MICROBIAL ECOLOGY AND ACTIVITIES IN THE RUMEN: PART II

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VI. GROWTH YIELDS AND ENERGY METABOLISM OF RUMEN MICROORGANISMS

One of the most eagerly pursued topics of rumen microbiology, because of its practical significance, has been the efficiency of conversion of food into microbial matter, the 'growth yield', of rumen microorganisms. This section deals with the microbial physiology and biochemistry of energy conservation during growth as it applies to these organisms and as it affects growth yields. Conclusions then follow, based on the experimental evidence reviewed. These differ somewhat from those of other authors cited; this reflects the controversy that exists in the calculation and interpretation of growth yields of microorganisms in general.

A. Theoretical Difficulties

Growth yield is usually defined as the dry weight of cells produced per mole of substrate fermented ($Y_{substrate}$) or weight of cells produced per mole of ATP formed in the fermentation pathway used by the organism (Y_{ATP}).

There are two easily defined central problems in the calculation and interpretation of the growth yields of microorganisms, and it will become clear that these problems are particularly acute in our understanding of growth yields of rumen bacteria. These are

1. Measured growth yields of bacteria (and indeed of yeasts and animal cells as well) are virtually always lower than the yield which would be expected by consideration of the ATP thought to be consumed by known biosynthetic pathways, and are usually substantially less than this value, which has come to be known as the 'theoretical growth yield' or Y_{ATP}^{theor}
2. ATP produced by breakdown of the energy source is derived not only from substrate-level phosphorylation, which is of known stoichiometry, but also from processes associated with electron transfer reactions and transmembrane vectorial metabolism of usually unknown stoichiometry. It is therefore impossible to be certain of the amount of ATP synthesized per unit of substrate metabolized in growing cells.

Although these problems are obviously closely related in their relevance to microbial yields, it is not always appreciated that they are quite distinct. Solution of (1) requires an improved knowledge of the ATP-consuming processes of cell growth and affects calculation of Y_{ATP}^{theor}, whereas (2) requires a better understanding of the ATP-generating reactions and causes difficulty in calculating the observed cell yield as a function of the ATP formed (Y_{ATP}^{obs}).

Y_{ATP}^{theor} for bacterial growth has been calculated by several authors.[237,243] It has been shown that this value is not constant, but depends on the composition of the cell, on the energy source, on the availability of cell monomers (amino acids, fatty acids, nucleotide bases, etc.) and other factors.[240,242] Some of these factors would be expected to influence the yields of rumen bacteria. For example, rumen bacteria tend to accumulate intracellular polysaccharide at certain periods during the feeding cycle, and since this costs less in terms of ATP than synthesis of other cell polymers, Y_{ATP}^{theor} would be increased at these times.[240,243] On the other hand, the lactate fermenters would be expected to have exceptionally low Y_{ATP}^{theor} because of the high (cf. glucose) energy cost of conversion of lactate to cell material. Nevertheless, the calculated net effect over the daily cycle of polysaccharide accumulation seems to be minor,[243] and the bulk of energy produced in the rumen is by the fermentation of hexoses, so that while some types of bacteria will have quite different values of Y_{ATP}^{theor}, most will have that associated with a hexose fermentation, which is in the region of 30 g/mol, a finding common to the different methods of calculation even with their different inherent assumptions. It should also be noted here that although rumen microorganisms use NH_3 rather than amino acids as their main source of N, the depression in yield due to this effect is likely to be fairly small.[240]

The concept of Y_{ATP} was introduced in 1960 by Bauchop and Elsden,[244] after finding that the yields of different species of microorganisms on different substrates were comparable when calculated in terms of the ATP produced by the metabolism of these substrates. The range of Y_{ATP}^{obs} they found, and most of the values which have been found subsequently, was in the region of 10 g/mol[242-247], much lower than Y_{ATP}^{theor}. Again the values vary somewhat according to organism, substrate, and growth conditions, but never so that Y_{ATP}^{obs} even approaches the value which is theoretically possible, although there are possibly some exceptions to this, and this is where the controversy begins.

