MICROBIAL ECOLOGY AND ACTIVITIES IN THE RUMEN: PART I

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I. INTRODUCTION

A. Herbivorous Animals and the Rumen

Nonpathogenic microorganisms are associated with all higher forms of life on earth. These microbes in many instances have a saprophytic or commensal relationship with the host animal and research with gnotobiotic animals has shown that, in such cases, the animals can live without associated microbes. In the herbivorous "animals" (and this term includes insects), though, a true symbiotic relationship between host and microbe exists and the microorganisms are essential to the life of the animal. This occurs because the structural polysaccharides of plants cannot be degraded by animal digestive processes and the herbivore relies on microbes in the gut to convert these polysaccharides to products which it can utilize for energy and growth.

Conditions suitable for microbial growth, particularly with respect to residence times of digesta, need to be provided in the herbivorous digestive tract. These are obtained in some cases by an enlargement of the large intestine, where microbial action takes place after the normal gastric digestive system. In the ruminant herbivores and some related animals, a separate organ has evolved in which the microbial action takes place before the gastric digestion (Figure 1). Further microbial growth occurs in the cecum and large intestine, but the nutrition of the animal microbial activity here is of minor significance compared with that in the rumen.

Food first enters the rumen-reticulum compartment. Although there are some differences in the digesta in the rumen and reticulum sections of the organ, the contents are intermixed and, as will be seen later, sampling methods are often imprecise in the location of the sampling point, so the whole organ is generally treated as one and referred to as the "rumen". This is large in relation to body size (approximately 5 to 10 ℓ in sheep and 100 to 150 ℓ in cattle) and, because passage from the rumen is selective by particle size, while the detention time for liquids is some 10 to 24 hr, large food particles can be held in the rumen for 2 or 3 days. Sufficient time is thus available for slow microbial degradation of resistant plant fibers. Flow of digesta through the rumen is mediated partly by water drunk by the animal, but more by a copious production of saliva, the flow of which is usually much greater than the flow of drinking water. The saliva contains salts, including bicarbonates and phosphates which, with ammonia, buffer the rumen fluid against the fermentation acids to a pH of about 6.5, which permits growth of the microorganisms. Heat of fermentation of food and, to a greater extent heat from the normal aerobic tissue metabolism of the animal, keeps the rumen contents near 39°C, and the contents are mixed by muscular contractions and relaxations of the rumen wall.

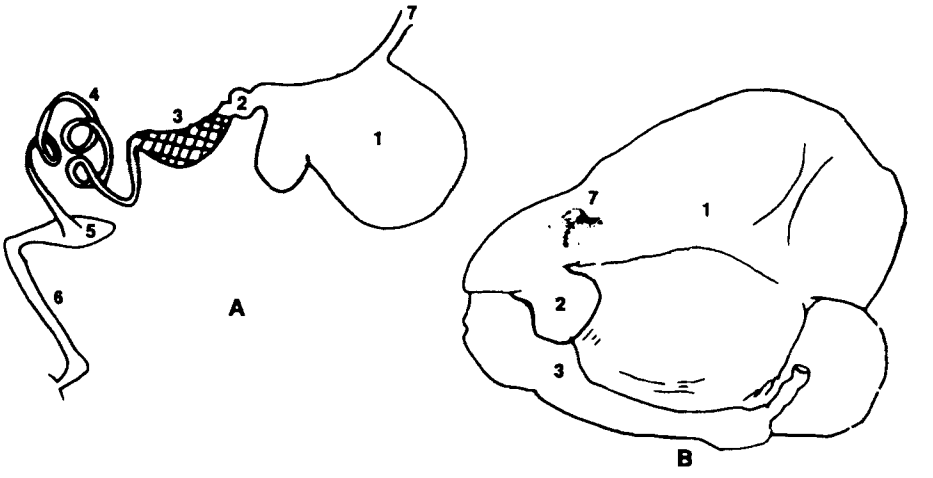

FIGURE 1. Ruminant digestive system (diagrammatic). (A) relative positions, but not sizes, of rumen-reticulum (1), omasum (2), abomasum (3), small intestine (4), cecum (5), large intestine (6), esophagus (7). The intestines are very much longer in proportion to other organs. (B) the rumen-abomasum size as in the animal.

The food residues, microorganisms, and rumen liquid flow onward to the omasum where salts and fatty acids and some water are absorbed, and the more concentrated solids pass on to the abomasum which is a true gastric organ and where normal digestive processes begin.

While the presence of the rumen and its microorganisms and something of the microbial products had been known for many years, it was not until the mid-1940s that the true role of the microbes in ruminant metabolism was elucidated.[1,2] The reactions occurring in the rumen are shown in Figure 2, and essentially there is an anaerobic fermentation with production of acetic, propionic, and butyric acids which are absorbed from the rumen into the blood stream and from there to the animal body, where they are converted to glucose and fats which the animal can use as sources of energy or body components. The fermentations produce excess hydrogen which is removed by conversion with carbon dioxide to methane, and eructated by the animal. Energy from the fermentations and methanogenesis is used for production of microbial cells and these pass on to the abomasum and small intestine where digestion of the microbial protein occurs. In essentials, then, the rumen microorganisms convert foodstuffs into volatile fatty acids which form the animal's energy sources and microbial cells which are its protein source. Excess ammonia formed by microbial action is absorbed and converted to urea. The urea can be either excreted in the urine and lost to the animal, or recycled in the saliva or through the rumen wall to be hydrolyzed to ammonia and again become available to the microorganisms. Minor products of microbial metabolism such as B vitamins become available to the animal as the microbes and rumen fluid pass through the abomasum and intestines.

The newborn and young ruminant is, in fact, a nonruminant. The rumen is small in relation to the abomasum and digestion of milk is by the same process as in any other young mammal, and a mainly lactobacillary-streptococcal, lactate-fermenting type of intestinal flora is present. With increasing ingestion of solid food as the animal grows, and finally with weaning on to a solid diet, the rumen and its associated complex flora develops and the metabolism of the animal changes to utilize microbial fermentation products rather than sugars. Since the development of the rumen and its flora depends on diet, with the different feeding systems and ages of weaning now prevalent on farms,

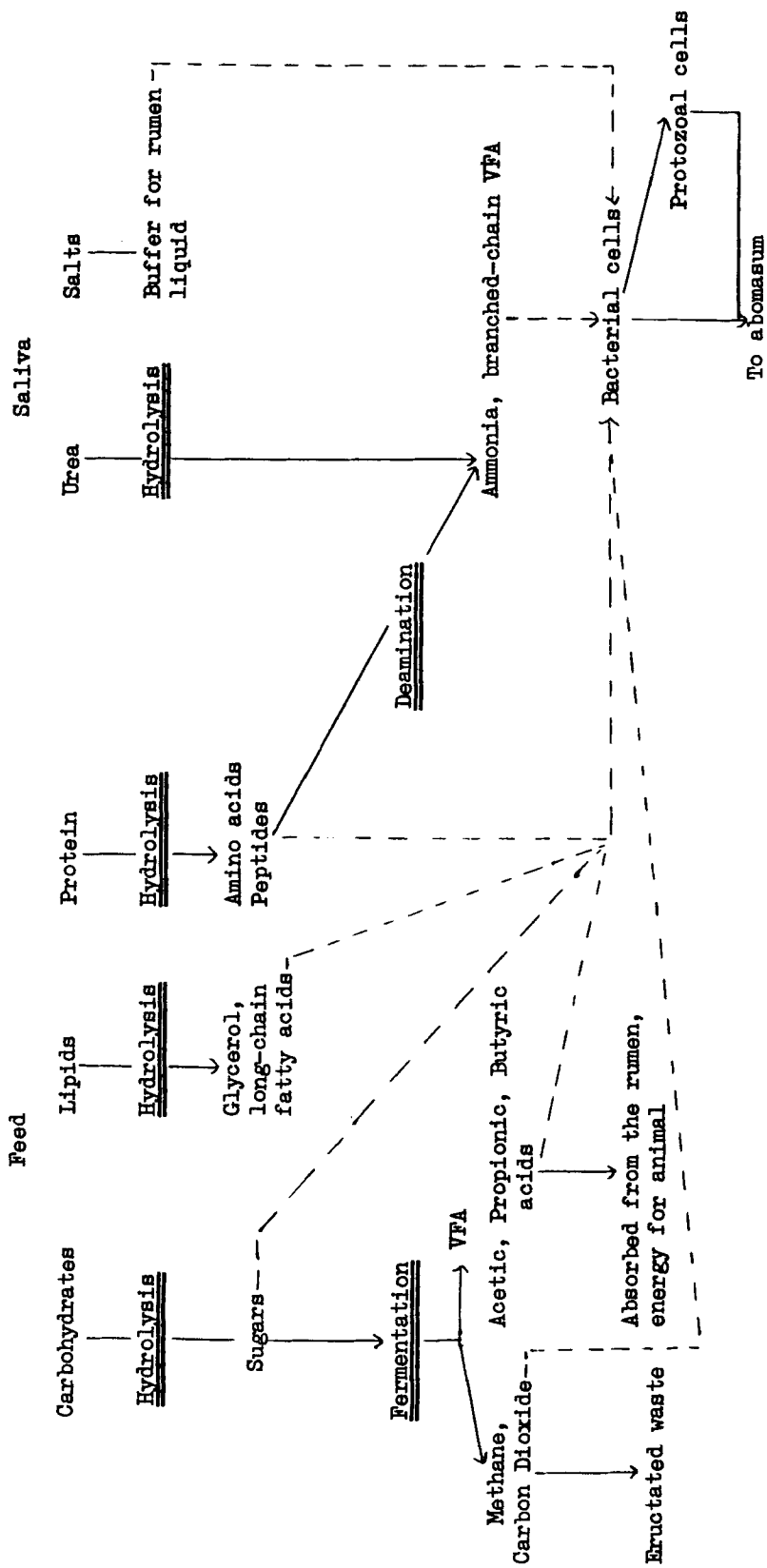

FIGURE 2. The principal reactions in the rumen. Long-chain fatty acids are hydrogenated if unsaturated. The acids are, in part, incorporated into bacterial cells and become available to the animal in the intestines, or pass to the intestines where they are absorbed. Some ammonia is absorbed from the rumen. VFA: volatile fatty acids; ---→ Incorporation into bacterial cells, in part at least; ——→ Metabolic pathways.

no particular age can be given for the final development of the adult rumen flora. While some work has been done on the rumen flora of young animals, the rumen microbiologist is principally concerned with the adult rumen and the reactions which enable digestion of food to take place. The interest in the rumen system has arisen principally because most animals of agricultural importance (except pigs) are ruminants or near ruminants. The emphasis in agriculture is on the intensive rearing of animals. To produce animals that mature quickly, grow to sizes, or give milk production, in excess of the wild ruminant means giving food in larger than natural quantities or in composition different from the natural diet. The rumen system and the microbes evolved to digest the wild ruminant diet (generally sparse, often of poor quality). Modern farm feeds are very different from this and can bring about digestive disturbances and illness in the animal. Knowledge of the rumen and its microorganisms can help to overcome these difficulties and to increase the overall efficiency of digestion in the ruminant. However, the rumen microbial ecosystem is not unique; not only are similar organisms and reactions found in the gut of nonruminant herbivores, but they are found in the intestines of omnivores such as man and anaerobic bacteria can be involved in sepsis and disease. Rumen-type microbial systems also occur in muds and waters, and in the anaerobic digester first used for sewage purifications and now projected as a source of biomass energy. The investigation of all these systems has developed from the techniques used in rumen microbiology and the realization that these techniques opened up a new field in microbiology, that of what might be called the "strictly" or "highly" anaerobic bacteria.

Just as the 1940s saw the discovery of the essential role of the fermentation acids in ruminant nutrition, so the same period saw the beginning of true rumen microbiology. Previously, bacteria had been isolated from the rumen but they were of common, previously encountered types. Although the microscopists had described bacteria attached to, and seemingly digesting, fibers and starch granules, the isolated bacteria did not seem to fit into these roles. With increased biochemical analysis of rumen contents it was realized that the habitat is highly anaerobic and the rumen gas is principally carbon dioxide and methane with only a trace of oxygen. About 1948, Hungate and Gall and co-workers in the U.S. and Sijpesteijn in Holland applied this consideration of a highly anaerobic habitat to culture, and isolated the first true rumen bacteria whose functions, such as cellulolysis, could be equated with reactions in the rumen. The Hungate medium simulated the rumen habitat better than the others and so this, and the associated techniques for preparing media and transferring inocula, became widely accepted as the basis for rumen microbiological studies.

In the 1950s and early 1960s there followed a highly productive period of research in which, although the number of laboratories concerned was small, bacteria with reactions representative of all those known in the rumen were isolated and grown in batch and continuous culture. These bacteria were of new genera or species and while type species were defined, continued research showed in most cases many variants and subspecies.

Typical of the bacteria isolated during this period were the cellulolytic bacteria, *Ruminococcus albus* and *flavefaciens* and *Bacteroides succinogenes*. The polysaccharides of fibers are, in general, difficult to hydrolyze, but xylan-fermenting *Butyrivibrio fibrisolvens, Bacteroides ruminicola, Eubacterium ruminantium* and pectin-hydrolyzing bacteria were also found. Feeds containing starch came into prominence with the intensive cattle rearing introduced in this period and a number of rumen bacteria were shown to hydrolyze starch, among them the new species *Bacteroides amylophilus*. The ruminal hydrolysis of feed lipids was discovered and a new genus of lipolytic and glycerol-fermenting bacteria, *Anaerovibrio lipolytica*, isolated. However, the bacteria responsible for the rumen hydrogenation of unsaturated long-chain fatty acids also found about this time had to await later discovery.

In pure culture these bacteria gave rise to one or more of the fermentation products

found in the rumen — carbon dioxide and acetic, propionic, and butyric acids. They also, however, produced other products seldom found in the rumen in any quantity: formic, lactic, and succinic acids, ethanol, and hydrogen. There also was found to be an extensive secondary fermentation, where bacteria such as *Veillonella alcalescens, Megasphaera (Peptostreptococcus) elsdenii* and *Selenomonas ruminantium* var. *lactilytica* converted lactate or succinate to acetic or propionic acid, carbon dioxide, and hydrogen, and where hydrogen and carbon dioxide were converted to methane by *Methanobacterium ruminantium*. Later it was found that pure culture actually led to artifacts in fermentation products that would not occur in mixed cultures.

Generally speaking, the bacteria found to perform other functions in the rumen, such as proteolysis and deamination, were identical or closely related, to these mentioned above. Cultural studies identified nitrogen sources of the bacteria and the growth factors present in the rumen fluid used as an additive to the original culture media.

