was produced or used by the sample in the rumen at the instant of withdrawal and therefore to be the actual rate in the rumen.

To the extent that the withdrawn sample is representative of the total rumen contents the measured rate applies to the whole rumen. Errors due to dilution of rumen contents by food and saliva, and the slowing down by accumulation of fermentation products are avoided.

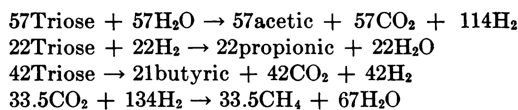
Manometric methods can be used to measure rumen activity (McBee, 1953) and in practice give values quite similar to those obtained by the zero time rate method. Manometry usually involves dilution of the contents with a liquid and thus modifies conditions from those in the rumen, chiefly in the direction of a more rapid and longer maintained activity *in vitro*, since the fermentation products are diluted. The manometric method has been used for an extensive study of the relative rumen fermentation rates in zebu and European cattle (Hungate *et al.*, 1960). The fermentation was estimated to account for 45 to 70 per cent of the food digested.

The manometric and zero time method both necessitate the estimation of total rumen volume in order to calculate total activity. Since the volume fluctuates according to the time of feeding, a considerable error is involved. This can be reduced by making numerous measurements and estimating an average volume.

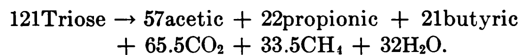
Another method for estimating the rumen fermentation is to measure the total methane formed in the intact animal. The proportion of methane to acid and carbon dioxide produced in the rumen can be determined in manometric experiments (Hungate *et al.*, 1960). On the assumption that the proportion of methane formed in the manometric vessels is the same as that in the rumen, the total acid and carbon dioxide production in the rumen can be calculated. The methane production in other parts of the alimentary tract is small (Hungate *et al.*, 1959) and can be neglected or subtracted.

The total methane production and the ratios in which the fermentation acids are produced have not been determined simultaneously on one animal, but on the assumption that they do not

differ markedly in different animals some estimates can be made. Blaxter and Graham (1955) found in a sheep that 1.69 moles of methane were produced per day. If the volatile acids were produced in the ratios, 57 per cent acetic, 22 per cent propionic, and 21 per cent butyric, as found for sheep by Halse and Velle (1956), methane constituted 16.8 per cent of the fermentation products and the acids 50.3 per cent. This theoretical value is obtained by assuming that the acetic, propionic, and butyric acids are derived from triose, and that methane and carbon dioxide are the other products. The theoretical stoichiometric relationships are:



$$57\text{Triose} + 57H_2O \rightarrow 57\text{acetic} + 57CO_2 + 114H_2$$
$$22\text{Triose} + 22H_2 \rightarrow 22\text{propionic} + 22H_2O$$
$$42\text{Triose} \rightarrow 21\text{butyric} + 42CO_2 + 42H_2$$
$$33.5CO_2 + 134H_2 \rightarrow 33.5CH_4 + 67H_2O$$



$$121\text{Triose} \rightarrow 57\text{acetic} + 22\text{propionic} + 21\text{butyric} + 65.5CO_2 + 33.5CH_4 + 32H_2O.$$

The proportions of the products exclusive of water are: total acid, 50.3 per cent; carbon dioxide, 32.9 per cent; and methane, 16.8 per cent. These ratios resemble those found in manometric measurements of fermentation products of rumen contents. On the assumption that they are applicable to the sheep studied by Blaxter and Graham (1955) the amounts of the various acids produced in the sheep which formed 1.69 moles of methane (from 1 kg of food digested) would be 2.88 moles (173 g) acetic, 1.11 moles (82 g) propionic, and 1.06 (93 g) butyric. These acids total 348 g and, with the CO_2, CH_4, and H_2O that are also formed, account for about 55 per cent of the food disappearing during passage through the sheep.

Similarly, Armsby and Moulton (1925) found that a steer fed 7 kg of timothy hay formed daily 8.9 moles of methane. The relative proportions of acids produced by rumen contents from cattle on a hay ration (Carroll and Hungate, 1954) were 60 per cent acetic, 21 per cent propionic, and 19 per cent butyric, in which case the acids would constitute 50.5 per cent of the total products on a molar basis and the methane 17.3 per cent. The actual percentage methane formed in cattle on a grass hay ration (Hungate *et al.*, 1960) was 18.8 per cent. The 8.9 moles of methane reported by Armsby and Moulton (1925) would thus correspond to 936 g acetic, 404 g propionic, and 435 g butyric acid, which, with the calculated amounts of CO_2, CH_4, and H_2O formed, would amount to a total of 2,790 g. The weight of food disappearing during passage through the steer studied by Armsby and Moulton (1925) was as much as 4 kg. In this case the calculated volatile acids and other fermentation products account for about 70 per cent of the food digested and metabolized in the host.

Activities of Individual Types

Individual activities can be considered from two standpoints, first, the magnitude of specific biochemical reactions and, second, the activity of particular organisms. The two are not unrelated in that if the total biochemical activity can be broken up into constituent parts, these may be referable to certain organisms.

Specific biochemical reactions. It has often been assumed that lactate is an important intermediate in the rumen fermentation of forage. This idea probably developed because propionate was known to be an important product of microbial lactate conversions and the presence of propionate as an important rumen fermentation product could thus be explained. In addition, there are a number of reports that lactate accumulated in the rumen soon after feeding certain fresh forages or when the animals received a ration of flaked maize. However, bacteria producing lactic acid and bacteria consuming it were not usually encountered in high numbers in the rumen (Gutierrez' 1953 paper is an exception), and the experiments of Johns (1949) indicated that production of propionic acid in the rumen might occur via a decarboxylation of succinate.