One possibility, *Bdellovibrio bacteriovorus*, a periplasmic parasite of Gram negative bacteria, had a calculated Y_{ATP}^{obs} of 25.9 g/mol, compared with a Y_{ATP}^{theor} of 35.6 g/mole, thus apparently actually growing near to the theoretically expected efficiency.[241] In another, Stouthamer and Bettenhaussen[248] extrapolated results obtained with *Klebsiella aerogenes* and suggested that the discrepancy normally seen was due to maintenance energy as defined by the Pirt equation:[249]

$$\frac{1}{Y_{ATP}^{obs}} = \frac{m}{\mu} + \frac{1}{Y_{ATP}^{max}}$$

where m = maintenance coefficient (mol/hr/g dry wt); μ = specific growth rate (/hr); and Y_{ATP}^{max} = extrapolated yield (g/mol) at $\mu = \infty$. Here the extrapolated yield Y_{ATP}^{max} was apparently similar to Y_{ATP}^{theor}, with values of 27.8 and 25.4 g/mol during anaerobic growth in the presence and absence of nitrate,[248] although the yields actually measured in the experiment did not approach this magnitude.

These experiments are unfortunately not convincing in helping to solve problem (1) however. The *Bd. bacteriovorus* system is less well understood than many others, and may suffer because of this. For example, although it was assumed that *Bd. bacteriovorus* used soluble compounds from the host cell, including nucleotides, the possibility that it obtained ATP intact from the host was not considered, and so the calculated Y_{ATP}^{obs} may be unrealistically high. The Stouthamer and Bettenhaussen conclusions were certainly not valid as one of the fundamental conditions of the Pirt relationship[249] was violated. Tryptophan, rather than the energy source, was the limiting nutrient, almost certainly leading to uncoupling[238,250-252] and consequently a nonvalid extrapolated value for Y_{ATP}^{max}. When the experiment was repeated under glucose limitation,[253] maintenance was small and Y_{ATP}^{max} was 14 g/mol.

Rumen bacteria and other strict anaerobes have been seen as a special case or 'anomalous', as they have yields intermediate between Y_{ATP}^{obs} for other bacteria and Y_{ATP}^{theor}. This phenomenon was first noticed in continuous cultures of *Ruminococcus albus*, which gave an average Y_{gluc} (g cells/mol glucose) of 51 after correction for intracellular polysaccharide[254], and *Selenomonas ruminantium*, with a maximum Y_{gluc} of 62.[255] These values were more than double those of other bacteria grown anaerobically,[244,245] and correspondingly high values have since been found for growth of *Bact. succinogenes* on glucose,[256] *Bact. amylophilus* on maltose,[257,258] *S. ruminantium* on glucose[80,259,260] and pyruvate,[80] *Bact. ruminicola* on glucose[256,259–261] and other sugars[262], *Strep. bovis* on glucose,[259,263] *A. lipolytica* on fructose[257,260] but not glycerol[257], *M. elsdenii* on glucose,[259,260] and *B. fibrisolvens* on glucose.[256,259] *Lactobacillus casei*[264] and *Bacteroides fragilis*[265] among anaerobes not normally encountered in the rumen, have similarly high yields. When these yields are converted to Y_{ATP}^{obs}, values in the region of 20 g/mol are usually obtained, double the yields obtained with other microorganisms.

The discrepancy between measured and calculated yields outlined above in (1) is often discussed in terms of factors which may be responsible for depressing Y_{ATP}^{obs} from the calculated maximum Y_{ATP}^{theor}. The rumen bacteria therefore seem to offer some prospect of at least a partial reconciliation with theory, and are of tremendous interest in this respect. Hespell and Bryant[243] discussed factors potentially responsible for the discrepancy, with reference to rumen bacteria. These factors included maintenance energy, nutrient transport, cell composition, availability of nutrients, and uncoupling. To this list might be added experimental imperfections such as dropwise feeds in continuous cultures, which lead to an artificial uncoupling since bacteria are not truly energy-limited all of the time.[266] Hespell and Bryant concluded that the main factor likely to depress yields of rumen bacteria is uncoupling,[243] but it seems to the present authors improbable that this will be significant for energy-limited chemostats of pure cultures, although undoubtedly it may be of greater importance in the rumen itself. We suggest that it is widely understood and even accepted that the discrepancy between Y_{ATP}^{obs} and Y_{ATP}^{theor} exists in microorganisms whose energy metabolism is far better understood than that of rumen bacteria. However, the reasons for this discrepancy are unknown. All that can be said is that Y_{ATP}^{theor} seems to be unobtainable. From what little knowledge is available it would seem that the biosynthetic pathways of the rumen bacteria are unlikely to be very different energetically from those of other bacteria, and yet the growth yields of the rumen bacteria seem generally to be high. So, it would seem more relevant to discuss this latter difference between Y_{ATP}^{obs} of rumen and other bacteria, rather than the former problem — the difference between theoretical and observed yields. Problem (1) as previously defined exists for all bacteria and seems at present unsolvable, but problem (2) is of particular significance in rumen studies and may be capable of explanation.