The metabolic properties of these bacteria, apart from the many instances of their extreme sensitivity to oxygen, did not seem to be unusual. Hexoses were converted to pyruvate by the usual Embden-Meyerhof route and pyruvate converted to the final fermentation products by pathways found in other, nonrumen, bacteria. By and large morphological and ultrastructural properties seemed no different from other bacteria, although it was early noted that the rumen anaerobes were preponderantly Gram negative.

The rumen ciliate protozoa presented quite different problems in their study. Because of their large size, their morphology had been described many years before the rumen bacteria were first cultured and their ability to ingest starch granules and fibers had also been noted. However, although because of their size they could be separated from rumen fluid, attempts to culture them were only partially successful in that, though some species could be kept alive for varying periods in vitro, this was only in media containing bacteria and undefined substrates such as grasses, grains, and flour. Washed suspensions were used to determine the fermentation products of, and substrates used by, the ciliates. These were found to be very similar to those of the bacteria, and the ciliates were also found to be very strict anaerobes. However, though antibiotics were included in washing solutions and suspensions to kill the bacteria always associated with the protozoa, the role of bacterial enzymes in the reactions attributed to the protozoa remained a source of argument, as did the assessment of the quantitative role of the ciliates in the overall rumen function. On the other hand, predator-prey relationships between protozoa and bacteria and between species of protozoa were described in this era.

In this very brief introduction it has been necessary to deal summarily with the many aspects of rumen microbiology and physiology investigated in the period to the mid 1960s. However, during this period the major metabolic pathways and the microorganisms concerned in them were determined, and the foundation laid for the more detailed studies of pure cultures, and of the interactions in mixed cultures which followed.

Hungate's book, *The Rumen and its Microbes,*[3] gives an extensive description of the findings up to about 1964, and other reviews[4-8] describe in more detail different aspects of rumen microbiology and of the microbiology of some of the related ecosystems determined during this period and up to about 1970. It seemed to the present authors that not only would it not be possible in a limited space to cover this "basic" rumen microbiology again, but indeed it was not necessary, as the reviews mentioned gave adequate coverage. The present paper attempts to summarize the developments of the last decade. Inevitably, even a review of the last decade or so will fail to discuss some important findings. We trust that these omissions will not be too numerous, however, and hope that the emphasis we place on different aspects, which naturally reflects to some extent our own particular interests, will be a fair account of the efforts of workers around the world who have contributed to this important subject.

II. METHODS FOR ENUMERATING RUMEN ORGANISMS

Since the ruminant depends on the rumen microbes for its energy and protein supply, it is obvious that the numbers and activities of the microorganisms are of great importance. The number of organisms is, however, extremely difficult to determine and it is probably true to say that the number, or even the total mass, of microorganisms cannot be determined because of the complexity of the system. What may be made is some estimate accurate enough for a particular purpose. For instance, if one is concerned with the amount of bacterial cell protein leaving the rumen, it is not necessary to know the proportions of viable and nonviable cells or the numbers of bacteria attached to the rumen wall. An assessment of the concentration of microbial protein in the rumen liquid and the volume of this liquid flowing from the rumen to the abomasum is what is required. Since the rumen is a kind of continuous culture, flow of liquid through the system is involved and measurement of this flow is necessary in many instances when the production of microbial mass is to be determined. As already pointed out, the largest proportion of the liquid flowing through the rumen is saliva and the production rate of this cannot be readily determined. The rumen turnover or detention time has to be measured by adding some internal marker to the system and measuring its rate of decrease in concentration in the rumen, or the time it takes to pass into lower portions of the gut. However, complications arise in that while liquid and small particles (e.g., free bacteria) have the same detention time, particles larger than a few millimeters are detained for varying times depending on particle size and rate of disintegration. Markers for liquid or particles are used depending on which particular flow rate is to be determined. Polyethylene glycol[9] is a common marker for liquid flow. Chromic oxide[10] for solids, ^{51}Cr EDTA for liquids, and ^{103}Ru-phenanthroline for solids are some of the other markers used. Lignin, present in plant fibers and assumed to be undegradable, also has been used as a marker for solids in digestion studies.[9,11]

Although the rumen is a continuous culture, it never attains the steady-state of the laboratory pure culture with one limiting substrate. The complexity of microbial substrates (in all except some synthetic laboratory feeds or specialized farm feeds such as urea-molasses), and the fact that different substrates will be degraded by different bacteria at different rates or by diauxic growth, is only one of the many factors normally leading to variations in numbers and activities of rumen microorganisms during the day. These and other factors influencing the continuous culture of the rumen have been discussed previously[5,12] and some will become apparent later in this paper, but they are mentioned here to show that determination of microbial mass in the rumen is difficult and many methods have been, and are, used.

A. General Considerations

Apart from complexities of the actual enumeration, problems arise in obtaining accurate samples as one is dealing with an animal. The rumen-reticulum is a large organ and although its contents are mixed by contraction and expansion of the rumen wall, there can (depending on diet and kind of animal) be some stratification of microorganisms and feed particles in the semifluid mass, and there can be differences between the rumen proper and the reticulum portion. However, the latter are, through necessity, usually ignored or attempts are made to mix samples from different areas or to mix the whole of the contents of the reticulo-ruminal sac. In general, as previously mentioned, the whole sac is referred to as the rumen and no attempt is made to distinguish the two portions; of course, natural movements continually intermix the contents.

While introduction of permanent cannulas through the rumen wall and skin of the animal can render the rumen contents accessible to sampling, such cannulation requires surgical skill and equipment and can be done only on a small number of animals usually

kept in pens or in other confined circumstances. A flexible stomach tube can, with care, be inserted into and positioned in the rumen, so rumen contents can be sampled in this way in normal animals. But the method is again limited in application; the animal must be of reasonable size and needs to be held still in some way while being sampled. There is also the possibility of contamination of the rumen sample with saliva.

The use of a cannula or stomach tube means that repeated samples may be taken from the same animal, but, while it was found possible during extended studies of animals in natural habitats to round up at intervals and sample by stomach tube the same sheep on the Scottish mountains, it was impossible either to round up or stomach-tube wild deer or reindeer. The only method was to shoot deer of approximately the same age and condition at the same time of day over the year and to dissect out, and sample from, the whole rumen.[13] Sampling from the dead animal has the advantage that the contents can be thoroughly mixed and the volume determined if necessary. On the other hand, dissection and sampling must take place reasonably soon after death or changes in the rumen contents may occur. A mobile laboratory van was used in the field experiments mentioned above and 20 to 25 min between death and processing of rumen contents was aimed for. As far as could be ascertained the rumen contents were representative of those in life. Under laboratory conditions, much less time between killing and sampling would be expected.

Although killing the animals and dissection of the rumen is probably the only way of ensuring that complete and representative samples of the whole rumen contents are obtained, the method is expensive and only one sample, representing the state of the rumen at one particular time, can be obtained. Sampling by tube through the mouth or cannula can be repeated, but the samples may be unrepresentative in that larger particles, particularly fibers, may not be taken into the tube and, indeed, may act as a filter on the aperture(s) of the tube. The location of the end of the tube may also be indefinite and even if a number of samples are taken they may not be representative of the average rumen contents. To overcome some of these difficulties, a method whereby the rumen contents were recirculated and mixed by an external pump attached to the cannula was used in some laboratory experiments,[14] but the system had mechanical difficulties and could be of only limited application.

Problems of sampling rumen contents thus exist, as do problems of sampling the rumen wall flora, but if samples are taken in the same way for one animal or for a number of animals on the same kind of feed, while absolute values may be in error, relative values may be assessed more accurately and, in many cases, relative values such as variation in microbial numbers with time are what is required. Measurement of rumen outflow may require cannulation for sampling at the abomasum or duodenum and this introduces further difficulties which mean that very few animals can be used in this type of experiment.

B. Counting Organisms as Individuals

"Total" counts of bacteria and protozoa are so-called to distinguish them from "viable" counts, but the total concentration of organisms in the rumen sample is not determined. Bacteria and protozoa exist in two phases in the rumen: in the liquid, and attached to particles of feed. While the former may be counted accurately, there is no method for entirely freeing the attached organisms.

In some early attempts to count free and attached bacteria, Minato et al.[15] used washing with a mineral solution by itself or with Tween 80® to detach the bacteria from feed particles separated from rumen fluid by filtration through gauze. In other experiments, blenders (Waring® or similar) have been used to break up feed particles and detach bacteria,[16] but too vigorous maceration will kill or tend to disintegrate bacteria, and protozoa are relatively easily broken by shearing forces. Various shaking methods also

have been used. The most generally used method for routine counts is to dilute the rumen sample in an anaerobic solution (e.g., the diluting fluids used in cultural counts) under carbon dioxide and often containing about 0.1% Tween®. The suspension is then vigorously shaken by hand or on a vibrating shaker for a few seconds to a minute. This removes at least the lightly attached bacteria, and suitable dilutions can then be made for counting, either total or cultural.

Total bacterial counts have been made using standard 0.02 mm deep counting chambers[17] or by stained, fixed film methods.[18] However, Hobson and Mann[19] introduced the use of the Coulter Counter® for counting rumen bacteria. This is much quicker and less fatiguing to the operator than visual counting, but it counts bacteria only by size while visual counting allows distinction by morphology. The method did not prove successful for ciliate protozoa.

For ciliate protozoa, a system of washing the organisms from entrapment in feed fragments held back on sieves and adding these to the separated rumen fluid was used in tests of a counting method,[20] but this is unsuitable for routine use and probably would not remove the protozoa now known to be actually in cavities in grass fibers. The ciliates generally have been counted in deep (1 mm) large counting chambers filled with a low dilution of rumen fluid in a glycerol solution which prevents these large organisms from settling during dilution and the filling of the chamber.[20,21] Another method is to count the protozoa as they are moved singly by a fine pipette from a drop of diluted rumen fluid of known volume.[22]

The total counts of bacteria count live and dead organisms, while viable counts can, of course, count only living organisms. Total and viable counts from pure cultures in the laboratory are usually within a few percent of each other if the bacteria are in the exponential phase of growth. Rumen viable counts, however, except in the case of some animals fed on all-grain diets,[23] are always only a fraction ($<25\%$) of the total count, and the lowest counts are usually from animals on all-fiber diets. The apparent low viability of rumen bacteria may be real (see Reference 5 for a discussion) or it may be an artifact due to deficiencies in media or methods, although by using a plating technique which overcame a temperature stress on the bacteria produced in the standard roll-tube methods, Leedle and Hespell[24] only increased the viable counts from 25 to 35% of the total count. The viable counts mentioned here as being compared with total counts are of the type done in media containing a variety of carbohydrate substrates which, it is hoped, will enable all metabolic types of bacteria to grow. These may be termed "total-viable" counts. Counts of bacteria with specific properties (e.g., xylan utilization) are attempted using media with only the one energy source, as discussed later.

Most viable counts of bacteria probably are still done by the anaerobic tube methods introduced and described by Hungate.[25,26] Modifications have been made in that threaded glass tubes with a screw cap and a central hole and a butyl rubber insert are now commercially available to replace the test tube and rubber bung. The former give better and more reproducible closure than the latter. The screw cap tubes also make it easier than with ordinary or hollow rubber bungs to autoclave basal media under CO_2 in the tubes and add other constituents including the inoculum by injection of solutions with a fine hypodermic needle pushed through the tube closure. Smith and Hungate[27] were probably the first to describe such a method in their isolation of *Methanobacterium ruminantium,* but it is coming into more general use not only for methanogens but also for other rumen bacteria.

An elaboration of the hypodermic needle injection method for the methanogens with a system whereby tubes of medium, before and after autoclaving, are evacuated and then filled with various gases or gas mixtures (e.g., $H_2 + CO_2$) under pressure from a manifold connected by multiposition taps to a vacuum pump or gas cylinders, was described by Balch et al.[28] This system has been adopted in some other laboratories.

The Hungate system and its modifications do not allow the use of plate cultures and this can be a disadvantage in some experiments. An anaerobic box technique in which all operations are carried out in a closed system with an airlock for entry and exit of apparatus and in which petri dishes of medium can be exposed for manipulation was used for human intestinal anaerobes by Draser,[29] and similar boxes have been used by others. Such boxes had an atmosphere of nitrogen or carbon dioxide, but it was difficult to produce the very low oxygen atmosphere needed for some anaerobes. More recently, the introduction of flexible plastic isolators has made the boxes easier to use than the earlier steel and wood ones, and the use of a carbon dioxide plus about 1% hydrogen atmosphere recirculating over a platinum or palladium catalyst has enabled very low oxygen tensions to be obtained and maintained. This permits the culture on plates of the most exacting anaerobes.[28] However, for much routine cultural work the tube technique is simpler and quicker.

The basal medium constituents have remained, in general, very similar to those of Hungate's original medium,[25] but with more knowledge of the requirements of the bacteria, rumen fluid as a source of growth factors has been replaced by known substances such as the straight and branched-chain volatile fatty acids and hemin.[30]

Reducing agents generally used are still sulfide or $-SH$ compounds, and of the latter, dithiothreitol has become a fairly widely used agent, usually with cysteine. A newer reducing agent is titanium (III) citrate. This was introduced by Zehnder and Wuhrmann,[31] who tested it with a *Methanobacterium, Clostridium formicoaceticum,* and *Bifidobacterium bifidum.* Used in concentrations of 0.6 to 2×10^{-3} *M*, it was nontoxic, but it did appear inhibitory to anaerobic growth of two facultative anaerobes. Jones and Pickard[32] compared cysteine hydrochloride, dithiothreitol, and titanium (III) citrate as reducing agents in a complex medium for growth for ten rumen anaerobes. The titanium gave the lowest E_h (-302 mV at 5×10^{-4} *M* and -404 mV at 2×10^{-3} *M*), but maximum growth of the bacteria was generally lower than with cysteine. The only exception was a strain of *Bacteroides amylophilus.* Growth using dithiothreitol alone was in most cases much lower than that with cysteine. The authors concluded that titanium (III) citrate can be used in media for some rumen bacteria when the exclusion of a sulfur-containing reducing agent is required. Brock and O'Shea[33] suggested ferrous sulfide as a reducing agent after testing it with a nonrumen *Methanobacterium* and a *Clostridium.* As the sulfide is insoluble, the quantity added is not critical, which could be an advantage in preparing media. Both these reducing agents need to be prepared in the laboratory.

C. Methods for Determining Microbial Mass

These methods can be used on whole rumen samples without separation of the microorganisms and should determine both free microbes and those attached to particles.