Application of radioisotope techniques to determine lactate turnover in hay-fed cattle has disclosed that lactate is an intermediate for less than 1 per cent of the total converted substrate (Jayasuriya and Hungate, 1959). In grain-fed animals it is more important, accounting for perhaps $\frac{1}{4}$ to $\frac{1}{10}$ of the total. The studies of Gutierrez *et al.* (1959) suggest that *Streptococcus bovis* is important in the primary conversion of starch to lactate in their animals. The further conversion, instead of occurring solely via a propionic fermentation, is by *Peptostreptococcus elsdenii*, with production of C_2 to C_6 aliphatic acids. Quantitative measurements on the extent

to which these species are responsible for the conversions would be extremely interesting.

Another biochemical intermediate in the rumen is formate. Beijer (1952) found that it was rapidly converted and that methane was formed in the amount expected if the hydrogen of the formate was used for production of methane. The experiments of Doetsch *et al.* (1953) suggested that formate was decomposed to hydrogen and carbon dioxide. Measurement of the rate of conversion of formate (Carroll and Hungate, 1955) showed a capacity on the part of rumen contents to convert added formate at a faster rate than it could be expected to appear as an intermediate. Hydrogen was shown to be utilized readily by rumen contents (Sijpesteijn and Elsden, 1952; Lewis, 1954).

Various rates concerned with methane production are shown in table 2. The capacity for conversion of added hydrogen is even greater than that for added formate and more than sufficient to account for the rate at which methane was formed in the rumen. The theoretical maximal amount of formate that could appear as an intermediate in the rumen fermentation, *i.e.*, the C atoms not accounted for in acetic, propionic, and butyric acids, is insufficient to account for the amount of methane formed. This indicates that hydrogen not arising from formate is also used in reducing carbon dioxide. The radioisotope experiments of Carroll and Hungate (1955) show that the rumen methane arises chiefly through CO_2 reduction but do not give information on the source of hydrogen.

TABLE 2

Conversions of methane precursors in the rumen

	μmoles/10 g/min
Measured rate of endogenous CH_4 production	0.92
Theoretical endogenous formate production	2.69
Measured exogenous formate conversion	6.73
Measured exogenous H_2 conversion	14.20
Measured rate of CH_4 production *in vitro* by pure culture with culture count equal to that in the rumen	>0.92

Individual microbial activities—direct estimates. Oppermann *et al.*, (1957) have demonstrated the presence in rumen contents of methanogenic bacteria capable of using some of the volatile acids as hydrogen donors for reduction of carbon dioxide but cells of these types do not appear to be abundant. The most numerous methanogenic bacterium found thus far in the bovine rumen is *Methanobacterium ruminantium* (Smith and Hungate, 1958). It produces methane from hydrogen and carbon dioxide and attacks formate with incomplete utilization but does not utilize any of a large number of other substrates tested. An unpublished preliminary test by Smith with *M. ruminantium* showed that the number of cells demonstrable by culture count of rumen contents was more than adequate to account for the rate at which methane was produced in the rumen. The rate per cell was calculated from manometric experiments with the pure culture, with numbers in the flask determined from a quantitative culture count. These preliminary experiments suggest that the energy-yielding reaction performed in the rumen by *M. ruminantium* is the reduction of carbon dioxide to methane with molecular hydrogen at a rate equal to the rate of total methane production.

Some experiments have tested the fermentative activity of the rumen protozoa. The holotrichs have been separated from accompanying rumen organisms and their production of carbon dioxide, hydrogen, and various acids has been measured (Gutierrez, 1955). The activity per cell has been determined and, on the assumption that the rate in the rumen is similar to that *in vitro*, the activity in the rumen has been calculated from the direct count of the holotrichs. Rough estimates indicate that the total protozoa perform 20 per cent of the total fermentative activity. Extensive studies on additional species are needed as a basis for more exact estimates. There is some question whether the protozoa studied display the same rate of activity in the Warburg vessels as in the rumen and there is need for additional experiments in which the protozoa are removed directly from the rumen and their activity is determined immediately without any added substrate.

Individual microbial activities—indirect estimates. Inability in the present state of knowledge to relate many types of rumen organisms to particular activities makes the direct determination of individual activity difficult. It seems possible that with further study the fermentative activity of bacterial and protozoan species can be related

to the rate at which they are produced and that this in turn can be determined from the numbers in the rumen. The number of microorganisms of a certain kind in the rumen is one parameter needed to measure its importance. The rate of turnover and the fermentative activity per cell needed to produce a new cell are the other measurements needed for calculation of the activity of that particular species.

The turnover of the bacterial population not serving as food for the protozoa is probably identical with that for rumen liquid and the small particles suspended in it. The turnover for small particles is slightly faster than for the large food particles in rumen contents (Castle, 1956). Because the larger protozoa settle rather rapidly when well fed, their turnover in the rumen may be slower than that of the bacteria. Reliable measurements of turnover of the rumen liquid are not available, but the turnover rate for hay, measured by the dyed particle method, is about 24 hr for cattle (Lenkeit, 1930). If the turnover rate for the microorganisms be assumed to be at least equal to this value, their growth each day must produce at least as many microorganisms as are contained in the rumen. On the assumption that the distribution of types among the noncultivable (dead?) bacteria is the same as the distribution among the cultivable ones, the proportion of viable bacteria of a particular type is a rough index of their activity. It is evident that differential rates of death, whether due to ingestion by the protozoa or to other factors, greatly influence the quantitative significance of viable culture counts. In these cases it is especially necessary to determine turnover rate for the individual bacterial strains.

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