B. Energy Conservation in Rumen Bacteria

The conversion of Y_{gluc}^{obs} to Y_{ATP}^{obs} requires a knowledge of the ATP produced per mole of glucose metabolized. Traditionally, bacteria growing anaerobically in the absence of exogenous electron acceptors had been assumed to be fermentative in the sense that ATP is produced by substrate-level phosphorylation only. There is now overwhelming evidence that ATP can also be made by electron transfer-linked phosphorylation in many anaerobes, and that ATP can form transmembrane electrochemical gradients and vice versa.[267,268] The findings also apply to rumen bacteria, for although ATP synthesis in response to electron transfer has only been directly demonstrated in one of these organisms, it is known that others contain functional electron transfer components similar to many bacteria known to conserve energy in this way.

The electron transfer chains of *M. elsdenii* and *V. succinogenes* have been studied in

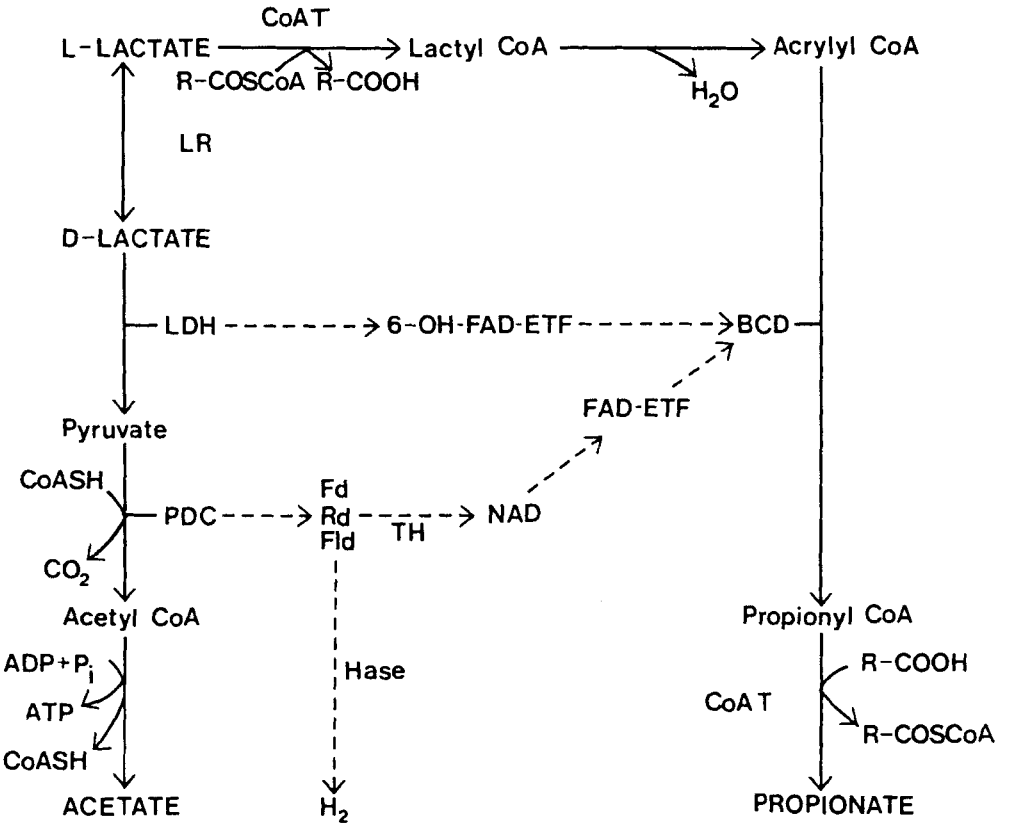

FIGURE 3. Lactate metabolism and electron transport in *M. elsdenii*. Abbreviations for enzyme names: LR, lactate racemase; LDH, D-lactate dehydrogenase; PDC, pyruvate dehydrogenase complex; CoAT, coenzyme A transferase; Fd, ferredoxin; Rd, rubredoxin; Fld, flavodoxin; Hase, hydrogenase; TH, ferredoxin; NAD oxidoreductase; ETF, electron-transferring flavoprotein: 6-OH-FAD, 6-hydroxy-7,8-dimethyl-10-ribityl-5'-ADP)-isoalloxazine; BCD, butyryl-CoA dehydrogenase. Solid arrows indicate reaction pathways, while dashed lines indicate the transfer of reducing equivalents. (From Brockman H. L. and Wood, W. A. With permission of the American Society for Microbiology.)