1. Protein Determinations

What is required for many experiments is a determination of microbial protein produced in and leaving the rumen. Protein determinations, as such, are complicated by the difficulty of distinguishing between feed and microbial protein, and only when the feed is synthetic with nitrogen supplied as urea and with purified carbohydrates, as in the experiments of Hume et al.,[34] can determination of protein in the digesta (in this case as nitrogen in a tungstic acid precipitate) be used as a measure of microbial protein. In early experiments on protein breakdown in the rumen, feed and microbial proteins were determined by using a purified feed protein which could be distinguished in some way, such as solubility, from microbial protein.[35,36] When normal feeds are used, some method of marking microbial protein is needed.

Diaminopimelic acid (DAP), found by Work,[37] appears to occur only in cell walls of bacteria.[38,39] Although the DAP content of different types of bacteria may vary, the amount of this acid in the mixed rumen bacteria was found to be fairly constant in animals on a fixed diet and was used to determine rate of synthesis of bacterial protein or amount of protein in the rumen.[40] The ratio of DAP to protein N is determined for a sample of mixed bacteria prepared from the particular rumen contents. Thus, in samples of whole rumen contents, the amount of bacterial protein may be determined. The accuracy of the method depends on the assumption that the proportion of DAP in the microbial protein remains constant whatever the growth rates of the bacteria, and that the proportions of the different species making up the population does not vary over the experimental period. Although this may be true for an animal on one feed as mentioned above, the proportion of DAP to nitrogen may change with type of feed as the types of rumen bacteria change. So, in feed-change experiments, separate standard determinations of the DAP content of the bacteria should be made for each feeding regime.

A marker for protozoal cells, 2-aminoethylphosphonic acid (AEP) was found by Horiguchi and Kandatsu[41] and has subsequently been used for determining the synthesis of rumen protozoal protein in the same way as DAP is used for bacteria. Ibrahim and Ingalls[42] used both markers in investigations of total rumen microbial synthesis, and Czerkawski[43] has more recently described methods for determining DAP and AEP which have advantages over previous methods.

Ling and Buttery,[44] however, have criticized the use of AEP as a protozoal marker as they found this acid in feed material and bacteria. They concluded that RNA was an adequate index of microbial protein in most cases. Gaussères and Fauconneau[45] used DNA as a determinant for microbial protein. Smith,[46] in a 1969 review, quoted some of his unpublished analyses for nucleic acid content of five species of rumen bacteria. They showed RNA nitrogen: total nitrogen ratios to be less variable between the species than DNA-N, and he suggested that RNA would be a better index of microbial nitrogen than DNA. In a later work, Smith and McAllan,[47,48] investigating nucleic acids and total and ammonia nitrogen concentrations in rumen digesta, used total nucleic acid N to calculate microbial protein N, but again suggested RNA might be better. They also showed that feed nucleic acids are degraded in the rumen, and this is a prerequisite for the determination of microbial protein. However, despite the results quoted above, RNA content of bacteria has been shown to vary with growth rate, while DNA is more constant.[49] Wolstrup and Jensen[50] used DNA for calculating microbial protein production in the rumen, and Slyter et al.[51] used DNA for measuring microbial production in an artificial rumen system in vitro.

Instead of using one amino acid specific to bacteria or protozoa, Evans et al.[52] used the amino acid profile of the whole digesta for determining microbial protein passing to the duodenum. The method involved the determination of the amino acid composition of microorganisms and feeds, with bovine pepsin used to represent endogenous protein secretions. These profiles were then computer mixed in different proportions until the amino acid profile of the whole digesta was simulated. A constant amino acid composition for the microbial protein was assumed. Earlier, Temler-Kucharski and Gaussères[53] and Ely et al.[54] had used the differences in lysine content between microbial and feed proteins to determine amounts of rumen microbial protein. These methods are obviously more difficult and probably subject to greater errors that methods which rely on estimation of a compound unique to microorganisms such as DAP or AEP, and they do not seem to be in general use.

Instead of determining a particular molecule contained in the microbial cells, the incorporation of an isotope into cell material being synthesized can be determined. The isotopes used have been ^{35}S, ^{15}N, and ^{32}P. The amount of isotope, measured as a specific

activity incorporated into the microbial mass, should then give a measure of microbial protein synthesized in any particular period. If the microbes in the rumen are labeled, then, in the absence of feed protein passing from the rumen, the specific (with regard to protein) labeling will be the same in rumen organisms and material leaving the rumen. Since the feed proteins will be unlabeled, undigested feed protein passing from the rumen will dilute the isotope activity. Although protozoa may not directly utilize the particular isotopic compound used in the labeling process, the fact that the protozoa ingest and utilize bacteria means that they will become labeled; therefore, no distinction between bacterial and protozoal protein can definitely be made by methods which label bacteria.

The use of ^{35}S incorporation into microbial sulfur amino acids as a measure of microbial synthesis was introduced by Henderickx.[55] Earlier studies with pure cultures of rumen bacteria showed that sulfate was reduced to sulfide in the rumen and that sulfur requirements of a number of rumen bacteria could be satisfied by sulfate.[56] Sulfate is the marker used for experiments in vivo, as Walker and Nader[57] showed that ^{35}S sulfide was lost as H_2S and only by using a completely sealed system could incorporation of sulfur from sulfide be used in vitro. Some bacteria require a sulfur amino acid such as methionine, but as there is continued death and lysis of bacteria, methionine used by one bacterium could become available to another, although the main source of intact sulfur amino acids is probably hydrolyzed feed proteins. Evidence from experiments in vitro and in vivo is rather conflicting about the proportion of microbial sulfur coming from the sulfide pool in the rumen.[58,59] But, as with all isotope experiments, if the proportions of different bacterial species (labeled and unlabeled) remain the same over the experimental period, then microbial protein should be quantified. However, experiments in vitro[60] cast some doubt on the measurement of microbial cell production, as it was found that ^{35}S was incorporated into bacterial protein when fermentable substrate was low and there was no net growth of organisms. Presumably, under these conditions, maintenance requirements played a part and, also, any growth of bacteria was derived from cell death and lysis. These authors suggested that incorporation of ^{35}S (and presumably other isotopes) is a reliable indicator of microbial synthesis only when bacterial growth rate is at a maximum.

Leibholz[61] and Harrison et al.[62] used ^{35}S incorporation to measure microbial protein passing from the rumen to the intestines, and Hume[63] tested the method with sheep on different diets. He found that according to the hypothesis mentioned previously, when microorganisms were labeled by introducing $Na_2{}^{35}SO_4$ into the rumen of sheep on a protein-free diet, the specific activities of sulfur in the rumen microbes and in duodenal contents was the same. When diets containing zein, casein, or fish plus lupin-peanut- or soya bean-meals were fed, the specific activity in the duodenal contents was reduced. Hume concluded from his own and other results[35,64] that where zein and casein digestibilities had been estimated by using the ethanol solubility of zein and the phosphate content of casein, the ^{35}S method gave a "realistic" measure of the proportion of microbial and dietary proteins leaving the rumen. Sulfur labeling has been used in a number of rumen experiments to determine amounts of microbial protein other than the examples mentioned here.

Singh et al.[65] used ^{35}S to determine production rates of bacteria in the rumen. Their calves were fed at 2 hr intervals, and for 5 days before the experiment the feed portions were sprinkled with a solution of ^{35}S sodium sulfate. This period was considered adequate for the bacteria to become uniformly labeled with ^{35}S. On the 6th day unlabeled feed was given, and fluid samples were withdrawn from various parts of the rumen some 8 hr after the last ^{35}S was given. A bacterial fraction was prepared from the samples by centrifugation and the specific activity per milligram of bacterial cells was determined. The rumen fluid volume was determined by the standard technique of adding a known

amount of polyethylene glycol and determining its concentration in the fluid. Samples for determination of bacterial specific activity were taken at intervals for 10 hr. The activity declined at a rate expected for growth of the bacteria (i.e., of the form $A_t = A_o e^{-rt}$), and from this and the concentration of bacteria and rumen volume a production rate could be determined.

The same workers later used a variation on this labeling method.[66] A 100-mℓ sample of rumen fluid was diluted with artificial saliva and was incubated in vitro for 12 hr with some of the animal feed and ^{14}C-labeled leucine or ^{35}S sodium sulfate. The labeled bacteria were separated from the incubation mixture, washed free of leucine or sulfate and injected as one dose into the rumen and well mixed into the rumen contents. Sampling at intervals showed the specific activity of the rumen bacteria declined as before and a bacterial production rate could be calculated as could the rumen dilution rate. Later experiments showed that labeling mixed rumen bacteria as above, or using a labeled culture of *Streptococcus bovis* originally isolated from the rumen gave the same results.[67] The rate of production of rumen ciliate protozoa was also determined by similarly using a suspension of mixed protozoa separated from rumen contents and incubated in vitro with ^{14}C glucose.[68]

The success of these experiments depends on the fact that the animals were fed every 2 hr. This makes the rumen approximate the theoretical continuous culture and attain a near steady state. Growth of bacteria will be balanced by passage out of the rumen and so the standard kinetics of bacterial growth and isotope dilution should apply.

Bucholtz and Bergen[69] studied incorporation of ^{33}P phosphate into rumen microbial lipids, but as the greater part of bacterial phosphate is in the nucleic acids, van Nevel and Demeyer[70] suggested that this nucleic-acid phosphorus be used by determining the incorporation of $^{32}PO_4$ into total microbial P, as the ratio of nucleic acid to total cell (and so protein) nitrogen had previously been reported to be reasonably constant. In the paper quoted, this method for determining microbial growth was critically examined in experiments in vitro. Microbial growth could be determined from incorporation of ^{32}P, but considerations like those outlined for ^{35}S again applied.

Experiments with whole rumen contents and with pure cultures have shown that the major source of nitrogen for bacterial growth is ammonia. The addition to rumen contents of ^{15}N ammonium salts is, therefore, another means of labeling to determine microbial growth and protein.[72] Urea labeled with ^{15}N has also been used by intravenous injection to study urea entry into the rumen and urea nitrogen incorporation into bacteria.[73] A stable isotope has some experimental advantages over a radioactive isotope. However, the proportion of microbial ^{15}N derived from $^{15}NH_3$, will depend on the availability of other nitrogenous nutrients such as amino acids and will also vary from species to species, so the provisos previously mentioned for other isotopes apply to ^{15}N.

Siddons et al.[74] compared ^{35}S, ^{15}N, RNA, DAP, and amino acid profile methods of determining microbial protein flowing to the duodenum. They found that the microbial protein flow per day in two groups of sheep on grass silage or dried grass feeds was the same when estimated by any method, but that the absolute amount varied with method, from approximately 15 g/day by the amino acid profile to approximately 69 g/day by RNA determination (exact figures and standard errors were given by the authors). The results showed that the amino acid profiles of forage feeds and microbes are similar and the profile method would seem unreliable. The high values of the RNA method suggested feed RNA was not degraded in the rumen. Some possible reasons for unreliability of the isotope and DAP methods were also suggested. Overall these authors concluded that there is no obviously satisfactory method of measuring rumen microbial protein, but that isotopic methods might be less subject to criticism than other methods, and in their hands the coefficients of variation between the different sheep in the two groups were less with ^{15}N than ^{35}S.

D. Indirect Methods for Determining Microbial Activity and Growth

Fermentative activity may or may not be coupled to growth of microorganisms, but in the ruminant (immediately after feeding for instance), rumen fermentation generally may be accepted as being combined with microbial growth although the relationship of growth to fermentation products may vary with feed or time after feeding. Thus, production of fermentation acids and gases has been measured as an indirect way of assuming cell production. In some methods, the rates at a particular time are determined by taking a sample from the rumen and immediately incubating this in a suitable anaerobic buffer solution. Acid concentrations are measured at intervals over the next few hours and the rate of acid production at zero time of the incubation is taken as the rumen rate.[75] El Shazly and Hungate[76] suggested a simpler method of determining microbial activity by incubating a sample of rumen contents in a bicarbonate buffer with an energy source (e.g., glucose) and measuring gas production over a short period (about 30 min). They suggested that a measure of microbial growth rate in the rumen could be obtained by taking a large rumen sample, subsampling this for a rate measurement as above, then incubating the remainder for 1 hr under CO_2 and taking another sample for a rate determination after this time. Increase in rate between 0 and 1 hr gave a measure of microbial growth rate. The gas production method was used extensively in field experiments on Scottish deer, sheep, and reindeer and gave a good indication of changes in overall rumen microbial activities with season of the year, and of adaptations in the rumen microbes to fermentation of the feeds available at different times of the year (by using these as substrates for the tests in vitro). However, the extension of the method to determine microbial growth rates did not give comprehensible results.[77] Later methods have used changes in specific activity of ^{14}C-labeled VFA injected into the rumen to measure rates of acid production.

A general product of fermentation in microorganisms is heat, and calorimetry has been applied to measurement of microbial activity. Microcalorimetry can only be applied to samples incubated in vitro, and requires special apparatus. Since rumen fluid contains many large particles, difficulties could be found with some commercial calorimeters made for use with bacterial cultures or thin suspensions. Walker and Forrest[78] used calorimetry to investigate rumen activity, but the method has not be adopted generally. The calorimeter held 250 mℓ of sample and was an adaptation for the slurry of rumen contents of the calorimeter built by Forrest[79] for examining fermentations in liquid media.

Adenosine triphosphate (ATP) is a constituent of all microbial cells and it has been used principally in material in which microbial numbers are very small (i.e., natural waters) to determine the number of bacteria. The method, like many others, depends on the correctness of the assumption that all bacteria always contain the same concentration of the measured constituent, in this case ATP.

Hobson and Summers[80] measured the ATP content of one rumen bacterium, *Selenomonas ruminantium,* in vitro and found that the ATP content of the cells was constant over much of the growth rate but decreased at very low growth rates, below 0.06/hr.

Wolstrup and Jensen[81] measured rumen ATP concentrations in heifers fed every 2 hr or every 12 hr. Bacterial and protozoal biomass was calculated from cell counts and average volumes and dry matter contents, while concentrations of VFA were used as a measure of microbial activity. The rumen ATP concentration was, at a mean of 55.3 mg ATP/ℓ rumen fluid in the heifer fed continuously, twice as high as that in the other heifer 12 hr after feeding. The ratio of biomass to ATP was 683 in the first animal and 1208 in the second. These values are similar to those found for various bacteria and also reflect, as expected, a lower ATP content in cells with a lower growth rate. Although there were variations in rumen ATP content not correlated with variations in the other parameters

measured, it seemed that ATP measurements would reflect rumen microbial activity; for biomass determinations, however, the considerations of the effects of microbial growth rate, etc., discussed previously still applied. Forsberg and Lam[82] measured ATP in rumen contents. They tested different methods of extraction of the ATP and arrived at a use of 0.6 N H_2SO_4 as the most suitable. Tests showed that ATP content of protozoal and bacterial fractions from rumen contents varied, as did the ATP contents of nine strains of rumen bacteria grown in pure batch culture. So while ATP could be used as a measure of biomass, variations in the rumen population would have to be taken into account. Wolstrup et al.[83] continued their investigations into the use of ATP as a measure of microbial activity using DNA concentration as a measure of the biomass. They considered DNA a good index of biomass and found, as might be expected, that ATP concentration varied independently of DNA and microbial count, and reflected the level of microbial activity rather than biomass.

E. Methods for Counting Specific Bacteria or Bacterial Types

Cultural counts may, of course, be adapted to count a particular species of bacterium by using some form of selective medium on which only the particular bacterium will grow. However, most selective media depend to some extent on use with an inoculum in which only a limited number of species occur, and production of selective media for a microbial habitat such as the rumen is difficult. A selective medium, using mannitol as sole (and selective) energy source for *Selenomonas ruminantium,* was devised by Bryant et al.[84] *Megasphaera elsdenii* also grows on this medium and while this may not interfere with *Selenomonas* counts in the normal rumen where *Megasphaera* are few (in the young animal or under some feeding conditions in older animals where *Megasphaera* are in numbers similar to *Selenomonas*) counting of the latter purely by growth on the selective medium is impossible.

Groups of bacteria with particular activities such as a polysaccharide hydrolysis or a nitrogen utilization can be counted more accurately than separate species by using media containing the particular substrate. Growth in a medium with only one added energy or nitrogen source cannot be taken as complete evidence for the presence only of bacteria utilizing the substrate, but growth combined with a chemical determination of breakdown or loss of the substrate in dilution culture can give a count of the numbers of particular "functional groups" of bacteria in the inoculum. Such methods have been used in many rumen experiments over the years, so particular examples will not be quoted.

An extension of the concept of using a particular fraction of the microbial cell as a measure of the numbers of that cell in the rumen was made by Cheng and Costerton,[85] who found that alkaline phosphatase seemed to be contained mainly in *Bacteroides ruminicola.* Measurement of the specific activity of this enzyme might then give a measure of the numbers of *B. ruminicola* in rumen contents. However, recent tests (Wallace, unpublished) suggest that the phosphatase is present in saliva, though whether this would survive in the rumen is not known.

No other cell fraction peculiar to a specific bacterium and easily measurable seems to occur, but Hobson et al.[86,87] early used fluorescein-labeled antibodies to visualize particular bacterial species in rumen contents. The method was successful to some extent, but work by these authors and others[88,89,90] showed that different serological types of many of the species occurred in rumen contents and that the proportions of the serological types varied with time in a constant numerical population of the species. Thus, to enumerate the total number of a species, it requires the isolation of all serological types and the preparation and use of labeled antisera to all types. This is a practical impossibility, but the use of labeled antisera to demonstrate relationships between cultural bacteria and those in the rumen, or to follow the fate of a specific bacterial inoculum introduced into the rumen contents, is possible.[88,91]

As previously mentioned, the determination of the numbers, or total or particular-component mass, or activities, of rumen microorganisms is important in analyzing rumen function. The number of methods described here show the difficulties of such determinations in a heterogeneous microbial habitat like the rumen, with large concentrations of microorganisms of very varied type and a heterogeneous feed, and with the system not generally in a steady-state. In fact, some methods are only applicable to specialized circumstances where the animal is fed on a synthetic, purified, diet or, if on a normal diet where the feeding is not normal, in being regulated to as near continuous as possible so that a good approximation to a steady-state in rumen activity is achieved.

The initial statement that determination of absolute mass or numbers of the microorganisms associated with the rumen and its contents is impossible still holds. However, the methods can give values useful in some investigations, and application of the methods will be illustrated in the work described in the following sections.

III. FIBER DIGESTION

The source of energy for the rumen microorganisms, and thus the ruminant, is carbohydrate. For the ruminant in natural surroundings, the carbohydrate is the mixture of mainly polysaccharides contained in leaves and stems of various plants. Starch is found in large amounts in seeds and tubers and is present in the ruminant diet only under conditions of farm feeding where concentrates prepared from cereal grains, rice, and in some cases tubers, are given.

The rumen microbiologist is, then, initially interested in the breakdown of plant fibers and the first investigations were microscopic observations. The light microscope and stains showed that bacteria were attached to fibers, and were apparently digesting pits into the fibers, and bacteria were digesting the inner portions of broken fibers. Similar observations were made on starch granules from concentrate feeds. Then came a period of isolation of bacteria presumed, because of their isolation in media containing preparations of the various polysaccharides, to be the bacteria concerned in the rumen degradation of the plant structures. Enzyme preparations were made from these bacteria and from ciliate protozoa which were seen to ingest and digest various types of plant fragments, and their properties were examined.

These investigations have been summarized in previous reviews (e.g., References 3,4,92,93) and will not be dealt with here in detail. As far as the isolation of bacteria presumed to be involved in the polysaccharide degradations are concerned, it is probably true to say that recent investigations have not definitely added to the genera and species enumerated in the reviews mentioned above. Bacteria of the genera *Bacteroides, Ruminococcus, Butyrivibrio,* and *Eubacterium,* particularly the first two, are the cellulolytic bacteria most commonly isolated and studied. The two clostridial species first isolated by Hungate[94] have seldom been found in later cultural examinations. Two newer rumen cellulolytic bacteria, *Micromonospora ruminantium*[95] and an unnamed, twisted filamentous, Gram negative bacterium,[96] are of uncertain status as they do not seem to have been reported since then. Two pectinolytic spirochetes,[97,98] where status is again uncertain, are described later in this section. The ciliate protozoa concerned are still those which early workers designated as amylolytic or as degrading polysaccharides of fibers. But in the last few years the probable importance in fiber degradation of some of the flagellate protozoa, now known to be phycomycete fungi, has been shown. The flagellates previously were largely ignored in rumen investigations.

The investigation of starch digestion became more important in the 1950s with the widespread use of grain feeding for rapid fattening of cattle, and investigations of the degradation of granular starch showed the process to have all the characteristics now being investigated in fiber breakdown (reviewed in Reference 93). Starch is, however, a

relatively simple molecular structure and a starch granule does not contain other polysaccharides. Also, starch granules are quite easily liberated from the grain, particularly in the case of pelleted or otherwise processed concentrates. It would seem from general rumen investigations and the gnotobiotic lamb experiments described in another section, that the bacterial degradation and fermentation of starch granules can be explained by the action of known bacteria and their enzymes. Similarly, the degradation of starch granules by ciliate protozoa can be explained by the protozoa ingesting the starch and subjecting it to the action of amylolytic enzymes similar to those of the bacteria.

The breakdown of fibrous plant material, whether it be in leaves or stems or the fibrous coatings of undamaged grains, is a different problem of greater complexity which still has not been solved. The feeding of fibrous materials has assumed greater importance with the suggestion that feeding grains, which can be used to feed humans, is wasteful when applied to ruminants which can utilize grasses and other vegetation of little or no immediate nutritive value to man. Ruminants can also make use of fibrous residues from processing of food for humans; straws, sugarcane bagasse, and so on. In the poorer tropical and subtropical countries such poor plant residues are principal sources of animal feeds. Increasing ruminant production by utilizing fibrous vegetation and improving the digestibility of plant residues then makes economic and ecological sense, as it uses a plentiful feed supply or more efficiently recycles waste materials. Investigations of the microbial degradation of fiber polysaccharides in the rumen may help in improving the utilization of fibrous feeds.

The advent of the electron microscope, and particularly the scanning electron microscope, has given the microscopist a new tool to investigate the importance of bacterial association with substrates, and E.M. work has figured prominently in recent investigations of fiber degradation. Investigations with aerobic cellulolytic fungi, which produce enzymes in greater quantities than the anaerobic bacteria, have shown the types of enzymes to be looked for in the bacteria, the modern chromatographic and electrophoretic techniques allow the use of much smaller amounts of enzymes than formerly. Detailed investigation of the enzymes concerned in fiber degradation is now being undertaken. The fact that fibers contain a number of polysaccharides as well as proteins and lipids, while bacteria are relatively limited in the substrates which they can attack, has led to perhaps more emphasis than hitherto being placed on the actions of a consortia of microbes as being involved in fiber digestion (see review, Reference 93). And it has, of course, long been realized that the bacteria and protozoa degrading fibers require nitrogen, sulfur and growth factors which are provided by other organisms and that all factors involved in microbial growth are therefore interconnected. Besides these factors, there is the basic problem of lignin and its action as a barrier to anaerobic digestion of fiber polysaccharides.

A. Interactions of Microorganisms in Fiber Degradation

Fibers such as leaves contain small amounts of sugars, but the main components are polysaccharides; pectins, hemicelluloses, and cellulose. Cellulose is a glucose polymer with no substituent groups; but the hemicelluloses, which can be 30 to 40% of the total carbohydrate, are complex polymers of xylose and arabinose, with substituent acyl and other groupings, while the pectins are polygalacturonic acids, again with substituents, methyl and other groups. In addition to the polysaccharides, the fibers contain proteins and small amounts of fats, waxes, organic acids, silica, salts, and the complex aromatic compound lignin. The amount of the latter varies with age of the plant, being greatest in older vegetation. Detailed analyses of plant fibers are given in References 99 and 100 and the review on Reference 101.

The rate of degradation of plant fibers depends very much on the state of maturity of

the plant and this affects the composition of the fiber, not only in lignin but in other components. So the enzymes and bacteria involved in fiber degradation will vary with the fiber being digested. There is little, if any, pectin in straws, and little protein, so degradation of these substances will play a smaller part in the total degradation than it would with fresh leaves. The work of Bateman[102] with plant-pathogenic fungi grown on plant cell walls showed that enzymes degrading pectic materials were synthesized first by the fungi; then came the hemicellulolytic enzymes and then cellulases. The importance of pectin degradation in fresh forages is shown by the fact that although some rumen bacteria and protozoa utilize pectins for growth, others will degrade pectins without utilizing the degradation products.[103-105] This hydrolytic activity would remove pectin and expose hemicellulose or cellulose utilizable by the particular organisms or by other bacteria associated with the pectinolytic ones. There also seems to be synergistic actions between pectin-hydrolyzing bacteria and pectin-utilizing bacteria. For instance, Gradel and Dehority[104] found that combination of a strain of *Ruminococcus flavefaciens* (which degraded pectin but did not appreciably utilize it) with a strain of *Bacteroides ruminicola* (which degraded and utilized pectin) resulted in increase in degradation and utilization of pectin in alfalfa from 30% with *B. ruminicola* alone to 82% with the mixed bacteria. Similar results were obtained with the ruminococcus and a strain of *Butyrivibrio fibrisolvens*.

The ability to hydrolyze xylans but not to utilize the hydrolytic products has been noted with a number of bacterial species and strains.[92] In some cases the xylan-degrading ability evidently allows the bacterium to expose cellulose which it can both degrade and utilize.[106] It also may provide substrates for non-xylan-degrading bacteria which can use xylose oligosaccharides. Such an action also can increase hemicellulose degradation by removing possibly inhibitory hydrolytic degradation products as, for instance, in the combined actions of strains of *R. flavefaciens* and *B. ruminicola* on the hemicellulose of brome-grass fibers.[106] There is, thus, evidence that one polysaccharide can provide a barrier to attack on another, but the noncarbohydrate constituents of plant cell walls may also have a similar effect. Forrest and Wainright[107] found that the β-glucans of barley endosperm cell walls were depolymerized by a proteolytic enzyme. The degradation of plant proteins in the rumen is described in another section of this review. The rumen hydrolysis of lipids, which also could act as a barrier to cell-wall polysaccharide degradation, is, again, dealt with in another section.

Silica and waxes, which could hinder the attack on plant fibers, are undegradable, at least so far as is known for the latter in the anaerobic rumen environment. Another of the fiber constituents previously mentioned, lignin, has always been regarded as undegradable in an anaerobic environment and as the major undegradable constituent governing the rumen digestion of fibers. There is no doubt that there is a general correlation between lignin content and digestibility in vivo of forages, but whether lignin is undegradable has been debatable. Lignin is a polymeric substance and the structure is poorly understood. A number of methods, largely based on solubility, are used in its analysis in feedstuffs and the material is to some extent defined by the method used. Small apparent changes in lignin content of feedstuffs are often noted in digestibility studies and these could be produced either by partial depolymerization of a large macromolecule or by the presence in the plant of lignin molecules of different sizes, the smaller of which are removed from the fiber structure during breakdown of some of the constituent polysaccharides. Lignin varies in structure in different delignified plant tissues,[108] and chemical bonding between the lignin-type phenolic acids, *p*-coumaric and ferulic, and glucans and xylans in rye grass has been shown.[109] Soluble lignin-carbohydrate complexes are present in rumen fluids.[110,111] Apparent loss of lignin could, then, result from the latter of the two previously-mentioned mechanisms[112,113] but the possibility of partial microbial degradation of lignin and actual removal of the

degradation products has been made more feasible by the recent isolation from rumen fluid of a filamentous, facultatively anaerobic bacterium.[114]

The electron microscope showed this bacterium to be attached to the lignified sclerenchyma of grass leaves in the rumen and in culture, and to be degrading the lignin. In anaerobic culture, the bacterium grew on cellobiose; there was a little growth on xylan but none on pectin or cellulose. Anaerobically it grew well on ferulic and sinapic acids but little on *p*-coumaric. Pretreatment of plant tissues in cultures of the bacterium rendered the structural carbohydrates more easily degradable by mixed rumen bacteria. The bacterium had a low growth rate and its optimum growth pH was higher (7.4 to 8) than the rumen pH, but it could compete in the rumen by utilizing substrates not used by other organisms, the insoluble lignin and soluble lignin-carbohydrate complexes.[114] The low growth rate could also explain the low overall degradation of lignin in the rumen. The numbers of bacteria in the rumen fluids were not ascertained, but this isolation appears to add another example to those mentioned in other sections of facultatively anaerobic bacteria with definite roles in the rumen metabolism.

B. Associations of Microorganisms with Plant Fibers

1. Bacteria

In the foregoing section some examples were given of interactions of bacteria and protozoa shown by culture experiments and analysis of growth and metabolism. These experiments, which involve known species of organisms, can be extended to studies using light or electron microscopy to determine spatial relationships of the organisms and fiber components. Similar studies can be done on fibers being degraded in the rumen, but problems in determination of bacterial species from morphology, particularly in thin sections, makes identification of the bacteria involved difficult. The protozoa can be identified more readily, particularly in scanning electron micrographs.