some detail. The latter organism is the one shown to synthesize ATP in response to electron transfer from H_2 and formate to fumarate, reviewed in References 269 and 270. The electron transfer scheme for transfer of electrons from lactate and pyruvate in *M. elsdenii* as proposed by Brockman and Wood[271] is given in Figure 3. The limited space available does not permit a full discussion of the reactions or the experimental evidence, but it is worth pointing out that electron carriers found in *M. elsdenii* include ferredoxin,[272] flavodoxin,[273] rubredoxin,[274] and two flavoproteins containing the novel prosthetic groups 6-hydroxy-7,8-dimethyl-10-(ribityl-5'-adenosine diphosphate)-isoalloxazine (or 6-OH-FAD) in ETF-LAC (Figure 3)[271] and 7-methyl-8-hydroxy-10-(ribityl-5'-adenosine diphosphate)-isoalloxazine (8-OH-FAD) in ETF-NADH.[276,277] Although these chains have been worked out in detail, their relevance to electron transfer-linked phosphorylation is not known. In view of the fact that the components are soluble[278] it seems unlikely that much electron transfer-driven ATP synthesis occurs.

In other rumen bacteria, cytochrome *b* has been found in *Bact. succinogenes*,[256] *Bact. ruminicola*,[256,260,278,279] *S. ruminantium*,[256,260,280] *A. lipolytica*,[260,280] and *V. alcalescens*,[280] cytochromes of the *a*-type have been found in smaller amounts in

A. lipolytica and *V. alcalescens*[280] and carbon monoxide-binding pigments occur in *Bact. ruminicola,*[278] *A. lipolytica,*[280] and *V. alcalescens.*[280] The nutritional requirement of some rumen bacteria for hemin or other metalloporphyrin chelates[278,281,282] reflects the requirement for the prosthetic group of the cytochromes. Oxidation of cytochrome *b* by fumarate was demonstrated in all of the above bacteria.[278–280] Electron donors to cytochrome *b* included NADH in all the bacteria above[278–280] and H_2 in *Bact. ruminicola, A. lipolytica* and *S. ruminantium,*[260] with glycerol-1-phosphate in glycerol-grown *A. lipolytica*[280] and lactate and pyruvate in lactate-grown *V. alcalescens.*[280] Flavoproteins seem likely to accept electrons from NADH in *Bact. ruminicola*[278,279] and inhibition studies with 2-n-heptyl-4-hydroxyquinoline N-oxide (HQNO) indicate that a quinone, probably menaquinone, might be an electron carrier prior to cytochrome *b* in *Bact. ruminicola* and the other bacteria listed above.[256,278–280] The role of cytochrome *b* is not certain, however. Inhibition studies with *Bact. ruminicola*[260,279] and *S. ruminantium*[260] indicate that cytochrome *b* may not be on the direct route from H_2 or NADH to fumarate. It has been suggested that there may be two cytochromes *b* in the electron transfer chain in these organisms.[260]

The clearest demonstration of the importance of electron transfer-linked reactions to a strict anaerobe was obtained, not with a rumen organism, but with *Bacteroides fragilis,* a predominant bacterium in the lower gut of man and closely related to *B. ruminicola.*[265] The growth rate of this organism was markedly increased by including hemin in the medium. This addition also increased Y^{obs}_{gluc} from 18 to 47 g/mol in a fermentation in which the products changed from the approximate molar proportions 3 fumarate : 2 lactate : 1 acetate : 1 formate : 0.2 succinate : 0.0 propionate to proportions of 0.1 : 0.2 : 2 : 2 : 2 : 1 in the presence of hemin. Cytochrome *b* was present only in hemin-supplemented cultures. Thus there is strong evidence that in *Bact. fragilis* much of the growth yield is derived from ATP produced by electron transfer-linked phosphorylation (ETP) associated with fumarate reductase.

Dinitrophenol and dicyclohexylcarbodiimide, both uncouplers of electron transport, were found to decrease Y^{obs}_{gluc} of *Bact. succinogenes, Bact. ruminicola* and *B. fibrisolvens,*[256] again indicating the value of electron transfer-linked ATP synthesis in rumen bacteria, but not conclusive proof as the depression in yield might have been due at least partly to an increase in maintenance, as was found with *K. aerogenes.*[283] It may be significant, however, that *S. ruminantium* strain pC18 had a very low yield of 28.9 g/mol glucose in the same study and this was not affected by the uncouplers.[256]