Lachnospira multiparus is a rumen bacterium known from culture studies to actively degrade and utilize pectin.[104] When white clover leaves which have a high pectin content[100] were incubated overnight in growing cultures of *L. multiparus,* the structure of the leaves was damaged and the leaves disintegrated when the culture was shaken. Electron micrographs of thin sections showed that the bacteria had grown into the intercellular species of the mesophyll tissue, but had not invaded the cells. The bacteria did not colonize the leaf surface and did not, apparently, gain access to the inner tissues through leaf stomata. Access seemed to be mainly through cut or damaged edges in the leaves. Although ryegrass leaves were similarly invaded by *L. multiparus,* these leaves (of a lower pectin content) were less disintegrated by the bacterial invasion.[115]

The ruminococci have been the bacteria most generally identified with rumen cellulolysis, and they can also hydrolyze other fiber polysaccharides. Latham et al.[116] studied the attack of pure cultures of strains of *R. flavefaciens* on ryegrass leaves. As with the entry of the *Lachnospira,* the sites of attack of the ruminococci were principally the cut or damaged edges of leaves, but the ruminococci adhered to the plant cell walls in these areas and cavities were digested in the walls. The ruminococci adhered most readily to the cell walls of the epidermis and sclerenchyma and phloem and mesophyll. Although the bacteria adhered to the sclerenchyma they did not degrade the cell walls, otherwise the presence of lignin inhibited both adhesion and digestion. The bacteria also adhered to cotton fibers, particularly at broken ends, and they digested the cellulose. In the ryegrass these bacteria could utilize both cellulose and xylan. The adhesion of the ruminococci to the leaf cells appeared to be mediated by a capsular polysaccharide, as with the bacteria which had been shown earlier to adhere to and degrade starch granules,[93] and the importance of capsular material in adherence of the rumen bacteria in general to plant material is evident in all micrographs of pure cultures or the rumen

mixed culture. Latham[117] found that chemical removal of the capsule of *R. flavefaciens* or change in its structure by addition of antibody prevented adhesion of the ruminococci to plant cells.

Minato et al.[15] showed by using fluorescent antibodies that cellulolytic bacteria, particularly *Bacteroides succinogenes,* were attached to plant fibers. Later electron microscopic studies[118] involved *B. succinogenes,* which may be more important in rumen cellulose degradation than the ruminococci although it has been much less frequently isolated in conventional cultures. *B. succinogenes,* while adhering readily to damaged edges of ryegrass leaves, also adhered to undamaged surfaces of mesophyll, phloem, and epidermal cell walls. The *Bacteroides* and *R. flavefaciens* in mixed culture exhibited preference for the damaged areas, the *Ruminococcus* predominating on the epidermis, phloem, and sclerenchyma, while the *Bacteroides* predominated on the mesophyll cells. In these experiments some degradation of the lignified walls of sclerenchyma cells was observed, but degradation was not nearly so rapid or extensive as on the unlignified walls to which the bacteria readily attached. A few *Bacteroides* adhered to meta- and protoxylem, but no degradation of these lignified walls was seen. The ruminococci retained their coccoid morphology and attached to the walls by a thick capsular layer. *B. succinogenes,* on the other hand, had a relatively thin capsular layer but appeared to have a flexible cell wall structure and altered its morphology to follow closely the topography of the surface to which it adhered. Hungate[119] suggested from the appearance of the growth of *B. succinogenes* in cellulose agar that the bacterium could move through the agar, and it might be that the *B. succinogenes* seen apparently attached to undamaged surfaces of the ryegrass were actually migrating to damaged and more easily degraded areas. The *Bacteroides,* unlike the *Ruminococci,* also appeared within the lumen of the plant cells, at some distance from the broken edge of the fiber.

Morris and Van Gylswyck[120] incubated teff *(Eragostris teff)* cell walls with various rumen bacteria with results largely similar to those of others. *Ruminococcus flavefaciens* seemed more strongly attached to cell walls than did *R. albus,* while *B. succinogenes* adhered closely and conformed to the cell wall. One strain of xylanolytic but not cellulolytic *B. ruminicola* also adhered to cells, but other strains did not do so. These and the nonadhering xylanolytic *Butyrivibrio fibrisolvens* strains and strains of an unidentified rod, were clustered around the plant material. Two strains of *Fusobacterium polysaccharolyticum,* which were both xylanolytic and cellulolytic, did not adhere to plant cell walls. This bacterium, isolated by Van Gylswyck,[121] is another of those of uncertain status as it has not been reported otherwise. The extent of solubilization of the cell walls was compared with the actions of the bacteria on filter paper powder and a soluble, commercial, xylan preparation. The cellulolytic bacteria also degraded xylan. They solubilized more of the cell walls, releasing arabinose, xylose, glucose, and uronic acid, than did the purely xylanolytic bacteria which released arabinose, xylose, and some uronic acid. All except *B. succinogenes* utilized the degraded polysaccharides: *B. succinogenes* did not utilize xylan. The xylanolytic bacteria which produced diffusible xylanase (shown by clearing in a xylan medium) degraded more of the cell-wall xylan than did the other bacteria. These studies tend to suggest that there is no relationship between adherence to cell walls and ability to degrade the component polysaccharides. However, Latham[117] showed that a number of factors such as pH and presence of rumen fluid affected adherence of ruminococci to cell walls, so that the results of one series of batch cultures under particular conditions may not be a complete guide to occurrences in the actual rumen. The ability of the bacteria in these experiments to degrade pectin in cell walls is in doubt, as it was not known how much uronic acid was associated with the hemicellulose structures.

Observations on fibers, such as these and others described later, and the early

observations on starch granules[122] (see Reference 93 for review) show the importance of the breakage of surface layers of plant materials in promoting the initial colonization by bacteria, and this can be related to the ruminant animal itself. The grazing or browsing ruminant tears off (rather than clearly bites) herbage, and this will severely damage the leaves and stems. Then while ruminating it again chews and crushes the digesta, producing more damaged areas on the partly digested fibers. With mechanically prepared feeds it is known that grinding of hay or grass can increase the rate of digestion (for instance, see Reference 123). Microbial degradation of fibers does not necessarily lead to visible loss of structure and fragmentation. The lack of breakage of fibers by rumination thus may be relevant to the rate and extent of degradation of fibers suspended in the rumen in porous bags.

The other point is that particular bacteria, to some extent at least, colonized particular parts of the plant fibers. With clover leaves incubated in a mixture of bacteria taken from the rumen, Cheng et al.[124] found that one morphological type of bacterium formed a population adhering to the surface of a particular type of cell wall, while mixed bacterial types were evident in the spaces around the cells. However, there were cases where the first adherent population seemed to have attracted other bacteria. Also, in earlier electron microscope studies of bacteria on plant fibers taken from the rumen, Akin[125] showed a bacterium with a thin capsular layer which appeared to have a flexible cell which conformed to the plant cell wall in the same way as the *B. succinogenes* in pure culture.

B. succinogenes has, as mentioned previously, seldom been isolated from rumen contents and its importance in fiber digestion probably has been underestimated (see for instance, Reference 126). The characteristics of the bacterium shown by the microscopic studies are relevant to recent cultural studies. Stewart et al.[127] isolated only ruminococci by the usual method of subculturing colonies forming clear zones in an agar medium containing HCl-treated and powdered filter paper. When dewaxed cotton fibers suspended in a liquid medium, inoculated with whole rumen contents, were used as the substrate and bacteria were isolated by subculturing particles of the degraded cotton, a large majority of the bacteria was *B. succinogenes*. Cheng and Hungate[128] found that higher counts of bacteria were obtained in media containing solid preparations from alfalfa fiber or green wheat than in media containing dissolved fiber polysaccharides and oligosaccharides.

There have been other electron microscope studies of bacteria attached to and attacking fibers suspended in the rumen, taken from feedstuffs in the rumen or incubated with mixed rumen bacteria. Dinsdale et al.[129] saw evidence of a spiral bacterium in grass cell walls. This might have been one of the pectinolytic spirochetes mentioned earlier. All show the preference of bacteria for attack of fibers through damaged areas. Colonization of fibers in the rumen is rapid, occurring in minutes rather than hours, and it is not random, so it presumably occurs through chemotaxis of particular bacteria to specific soluble sugars diffusing from the broken fibers. The formation of associations of bacteria on the fibers probably will be between bacteria able to utilize degradation products of the fiber polysaccharides, either mono- or di-saccharides, or large soluble molecules formed by partial degradation of the polysaccharides, as seems to occur in the case of starch granule degradation.[93] That the bacteria adhering to feed particles in the rumen form a large proportion of the population is borne out not only by microscopic observations but by other tests. Forsberg and Lam found some 75% of the ATP (see Section II.D) to be associated with feed particles.[82] Minato and Suto[130] and, earlier, Minato et al.[15] showed by treating digesta particles to detach bacteria, that at least as many bacteria were associated with the feed particles as were free in the rumen liquid (see Reference 131 for earlier work). The association of bacteria with particulate matter in the rumen has been

known for many years, and the rumen bacteriologist has realized that he has been culturing only a portion of the total bacteria — those free in the liquid and those dislodged from particles in the preparation of his initial dilutions of the rumen sample (e.g., References 5 and 131). Nevertheless, counts done in the same way should give a relative measure of changes in bacterial numbers in the rumen at, say, different times, if the proportions of fixed and free bacteria are the same at these times. Since the residence time of larger fiber feedstuffs in the rumen is longer than the liquid residence time and there is always fiber in the rumen between feeds, the former supposition is probably true. It is possible, however, that the slight fall in numbers of bacteria often noted just after feeding could be caused in part by rapid transfer of bacteria from the liquid to the solids as the latter are colonized, as well as by increased salivary flow or other factors. What has been assumed is that, because bacteria must be liberated into the liquid as fibers become completely digested or bacterial colonies grow to cover all available surfaces, the bacteria in the liquid are representative of all the bacterial types in the rumen population and that these will grow in the media provided. The culture experiments referred to above suggest that this is not true and that some bacteria are so adapted to life on particular types of solid substrates that they remain viable for only a short time away from the substrate and must transfer almost immediately from one particle to another, and/or that they only have sufficient carbohydrate nutrients when attached to their particular substrate, perhaps because of localization of enzymes. However, the *Bacteroides* use ammonia, so they must be able to utilize a dissolved nitrogen source and also dissolved growth factors. It is possible that the infrequent isolation of other bacteria, such as those referred to at the beginning of this section, is for similar reasons and that they may yet be found to be of importance in rumen digestion.

2. Protozoa: Ciliates and Flagellates

Light microscopy showed starch granules or small pieces of grass or other fibrous animal feeds inside some of the ciliate protozoa, and the presence of appropriate enzymes in protozoal extracts added further evidence that the entodiniomorphid protozoa digested this particulate matter. Experiments mentioned in a previous section suggested that many of the ciliates did not leave the rumen and could have been held in the mass of fibrous digesta in the rumen. Recent tests[132] have shown that holotrich protozoa also may live for much of the time attached to the rumen wall. That the protozoa exhibit a preference for the solid material in the rumen rather than the liquid has also been shown by artificial rumen experiments. In these artificial rumens one or more mesh bags of hay or other feed is moved slowly up and down in a cylinder of artificial saliva-type medium inoculated with whole rumen contents. A feed pump or similar system keeps a slow flow of medium through the cylinder and the whole system is, of course, enclosed and anaerobic. At suitable intervals, similar to those between animal feeds, a bag of "feed" is removed and a fresh one substituted. A number of workers have used this type of artificial rumen[133–136] and have found that populations of bacteria and of ciliates similar to that of the normal rumen can be maintained for some days or weeks.[136] In all cases, though, the concentration of protozoa in the feedstuff in the bag is much higher (by a factor of 10^{136}) than it is in the surrounding liquid.

The scanning electron microscope has shown that ciliates actually become attached in very large numbers to the broken ends of fibers. The entodiniomorphid *Epidinium,* a cellulolytic protozoan, was seen in scanning electron microscope pictures of grass and other fibers taken from the rumen to be ingesting plant material and degrading the fibers.[137–139] As with the bacteria, colonization was rapid and occurred mainly at damaged ends of plant stems. The protozoa formed a ring around the borken end of a stem between the epidermis and vascular cylinder, and cortex, phloem, and pith tissues

were degraded. The undegraded epidermis peeled away as attack on the stem continued. It was not apparent from these studies whether the protozoa rapidly digested ingested plant tissue or became detached and then slowly digested the tissue. If the latter, detached protozoa were rapidly replaced.[138]

The holotrich protozoa do not ingest large particles but obtain energy by fermentation of soluble carbohydrates. Nevertheless, both *Dasytricha* and both species of *Isotricha* colonize and invade fibrous plant particles in the rumen.[140,141] Orpin and Letcher[141] showed that *Isotricha* had a chemotactic response to glucose, fructose, and sucrose, as presumably has *Dasytricha*. Soluble sugars are known to be present in fresh grasses and other herbage, and glucose and fructose could be released as the end products of bacterial degradation of glucosans or fructosans in the plants. Fructosans are also degraded by the holotrichs[142] so an initial colonization of a fiber by a few holotrichs could release fructose which would attract others. As might be expected from knowledge of their enzymic content, the holotrichs do not digest the structural material of the plant particles.

Flagellate protozoa were among the larger organisms observed by the light microscopist. More recently, however, Orpin has shown that some of these flagellates are actually stages in the life cycles of phycomycete fungi. *Neocallimastix frontalis*, *Sphaeromonas communis* and *Pironomas communis* are all zoospores of fungi.[143-145] The zoospores of *N. frontalis* and *P. communis* were seen to be attracted to inflorescence tissues of grasses by a chemotaxis to soluble substances leaking from the damaged areas which were preferentially invaded. The flagellates then went into a vegetative stage, with sporangia attached to the plant material. Flagellates released from the sporangia subsequently invaded the plant tissue. The zoospores were released from the sporangia of all three species in response to some components which seemed to be acidic in nature, dissolving from damaged plant tissues.[146] Tests with ^{14}C-labeled barley awns showed that *P. communis* sporangia took up ^{14}C from the barley, so that the organism was using the vegetable tissue as a nutrient and not just as an anchorage point. However, culture of this organism demonstrated that it could utilize dissolved nutrients and that solid plant material was not essential. In further tests *N. frontalis* flagellates were found to be attracted to carbohydrate mixtures representative of the soluble sugars of barley awn and inflorescence tissues, but not to amino acids, purines, pyrimidines, or vitamins.[147]

Scanning electron microscope studies by Bauchop[148] showed that zoospores rapidly attached to plant fragments in the rumen and developed into sporangia. Very large numbers of organisms, compared with those seen free in the rumen fluid, were found on the plant material. It would appear that the organisms could be much more important in the rumen metabolism, particularly in fiber breakdown, than was suggested by previous observations of the occurrence of organisms in the fluid. The life cycle of the organisms appeared to be about 24 hr. The fungi attach by rhizoids to the plant material and extensive invasion of tissues by mycelium was observed in the electron microscope. Primary invasion was, as with other organisms, mainly at damaged areas of plant material although grasses were colonized on the inside surfaces of the hollow stems. Mesophyll and long epidermal cells in wheat-straw leaf were digested from the inside, but the short, silicified, epidermal cells were not digested. Cellulolytic activity was demonstrated by the destruction of filter paper strips in fungal cultures.[140]

C. Straws and Treatments to Enhance Their Digestion

As previously mentioned, there is now much interest in using waste plant material as ruminant feeds, and of these materials the straws are the most abundant. Straws are the dry mature stems of various annual crops. In Great Britain, straw is associated with wheat, barley, or oats, but straws from other plants (e.g., rice) are used as ruminant feeds. These straws are essentially hollow cylinders of lignified polysaccharides, predominantly cellulose and hemicellulose, with very little protein (see References 101 and 149). Straws

also contain relatively large amounts of silica. The low protein content (usually less than about 5%) makes straws a poor ruminant feed, but even if the protein content is increased by feeding them as part of a mixed diet, the resistance to rumen degradation of the lignified polysaccharides makes the straws a poor energy source. Since protein can be added, as such, or as urea or ammonia, the present main interest in straws as ruminant feeds is in improving them as an energy source. This involves breaking down the lignin-polysaccharide structure, and possibly removing silica or other materials impeding degradation. The methods used have been chemical treatments with sodium hydroxide, liquid ammonia, aqueous ammonia, urea (which is hydrolyzed to ammonia by plant or contaminant microbial enzymes) or urine, which is a cheap source of ammonia and urea in underdeveloped countries. There are very many papers on these treatments and their effects, and caustic soda treatment, for instance, was first tried almost a hundred years ago; so no attempt at providing references will be given here. On-farm and factory processes have been used, but in the former case widespread use probably is inhibited by the danger in use of caustic and gaseous chemicals. Microbiological processes using lignin-degrading fungi have also been tried, but so far as we know this has not been done on a large scale. Similar effects on straw digestibility are produced by all treatments, but ammonia treatment also improves the digestible nitrogen content of the straw.

A number of experiments have been done to determine the chemical nature of alkali attack upon straws. The main effect is probably that alkali removes phenolic acid residues acting as barriers to microbial degradation of the polysaccharides; the removal of these barriers leaves a polysaccharides structure similar to that existing before the barrier. The straw digestibility can be improved to a certain extent, but there finally remains a lignified structure which cannot be further opened up by the low temperature alkali treatments. Evans[150] recently has given a more detailed review of chemical and physical aspects of alkali treatment.

Microbiological experiments similar to those described for untreated fibers have also been made. Latham et al.[151] used electron microscopy to observe the adherence of *Ruminococcus flavefaciens* and *Bacteroides succinogenes* to sodium hydroxide-treated barley straw and the less lignified stem of the grass *Lolium temulentum*. Before alkali treatment, while both bacteria adhered to and digested the unlignified walls of the innermost parenchymal cells, there was very little or no adherence to other areas of the stems. After sodium hydroxide treatment there was a much more general adherence, bacteria being associated with most types of cell walls, and differences in degree of adherence of the two bacteria to different plant cells was less marked than in untreated fibers (see earlier in this section). Labeling (^{14}C) of the bacteria showed that 2 to 5×10^8 adhered to a gram of untreated stems, while 27 to 33×10^8 were found on treated stems. The bacterial digestion of the stems was increased by alkali treatment, but while the ruminococcus digested twice as much of the untreated stems as the bacteroides, both bacterial species could digest equal amounts after treatment. On the other hand, although the bacteria adhered to cell walls shown by histochemical methods to be of reduced lignin content, this did not necessarily result in polysaccharide degradation.

Stewart et al.[152] incubated untreated barley straw with various cellulolytic, xylanolytic, or pectinolytic rumen bacteria. The cellulolytic *Ruminococcus albus, R. flavefaciens,* and *Bacteroides succinogenes* caused loss in weight of straw, the latter two bacteria causing the greatest loss. When the straw was incubated with pure culture or a mixture of the bacteria, the ruminococci and bacteroides were closely associated with areas of stems showing degradation, while the xylanolytic *Butyrivibrio,* although seen in large numbers, were not closely associated with the cell walls. As might be expected from the composition of straws, the pectinolytic *Lachnospira multiparus* did not by itself cause any apparent disintegration of the straw, and was not associated with the cell walls (cf. effect on pectin-containing leaves described previously). In these experiments,

contrary to the results of Latham et al.[151] with untreated barley straw, *Bacteroides succinogenes* digested more of the straw than did *R. flavefaciens*. However, both these species digested more of the straw than did *R. albus* which could digest powdered filter paper but not cotton. The former bacteria degraded cotton.

Straw is degraded by rumen organisms in the same way as other plant fibers by bacteria attached to, or closely associated with, the plant cell walls, but bacterial attachment is much more impeded by the large amount of lignin and possibly silica or other undegradable materials associated with the straw. Alkali treatment removes some of the lignin and allows more extensive bacterial colonization and degradation.

D. Some Enzymes Concerned in Fiber Digestion

Over the years a number of enzymes able to hydrolyze preparations of the polysaccharides of plant cell walls have been isolated from mixed and single rumen organisms. "Preparations" is used here because the substrates used have been prepared, either commercially or by the workers concerned, from plant material and this involves physical or chemical (or both) treatments that will result in some change in the structure of the molecule from that in the intact plant. In addition, most commercial preparations will be from plants other than those used in ruminant feeds, e.g., cellulose from wood or cotton (papers) or pectins from fruits. The ability of enzymes to attack these substrates may not be entirely indicative of their attack on intact feedstuff carbohydrates. However, it is difficult to overcome such problems.

Earlier work on enzymes is described in some of the reviews mentioned at the beginning of this section and Prins[153] has also listed many of the experiments up to about 1976. In this section, mainly more recent experiments will be quoted briefly to illustrate particular points.

The work discussed previously has shown that the cellulolytic bacteria, in general, attack both cellulose and hemicellulose in plant cell walls, as well as, in some cases, pectin. Pettipher and Latham[154] investigated in some detail the fiber-digesting enzymes of a strain of *Ruminococcus flavefaciens*. In culture supernatants of bacteria grown with cellobiose or filter paper as the energy source, enzymic activity against the following substrates was detected. The activities are expressed as total reducing sugar released from each substrate with ball-milled filter paper arbitrarily assigned the value 1.

1. Larch-wood xylan, 9.9
2. Oak sapwood hemicellulose, 5.8
3. Ryegrass mesophyll cell walls, 3.4
4. Carboxymethylcellulose, 9.3
5. Filter paper, 1.0
6. Solka-floc, 0.5
7. Avicel, 0.4
8. Scoured native cotton, 0.03

Pection lyase and pectin methylesterase were detected using citrus pectin and weak aryl β-glucosidase, and xylosidase activities were found with the o- and p-nitrophenyl derivatives.

These results show that enzymes commensurate with the properties of the bacteria are liberated into the culture medium and so could be liberated into plant cells near to the bacteria in the rumen, but they also emphasize the previous point about diverse substrates. The cellulase, xylanase, and aryl β-glucosidase all had pH optima at 6.4, about the normal rumen pH when forages are fed, and temperature optima of 39 to 45°C. A cellulolytic enzyme mixture from rumen contents had a pH optimum of 6.9.[155]

The pH optima of the enzymes is related to the pH at which the bacteria can grow. For

instance, Stewart[156] had previously found that reducing the pH of rumen contents to 6.0 from 7.0 almost completely destroyed cellulolytic activity (cotton threads) and reduced the numbers of culturable cellulolytic bacteria. The effect of addition to the feed of starch, such as barley, on lowering digestion of fibers seemed from this study to be mainly an effect of the lowering of rumen pH by rapid fermentation of starch. Gel filtration showed that although the enzymes were recovered from the culture supernatant, a large proportion of the polysaccharase activities was associated with a high molecular weight material that could have originated in the bacterial cell wall.[154] This material may have come from lysed bacteria, but it is interesting to note that Stewart et al.[152] observed small particles associated with *B. succinogenes* attached to plant fibers. In earlier work Henderson and Hodgkiss[157] found the extracellular lipase of *Anaerovibrio lipolytica* to be associated with similar membranous particles. This latter bacterium probably acts at the interface of water and oil droplets in breakdown of lipids.

The degree of order in cellulose fibers is decreased by mechanical and chemical treatments such as are used to produce many of the substrates used in determining the activities of cellulolytic organisms and enzymes. Wood and McCrae[158] found in some fungi an exoglucanase and an endoglucanase which, acting together (either as the initial mixture or separated and recombined enzymes), could degrade the most highly ordered cellulose. Many cellulolytic organisms contain only the endoglucanase, and enzymes that can degrade highly ordered cellulose have not been isolated from any cellulolytic bacterium.[159] The extracellular cellulase of *R. albus* was an endoglucanase which could degrade the soluble carboxymethylcellulose or solubilize acid-swollen cotton but not solubilize native cotton. A mixture of this enzyme with an exoglucanase from *Trichoderma koningii* did not attack cotton, but a mixture of endo- and exoglucanases from *T. koningii* was highly active.[159] Either there are stereo or other specific effects in the actions of the glucanases or the action of the ruminococci on cellulose differs from that of the fungi. However, the situation is in general complicated, in that strains of cellulolytic rumen bacteria differ in their ability to attack highly ordered cellulose.[160]

Yu and Hungate[161] examined extracellular cellulolytic enzymes from culture fluids of a number of strains of *R. albus*. At least four cellulases, differing in molecular weight, were found, but strains varied in cellulase activities. All four enzymes produced soluble carbohydrates from ball-milled cotton, Avicel, and alkali-treated alfalfa cell walls, but the enzymes differed in their rate of attack on the same substrates and each enzyme had a different rate of attack on the different substrates. The rate of attack on each substrate in all except one case decreased with decreasing molecular weight of the enzyme. Avicel generally was attacked at the lowest rate. However, extent of degradation was not reported. The soluble products of hydrolysis of ball-milled cotton differed for the four enzymes.

The ruminococci degraded pectin and contained pectinolytic enzymes, but did not utilize the products of pectin hydrolysis. *Lachnospira multiparus* has previously been discussed as a bacterium with pectinolytic activity and which utilizes pectin for growth. A strain of this bacterium produced an extracellular enzyme complex in culture. The crude enzyme precipitate contained an exopolygalacturonase producing galacturonic acid and unsaturated galacturonic acid. The major enzyme in the mixture was a polygalacturonic acid lyase producing unsaturated galacturonates, mainly the dimer. The optimum pH of the enzymes was 8.0, tested over the range 7.0 to 9.6, which is above the normal rumen pH, but the optimum temperature was 40°C. The enzyme required calcium ions. A similar mixture of enzymes was said to be present in some strains of *Bacteroides ruminicola*.[162]

The large pectin-fermenting spirochete previously mentioned produced pectinolytic enzymes in the culture liquid. Partially purified enzyme, tested on the basis of production of reducing sugars from polygalacturonate, had a maximum activity at pH 8.5, but a

shoulder on the activity curve at pH 6.5 suggested the presence of another enzyme. Tests showed that the predominant enzyme in the mixture was a polygalacturonate lyase, most active at pH 8 to 9. The other enzyme acting below pH 7 was probably a polygalacturonase, but the pH range of activity was higher than that of nonrumen bacterial enzymes. The mixture of enzymes degraded polygalacturonate to saturated and unsaturated monomers. A pectinesterase was also present in the culture liquid.[163] The small pectinolytic spirochetes produced a polygalacturonic acid lyase and a pectinesterase.[98] *Streptococcus bovis,* generally regarded in the rumen context as a starch-degrading bacterium, is also pectinolytic and contains a lyase, but no pectinesterase or polygalacturonase.[164] This bacterium does not utilize pectin degradation products, but it can grow on the pentoses or galactose that occur in natural pectins and which are released on pectin hydrolysis.

Coleman et al.[165] examined nine species of entodiniomorphid protozoa for pectinolytic activity. *Entodinium caudatum* had no activity, and other starch-feeding protozoa had little. The highest activities were found in the grass-digesting protozoa. However, none of the protozoa actually utilized the degradation products of pectins. The enzyme in all the protozoa was a lyase, again with a pH optimum of about 8 to 9.

Hemicellulolytic activity in *R. flavefaciens* was mentioned previously and the enzyme produced a mixture of xylose and the biose, triose, and tetraose from larchwood xylan.[154] However, recent investigations of the hemicellulases of rumen organisms have been few. Williams and Withers,[166] in a preliminary report on the enzymes of a number of strains of hemicellulolytic bacteria, said that the hemicellulases were constitutive, but growth of the bacteria with glucose or glucose-containing disaccharides reduced enzyme activity. The hemicellulase of *R. flavefaciens* was constitutive,[154] but earlier work had shown that the xylanases of a *Butyrivibrio* and a bacterium then named *Bacteroides amylogenes* were inducible.[167]

Many enzymes are involved in fiber polysaccharide degradations and the multiplicity of enzymes is a reflection of the complex physical and chemical structures of the polysaccharides themselves, as well as the heterogeneous structures of the fibers as a whole. The action of some enzymes seems to be to expose a growth substrate to the organism — not to provide soluble sugars which can be utilized. This is probably particularly the case with pectinolytic enzymes. Pectin is a matrix in which plant cells are embedded so the possession of pectinolytic enzymes is obviously of use to bacteria which require hydrolysates of the wall polysaccharides, or other plant sugars, for growth. But one might think that it would have been more efficient if these organisms also had evolved a mechanism for fermenting galacturonic acid, as have other organisms.

IV. INTERRELATIONSHIPS BETWEEN RUMEN BACTERIA AND CILIATE PROTOZOA

Recently, two reviews have been published which deal with the rumen ciliates.[168,169] These give references to papers on the biochemistry of particular species of protozoa. In a more general review, all aspects of a subject cannot be covered as they can in a specialized review. So one particular aspect of the role of the ciliate protozoa has been emphasized in this section, the interactions of protozoa and bacteria. Some of the metabolic reactions of the protozoa are referred to in other sections.

A. Defaunation

The ciliate protozoa were the first rumen organisms to be described in some detail by Gruby and Delafond in 1843.[170] Because of their large size they could easily be distinguished from the bacteria and food debris. Becker and Talbott[171] in 1926 and

Dogiel[172] in 1928 described and illustrated the species of protozoa, and as early as 1930 attempts were made to culture them.[173]

Even at that time there was argument about the role of the protozoa in the ruminant, and Becker[174] in 1929 described experiments in which copper sulfate was used to kill the protozoa without, apparently, affecting the bacterial population or the life of the host animal. In the later period of rumen microbiological activity in the 1950s and 1960s, the determination of the rumen metabolic pathways and the biochemistry of the bacteria and protozoa, which showed that protozoa and bacteria carry out similar carbohydrate fermentations, led to more experiments on the role of the protozoa in the life of the ruminant.

Following Becker's ideas, the object of the experiments has been to produce an animal with a "normal" bacterial flora but no protozoa. The flora might, of course, differ from that associated with protozoa, but the defaunating method itself should not influence the bacteria. Thus, an agent is sought which will selectively kill the protozoa on one or two additions to the rumen and then the animals can be kept protozoa-free by isolation from other, normal, animals. Such an agent, so far does not seem to have been found. The copper sulfate used by Becker and later workers is toxic to all cells. Recent agents have been suface-active chemicals, including dioctyl sulfosuccinate and Terics.[175,176] These latter almost certainly affect the bacteria,[176,177] and an effect on the animal itself in the days after dosing has been mentioned sometimes,[176,177] so the dosage must be determined carefully. The ciliate protozoa are in relatively small numbers (usually less than 10^6/mℓ) in the rumen and the defaunating agent can reduce them to numbers from which the population cannot regenerate, particularly as natural inocula come only from close association with faunated animals. However, because of the large numbers of bacteria (approximately 10^9/mℓ), even if they are damaged to some extent, sufficient will remain to regenerate the population. Inocula to help regeneration will come from the air, feedstuffs, and other sources. Regeneration of a full bacterial flora by natural inoculation may take some weeks, however,[176] and some experiments with defaunated animals have been short-term. Changes in the bacterial population are not easy to determine and doubts have always been expressed as to whether a chemically defaunated animal is truly comparable with the animal with its ciliate population.

The method of rearing unfaunated animals by keeping them isolated from other ruminants from birth would seem to offer a better comparison between the faunated and unfaunated rumen. Airborne bacteria should inoculate the isolated animals, since the animals can be kept in the same building as normal ones and rumen bacteria have been found in the air of animal houses.[178] The procedure, though, demands long-term care of the animals and long-term planning. Another method of defaunation is to temporarily change the feed to one giving rumen conditions (e.g., pH below 6) inimical to the protozoa and then isolate the animals.[179,180] To try to ensure full regeneration of the bacteria after any of these defaunating procedures, inocula of protozoa-free (differentially centrifuged) rumen fluid from normal animals sometimes have been given, but obtaining truly comparable rumen contents remains a big problem in studies of the effects of the protozoa. This may be the cause of some of the varying experimental results obtained.

B. The Effect of the Protozoa on the Animal: Growth and Protein Utilization

There is no doubt that protozoa, as such, are not essential to the ruminant digestive system. Not only do numbers and types of protozoa vary with the diet, but some diets, particularly the modern ones high in starch concentrates, can produce rumen conditions (as mentioned above) in which the protozoa cannot grow, and yet the animals thrive. But on diets more akin to those of the ruminant in natural surroundings, the protozoa are an integral part of the rumen system — have they a special role in this system?

Bacteria-feeding by the protozoa was noted early on, as described later, and Eadie and Hobson[181] found that the numbers of bacteria in faunated sheep were very much smaller than in unfaunated animals. Since the metabolic activities of the microorganisms were similar, it was suggested that the bacteria and protozoa were, in effect, "interchangeable", lack or low numbers of one being made up by more of the other. If this is not so, and if the protozoa are to have any measurable effect on the animal's digestive processes, then this should be discernible in the weight gains of growing animals.

In experiments in 1930, Becker and Everett[182] found that faunated animals gained slightly less weight over a 3-month period than did companion defaunated animals. On the other hand, 35 years later Christiansen et al.[183] reported that faunated animals gained weight more rapidly and more efficiently than did defaunated ones, and Abou Akkada and El Shazly[184] arrived at the same conclusion.

A factor affecting weight gain is the digestible protein supplied to the animal; in the case of the ruminant this is largely the rumen organisms. Measurements of the proportions of bacterial and protozoal protein in rumen digesta have given variable results (e.g., 20[71] to 58%[185]) as might be expected since the numbers of ciliates visibly vary with the ruminant diet. McNaught et al.[186] found that the protein of a dried preparation of protozoa from rumen contents was better digested by rats and of higher biological value than that of dried rumen bacteria. Weller[187] also showed that protozoa have a higher content of the essential amino acids than have bacteria. The bacterial concentration in the faunated rumen is lower than that of the unfaunated rumen, but if the bacteria are replaced by protozoa of higher value as protein, then this is a positive effect and the more protozoa the better. Later analysis has suggested that protozoal protein is not of higher value than bacterial protein.[188] However, whether or not protozoal and bacterial proteins differ, the value of the protozoa as protein for the host depends on the protozoa leaving the rumen to be digested and absorbed in the intestinal tract.

The work of Weller and Pilgrim[189] cast doubt on the value of the protozoa. They found that only 20% of the protozoa in the rumen actually passed from the rumen to the omasum, and so to the abomasum and intestines, and that the protozoal nitrogen leaving the rumen was only 2% of the dietary nitrogen. Since the protozoa convert bacterial and probably other particulate protein (see later) to protozoal protein, the protozoa are effectively recycling within the rumen protein which could be of value to the host. If this is so, it should be reflected in the nitrogen balance and growth of young ruminants.

Bird et al.[190] confirmed the results of Weller and Pilgrim, finding that only about 10% of the protozoa in the rumens of sheep and steers taken from pasture feeding left the rumen. (Weller and Pilgrim's experiments were on sheep fed at hourly intervals on different hays or grain.) They also found that on low-protein diets defaunated lambs gained weight quicker than faunated ones. The results were confirmed in more detailed experiments[191] and were also found with cattle.[192] In these animals increased growth was obtained by greater efficiency of utilization of the feed, not by increased intake. The experiments suggested that when protein intake is high, undegraded feed protein bypasses the rumen (see later) and this, with microbial protein, is more than sufficient for the animal's needs. So a loss of feed nitrogen as protozoa remaining in the rumen has no apparent effect on growth. When feed protein is low it is all degraded in the rumen and the animal must rely entirely on the microbes for its protein supply. A loss of microbial protein thus leaves an insufficient amount for optimal animal growth.

However, Harrison et al.[193] found with sheep fed on a semipurified diet of starch, Solka-floc® cellulose, Molassine® meal, and urea as the only sources of nitrogen, that a higher proportion of rumen protozoal protein (41%) reached the duodenum, and protozoal nitrogen was 24% of the microbial nitrogen at the duodenum. Thus, although

the overall conclusions appear correct, quantitatively results seem to differ in different experiments.

A principal source of quantitative difference may lie in the nature of the diet, as experiments in vitro with artificial rumens containing fibers (grass, hay, etc.) not allowed to leave the rumen vessel have shown that such masses of fibers sequester large numbers of ciliates and the concentrations of the protozoa in the fibers is much higher than in the liquid flowing through the rumen.[135,136] Electron micrographs of fibers from the true rumen digesta have shown large numbers of attached protozoa.[137-139] In the rumens of Red deer which contained larger amounts of slowly digested and fibrous heather and other shrubs, higher numbers of protozoa were found than in sheep which ate the softer and more easily digested heather tips and roots. In reindeer, feeding on lichens and grass where the rumen contents were a "solid" mass from which little liquid could be expelled, protozoal numbers were extremely high.[13] Solid, relatively slowly digested feed particles remaining in the rumen for longer than the liquid detention time may not only attract by chemotaxis and retain protozoa which can digest the particles, but (particularly if they are fibrous), also physically entrap the protozoa and so prevent or delay their passage from the rumen. Such effects obviously would vary quantitatively between ruminants on different feeds, and together with the effect of protein content of the feed could explain the various growth rates of faunated and unfaunated animals.

C. Effects on Rumen Metabolic Activities

Even if a proportion of the protozoa never leaves the rumen, they still may form a significant proportion of the microbial mass and replace some of the bacterial population. If their metabolism differs from that of the displaced bacteria, a difference in microbial metabolites might be expected between the rumens of faunated and unfaunated ruminants. Unfortunately, or perhaps as might be expected, experimental results are by no means clear cut. Again, this may be due to differences in diet and numbers and types of protozoa in the experimental animals.

There seems to be general agreement that rumen ammonia concentrations are higher in faunated than unfaunated sheep. However, a number of workers reporting this have mentioned that it is difficult to determine whether the higher ammonia level can be related directly to protozoal activity.[194-197] Abou Akkada and Howard[198] found that washed suspensions of one ciliate, *Entodinium caudatum,* lost about 1% of cell nitrogen per hour when incubated in buffer, and more than half of the nitrogen was in the form of ammonia. However, although the protozoa hydrolyzed casein, they did not deaminate the resultant amino acids. Onodera and Kandatsu,[199] in later studies, also found that about 40% of the nitrogen compounds liberated by endogenous metabolism of mixed ciliates was ammonia and 40% was amino acid. When particulate or dissolved casein was ingested by Ophryoscolecid or Holotrich protozoa, part of the casein was incorporated into the protozoa and the rest was liberated into the medium, mainly as peptides.[200] Coleman[169] has shown that proteins of bacterial or other origin, digested by Entodiniomorphid protozoa, are excreted partly as amino acids. The higher ammonia levels in faunated animals could, then, be due to excretion of ammonia during periods of endogenous metabolism of the protozoa or by an increase in the amounts of peptides and amino acids available for bacterial deamination.

Similarly, reports on the effects of faunation on the total concentration of VFA and the proportions of the three main acids have varied. An increase in the proportion of butyric acid[23,195] might be expected as butyric acid is a fermentation product of a number of protozoa (see Reference 4). An increase in the molar proportion of propionic acid is more difficult to understand as propionic acid is not a large product of protozoal fermentations.[4,201,202] Luther et al.[196] found higher propionic acid only in faunated lambs

fed a mainly concentrate diet, as were the lambs in the previous work quoted, but Males and Purser[194] said that although ammonia levels and the proportions of the three VFA were interlinked, they were not necessarily related to the protozoa. In the same way, although ciliates have been shown to hydrogenate unsaturated long-chain fatty acids in vitro,[203] the effects of defaunation on lipid hydrogenation have been variable (see Reference 169).

D. Protozoal and Bacterial Ecology

Whatever the gross results of the presence or absence of protozoa in the rumen, and it is obvious that they do not directly replace bacteria in metabolic function, there is no doubt that in the mixed culture of the normal rumen the ciliate protozoa and bacteria interact at the microscopic level as do protozoa and bacteria in other habitats. Detailed quantitative studies of the interactions of the rumen protozoa and bacteria have not been so far possible in the ways in which the relationships in other habitats (both protozoa and bacteria from which can be grown in pure or defined mixed, continuous cultures) have been studied in vitro.

1. Ingestion of Bacteria by Protozoa In Vitro

Granules of various kinds were noted in early studies on the protozoa, and later work has shown that rumen protozoa, like those from other habitats, ingest bacteria. Van der Wath and Myburgh in 1941[204] said that the so-called glycogen granules in some rumen protozoa (mainly *Entodinium* and *Diplodinium*) were actually bacteria, and that the digestion of starch and cellulose in the protozoa was due to bacteria. The argument about whether the protozoa used bacterial hydrolytic enzymes or possessed enzyme systems of their own continued for some time. However, experiments by a number of workers with washed and antibiotic-treated ciliates have shown that the protozoa possess their own hydrolytic enzymes, and that bacterial enzymes play little, if any, role in their metabolism. The cellulolytic activity of *Eudiplodinium maggii* was one enzymic activity about which there was originally doubt,[205] but Coleman[206] has shown recently that 70% of the cellulase activity he determined was not of bacterial origin, although some bacterial cellulase could have been associated with the protozoa. The amount of bacterial enzymes present in protozoa may be quite fortuitous, because it now seems that the principal role of bacterial ingestion is as a nutrient supply for the protozoa and bacteria are lysed and digested.

In early experiments on the culture of rumen ciliates, ingestion of bacteria by at least some of the protozoa was noted. The holotrichs, *Isotricha prostoma* and *Dasytricha ruminantium,* and the entodiniomorphs, *Entodinium* and *Diplodinium,* ingested bacteria (both rods and cocci) in culture but, while viable bacteria were necessary for the continual growth of *Epidinium ecaudatum* in culture, whether the bacteria were ingested was uncertain.[207-210] Mah[211] noted that *Ophryoscolex purkynei* ingested a large streptococcus from the culture medium.

Later experiments have shown that all the protozoa ingest bacteria, and have confirmed the general results of the earlier observations. Like the experiments mentioned above, later experiments on bacterial ingestion have been carried out either by observing nonaxenic cultures, or suspensions of protozoa taken from culture or the rumen and presented with bacteria. The bacteria available to the protozoa thus have differed from experiment to experiment and this makes results difficult to interpret. Since nonsterile conditions are used, most protozoal cultures contain a larger proportion of common, contaminating types of facultative bacteria than does the rumen habitat of the protozoa. It has not been shown conclusively that any species of protozoon is uniquely dependent on one particular species of bacterium; nonrumen and rumen bacteria, and yeasts have been ingested in experiments on various protozoa.

Coleman[212] found with *Entodinium caudatum* that different species of bacteria survived for different times in the protozoon before being killed and digested. With Gram negative bacteria all except the lipopolysaccharide component of the membrane system were digested, but the Gram positive bacteria tested varied in susceptibility to lysis. The cell walls of *Bacillus megaterium* were completely digested, but those of *Staphylococcus aureus* and *Streptococcus faecalis* were resistant. Residues of bacterial cells were extruded by the protozoa.[213] These three bacteria are only minor components of the rumen flora, if found there at all, and as with some other results of experiments on rumen ciliates it is possible that culture conditions may have some effect on the protozoa. In the experiments mentioned above, *Escherichia coli* was one of the bacteria seen to be engulfed by cultured protozoa, and Hino and Kametaka[214] grew *Entodinium caudatum* in culture with *E. coli* as the only bacterium present. The cultured *Entodinium* ingested 12,000 *E. coli*/ protozoon/ hr, but entodinia grown in vivo and then tested in vitro seemed to ingest only about 40 rumen bacteria/ protozoon/ hr.[215,216] *Entodinium longinucleatum* in culture ingested a variety of bacteria,[217] but showed a preference for *Klebsiella aerogenes* and *Proteus mirabilis,* nonrumen bacteria, while it did not ingest the rumen *Bacteroides ruminicola.* However, it also rejected the nonrumen *Pseudomonas* sp., so in this case whether artificial growth conditions were affecting the preference for bacteria is difficult to say. *Polyplastron multivesiculatum* showed a preference for engulfment of *Proteus mirabilis,* a bacterium in the culture medium, rather than for rumen bacteria.[218]

Further experiments (see review, Reference 169) have shown engulfment of rumen and nonrumen bacteria by other entodiniomorphid protozoa. The rate of engulfment, and the number of bacteria taken up, varies, the latter probably being a function of the volume of the protozoon, although there may be other factors involved; experimental results are indefinite. The protozoa seem to pass the liquid (in which they are growing continuously) through their bodies and with it particulate matter. The work of Onodera and Kandatsu[219] suggested that the protozoa were not discriminatory with regard to the nutritional value of particles ingested as the entodiniomorphid protozoa, in their experiments, ingested bacteria, yeasts, and cellulose or carbon particles. Coleman and Hall[220] also noted that *E. caudatum* would ingest any particles, provided that they were not too large. Onodera and Henderson[221] found that *E. caudatum* could most effectively assimilate a soluble nutrient prepared from bacteria when the nutrient was absorbed on to small particles of activated charcoal which the protozoa could ingest.

Investigations of bacterial ingestion by the two rumen genera of holotrich protozoa have not been as extensive as those on the entodiniomorphids. Apart from the previously mentioned observations and those of Onodera and Kandatsu,[219] Wallis and Coleman[222] showed that *Isotricha* spp. could engulf 3000 *E. coli*/ protozoon per hour and that the bacteria were killed and digested.

2. Bacterial Ingestion In Vivo

The experiments in vitro do not, overall, suggest that a mixed rumen protozoal population would by ingestion change the numbers of only particular types of bacteria. Early experiments showing an overall decrease in numbers of small bacteria when a protozoal population developed in previously unfaunated sheep did not distinguish morphological types.[17] There was little, if any, change in numbers of large bacteria (Quin's ovals, large selenomonads, etc.), and later experiments where counts were done over longer periods showed that the numbers of large bacteria varied considerably over the weeks without reference to the presence, or absence, of protozoa.[223] It is possible that these bacteria are too large to be easily engulfed by the protozoa.

Visual separation under the microscope of bacteria into the smaller and larger types is relatively easy. However, visual differentiation of the small bacteria is very difficult because of pleomorphism in particular species and morphological similarities between

different species, so it is difficult by microscopic counts to determine whether selective protozoal ingestion of particular types of small bacteria takes place in the rumen. Cultural counts may provide evidence, but, again, there is the problem of whether all viable bacteria are being cultured. Whatever method of assessing the bacterial population is used, because of the apparent day-to-day as well as diurnal variations in bacterial numbers, many counts over long periods are needed to obtain meaningful results and relatively few experiments have been done.

The results of Kurihara et al.,[223] where total (microscopic) and viable (cultural) counts of bacteria were made, and a number of media were used in the viable counts, suggested that there was some selective removal of starch-hydrolyzing bacteria by *Entodinium caudatum* and *Polyplastron multivesiculatum,* but this could have been due not to ingestion of the bacteria as such but to ingestion along with the starch granules, used by the protozoa as food, of adherent amylolytic bacteria. These sheep were fed on natural diets. Later work by Kurihara et al.,[224] where sheep were fed on a purified diet containing wood-pulp cellulose and corn starch, confirmed the previous observation that in unfaunated sheep amylolytic bacteria were about 65% of the viable bacteria, whereas in a comparison sheep with a protozoal population consisting mainly of mixed *Entodinium* species amylolytic bacteria were only about 6%. The suggestion was made many years ago that protozoa modified the rumen fermentation by ingesting starch granules, which they fermented slowly so preventing rapid bacterial fermentation of free starch leading to an acid rumen, and some later observations on rumen VFA concentrations seemed to confirm this.[225] Examination of some 2000 slides of the bacteria cultured from the faunated and unfaunated sheep in the former experiment[223] did not lead to conclusive results so far as species were concerned. Curved, Gram negative rods (*Butyrivibrio*-like) decreased in proportionate numbers in faunated sheep compared with those in the unfaunated animal. Coleman and Sanford[216] later noted that rumen-grown *E. caudatum* rapidly digested *Butyrivibrio* in vitro, but these were not the only bacteria ingested. On the other hand, the presence of rather more *Streptococcus bovis* type bacteria in the sheep faunated with *E. caudatum* alone than in the unfaunated sheep suggested that the ingestion of *S. bovis* in vitro by *Entodinium* previously reported[209] was not selective. *S. bovis* was largely eliminated when *Polyplastron* was added to the *Entodinium* population or when a sheep was faunated with a mixed protozoal population. In these experiments there was no observable selective ingestion of cellulolytic bacteria by the cellulose-digesting *Polyplastron,* and there seemed to be no synergism or competition between the cellulolytic organisms.

In experiments on the development of rumen bacterial populations in young calves Bryant and Small[226] noted that counts of cellulolytic bacteria increased markedly when isolated animals were inoculated with protozoa-free rumen fluids, and then decreased to "normal" levels when the calves were reinoculated with whole rumen fluid. Total viable counts and counts of lactate-utilizing bacteria showed the same trends, but to a lesser degree. However, the effects of rumen protozoa were not being investigated, and only a few counts were done. Since their isolated calves had rather abnormal bacterial populations before inoculation, it is difficult to tell how much of the decrease in the cellulolytic bacteria is related to the protozoa and how much to the "overshoot" before settling to a steady-state population which occurs when a microbial population is developing in a continuous culture or in the rumen.

On the other hand, Kurihara et al.[224] in the experiments with sheep on a purified diet found the numbers of cellulolytic bacteria were higher in the faunated sheep than in the unfaunated one. Yoder et al.[227] found that cellulose digestion by washed rumen bacterial suspensions incubated in vitro was increased when washed, mixed protozoa were added and the digestion was greater than the added value of the protozoal cellulolysis.

However, cellulolysis was also stimulated by dead, autoclaved, or frozen-thawed protozoa, though not to the same extent as by the live organisms. The reason for stimulation remained unclear, but it was not due to addition of protozoal fermentation acids, B vitamins, or amino acids.

Bauchop and Clarke[137] and Akin and Amos[139] observed by electron microscopy that the cellulolytic *Epidinium ecaudatum* form *caudatum* were attached to or in forage fibers being degraded by the protozoa, and it is possible that a partial degradation and physical breakdown of plant fibers by the protozoa exposes more surfaces for bacterial attack and so increases the numbers of cellulolytic bacteria. On the other hand, as the protozoa ingest fragments of fibrous material they could also be ingesting cellulolytic bacteria attached to the fibers. This might decrease overall cellulolysis or numbers of cellulolytic bacteria.

Apart from ingestion of bacteria the protozoa may affect number or types of bacteria by altering the concentrations of bacterial substrates through their own metabolism, though what is cause and what is effect is difficult to determine. With the sheep fed on natural diets Kurihara et al.[223] found that *Bacteroides*-type rods and selenomonads seemed to predominate over *Butyrivibrio*-type rods in the faunated sheep, whereas the reverse seemed to occur in the unfaunated sheep. This was ascribed to increased nitrogen sources for the particular bacteria provided by the protozoa. It was suggested that an increase in selenomonads and bacteroides types could have accounted for the increased propionic acid concentrations found in faunated sheep by other workers. However, in the later experiments with sheep on a purified diet *Bacteroides* were the major part of the flora of the unfaunated sheep.[224]

The rumen bacteria show a diurnal variation in numbers related to the method of feeding of the animal and, as might be expected, the more continuous the feeding, the less the variations. Authors of a number of papers have reported on diurnal variations in either bacteria or protozoa, but in less have the variations been related by use of faunated and unfaunated animals.

In the experiments with sheep on the natural feed[223] it was shown that as numbers of protozoa increased after inoculation of the rumen, numbers of small bacteria decreased until a new steady-state was reached. Calculations showed that in the different sheep and at different times, the concentration of small bacteria was related to the total volume (per some unit volume of rumen fluid) of protozoa calculated from the numbers and dimensions of individual species. The results suggested that the smaller entodinia were ingesting bacteria to almost the full volume of the protozoon while the larger protozoa filled only part of their total volume with bacteria; a result in accordance with observations in vitro.[215] The protozoa affected the pattern of diurnal variation of the small bacteria, but not that of the large bacteria which they did not ingest. The sheep were fed twice a day and bacterial multiplication was rapid after the feed. The protozoa showed a longer-term diurnal variation not necessarily related to the feeding of the sheep. Mixing of protozoal species in some cases altered the diurnal pattern of particular protozoa, and the various protozoa had different effects on the bacteria depending, probably, on whether the former were in an actively multiplying or "resting" state at the times of maximum bacterial growth. Calculations from the numbers and growth rates of the bacteria and numbers of protozoa showed that (using the figures for rates of bacterial ingestion by protozoa in vitro then available), when most active, the protozoa could nullify the potential increase in bacterial population.

Considering all the information available there is no doubt that by ingestion of bacteria the protozoa do influence the bacterial population. However, since this influence appears to depend on numbers as well as species of protozoa, the effect varies. While the concentration of bacteria seems to be of the same order of magnitude in

different animals on different diets, the concentrations of protozoa seem to vary by several orders of magnitude, sometimes even between animals on the same diet. While this may affect overall quantitative effects of the protozoa on rumen metabolism, the conclusion that the protozoa do influence the bacterial population is valid. It would seem that the protozoa, while some at least are at times attached to feed particles, swim through the rumen fluid ingesting both live and dead bacteria and small bacteria-size particles. On the whole there is little evidence for selection of bacteria, except perhaps by size, although ingestion of plant particles with attached bacteria which are attacking the plant material fortuitously may produce an apparent selection of types. And, except for this latter fact, there seems to be no evidence that the protozoa ingest bacteria with enzymic activities which could help their metabolism. The ingested bacteria are killed and used as nutrient sources by the protozoa, although, as might be expected since bacterial death is not instantaneous, viable bacteria can be cultured from broken protozoa.[228]

3. Bacteria on Protozoal Surfaces

In addition to the bacteria ingested by the protozoa, bacteria are also seen attached to the outer surfaces of the organisms. There is, so far, only a little experimental evidence about the interactions of the surface bacteria and their protozoal hosts. Coleman and Hall[229] examined autoradiographs of sections of *Entodinium caudatum* and *Epidinium ecaudatum caudatum* which had been incubated as washed suspensions with tritium-labeled glucose, amino acids, and bacteria (*B. megaterium* and *E. coli*) to determine where the labeling was deposited. They noted two types of bacteria, a small and a large coccus, attached to the *Epidinium,* but no bacteria attached to *Entodinium*. The labeling from the materials metabolized by the epidinia appeared in the surface bacteria after various times. The bacteria in this case could be metabolizing labeled soluble compounds from the medium and labeled lytic products of bacteria released by the protozoa. So the association would benefit only the bacteria.

Imai and Ogimoto[230] also found spherical bacteria on the surface of epidinia and these bacteria reacted with fluorescein-labeled antisera to *S. bovis* and *R. albus*. From the known properties of these bacteria it is difficult to see any benefit to the protozoa from this association and presumably the bacteria were metabolizing substances excreted by the protozoa.

On the other hand, Vogels et al.[231] demonstrated an association of bacteria and ciliate protozoa which could be of mutual benefit to the organisms. Using the fluorescence of methanogenic bacteria caused by the presence of the coenzymes F_{420} and F_{350} they showed that methanogenic bacteria were associated with the outer surface of all 11 species of entodiniomorphid protozoa they found in rumen samples, but not with the holotrich protozoa. The frequency of association varied between the different species and also in particular species in rumen samples from different cows. Hydrogen is produced by protozoal fermentations, as by bacteria, and removal of this hydrogen by methanogenesis is an important factor in maintaining the rumen biochemical equilibrium. If fermentation hydrogen is excreted through the cell surface of the protozoa, a close association with methanogenic bacteria could increase the rate of protozoal fermentations. The bacteria would benefit by having a convenient source of hydrogen, as well as, possibly, fermentation carbon dioxide, their only substrates for growth. Long chains of methanogenic bacteria suggested active growth. The reasons for variation in frequency of association of bacteria with the different species of entodiniomorphid protozoa were not apparent, although the structure of the protozoal surface could be a factor. There would seem little possibility of bacteria adhering to the cilia-covered surface of the holotrichs.

Again, association with surface bacteria is not peculiar to the rumen protozoa; bacteria associated with the surface of protozoa in other habitats have been described.

V. PROTOZOAL-PROTOZOAL INTERACTIONS AMONG THE CILIATES

This review has concentrated on the interactions of the bacteria and ciliate protozoa, particularly the predator-prey actions. Predator-prey actions have also been observed between some species of rumen ciliate protozoa in vivo and have been confirmed in cultures.[232-234] It originally was suggested that certain ciliate populations[232,235] could not permanently coexist, but there are observations of coexistence although the timespan is indefinite, and certainly the prey species of protozoa continue to be rumen inhabitants so the overall interactions must be involved.

Eadie[232,235,236] found that the ciliate populations in all sheep contained a mixture of *Entodinium* spp. and holotrichs, but that the larger entodiniomorphid protozoa were either of Type A, in which *Polyplastron multivesiculatum* was a major component with other species such as *Diploplastron affine* and *Ophryoscolex tricoronatus,* or of Type B in which *Eudiplodinium maggii* and *Epidinium* spp. were together or alone. There seemed to be possibly some host effect as in the range of animals studied, Type A seemed to be more prevalent in sheep whereas Type B were more common in cattle. The "spontaneous" change of a Type B population in a sheep, penned near other sheep with Type A populations, to a Type A population was noted, and experiments showed that this change could be brought about in other sheep by inoculation with Type A protozoa. No gross rumen bacterial change was observed in these and other experiments with cross-inoculations and although some protozoal parasites were observed, these did not appear to be the causative agents.

Predation was a possibility and later experiments in vitro showed that this definitely occurred. In these experiments,[233] although *Epidinium ecaudatum caudatum* could be cultured in the presence of only bacteria, *Polyplastron multivesiculatum* could grow in the usual bacteria-containing culture media only when the *Epidinium* or *Eudiplodinium maggii* were also present. The *Polyplastron* engulfed and digested the other protozoa and each *Polyplastron* seemed to require at least one prey protozoon per day to grow. The *Polyplastron* could grow without prey in a medium with a higher sodium chloride concentration than that needed by other nonpredatory protozoa, but protozoa grown thus became predatory under the correct conditions. Prey specificity varied with cultures derived from different inocula of *Polyplastron.*

Culture experiments[234] also showed that *Entodinium bursa* had an obligate requirement for *E. caudatum,* but only for the spineless form. The *E. caudatum* is usually in the spined form in sheep, but spineless forms develop in vitro and also in vivo. The spineless form is engulfed by *E. bursa* and so in sheep containing the two protozoa only the spined form visibly survives. Coleman[234] suggested that the spine requires extra energy for growth and so spined variants are normally at a disadvantage, but the spine obviously protects the protozoon against predation.

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