From standard redox potentials (E^1_0), it can be seen that there is ample energy available from several metabolic redox reactions to drive ETP in rumen bacteria, especially if some of the redox couples are displaced from equilibrium.[267,268] Hobson and Summers[80] calculated from yields of *S. ruminantium* on glucose (62 g/mol) and pyruvate (21 g/mol) that there was a yield of about 20 g/mol from conversion of glucose to pyruvate. As 2 ATP are formed in the Embden-Meyerhof-Parnas pathway from glucose to pyruvate, a 'normal' Y^{obs}_{ATP} of 10 g/mol seemed to apply to that part of the pathway. Approximately equal amounts of acetate and propionate were produced in the fermentation, so assuming that 1 ATP is produced in acetate production, 3 ATP would have to be derived from propionate production to obtain a Y^{obs}_{ATP} of near 10 for VFA production. Propionate in *S. ruminantium* is produced by the succinate, or randomizing, pathway.[284] Comparison of E^1_0 for the NADH/NAD and fumarate/succinate couples indicates that thermodynamically at least 1 ATP and possibly 2 ATP could be conserved by electron transfer-linked phosphorylation.[267,268] Energy might also be available from malic enzyme[80] and is very likely produced by substrate level phosphorylation during decarboxylation of methyl-malonyl CoA.[237] Thus a yield of 3 ATP from pyruvate → propionate seems feasible, both thermodynamically and, as discussed above, in terms of

the electron transfer components known to be present. Y_{ATP}^{obs} therefore could be calculated to be near 10 g/mol for *S. ruminantium*, assuming that ETP took place,[80] in contrast to earlier calculations with no allowance for other than substrate level phosphorylation.[255] The yield of *S. ruminantium* would then no longer be 'anomalous'.

Howlett et al.[261] measured growth yields and fermentation products of *Bact. ruminicola*, and concluded that if ATP were made only by substrate level phosphorylation, Y_{ATP}^{obs} would be 23 g/mol. It can be calculated from these results that if 2 ATP were produced by ETP during succinate formation, Y_{ATP}^{obs} would be 13 g/mol, again not so 'anomalous', especially considering other complicating factors discussed below. Calculation of Y_{ATP}^{obs} from yields of other rumen bacteria is made difficult because of insufficient information on the carbon balance and fermentation products, but it does seem likely that similar arguments could be applied and similar conclusions drawn from them.

Another factor which has so far not been investigated in rumen bacteria is that the outflow of fermentation products from the microbial cell may be linked to proton efflux, thereby generating an electrochemical gradient. A theoretical formulation has been presented for energy coupling to lactate efflux[285] and experimental evidence for energy conservation by this method has been obtained in *Streptococcus cremoris*.[286] This was shown to have a profound influence on growth yield; removal of lactate by coculture with a lactate fermenting pseudomonad increased the yield of *S. cremoris* by 70%.[287] Clearly the implications of this for the rumen ecosystem are enormous. In addition to lactate, the excretion of several other fermentation products might well be linked to the conservation of energy by analogous mechanisms. If this were true, all Y_{ATP} calculations to date would be quite considerably in error.

C. Experimental Limitations

Thus far only factors related to the efficiency of energy conservation have been discussed. Several other difficulties have been found in the measurement and calculation of growth yields of rumen bacteria. These include changes in cell composition and fermentation pattern according to growth conditions, problems in identifying all sources of carbon incorporated into cell material, and in accounting for that carbon in fermentation products, and often simply that dry weight determinations are bedeviled by cell lysis and other technical difficulties.

Several rumen bacteria have been found to form large quantities of intracellular storage polysaccharide, including *R. albus*,[254,288] *Bact. ruminicola*,[261] *Bact. succinogenes*,[127] *M. elsdenii*,[289,290] *Bacteroides amylogenes*,[291] Eadie's oval,[292] *S. ruminantium*,[293] and rumen streptococci,[294] and exopolysaccharides such as the slimes or capsular materials of *Strep. bovis*,[263,295] *M. elsdenii*,[296] *B. fibrisolvens*,[297] *R. albus*,[298] *Bact. amylophilus*,[258] and other unidentified rumen bacteria.[299-301] Typically, reserve glucans accumulate during exponential growth, to be used as a source of energy in stationary phase.[127,254,261,288,289,292] The degree of accumulation probably varies with growth rate, as seen in *S. ruminantium*,[293] where the glycogen content increased as μ increased under glucose limitation, and in mixed continuous culture.[302] Similarly, the quantity of exopolysaccharide varies according to nutritional conditions.[263,293,295] The carbohydrate content of rumen bacteria can be as high as 75% of the dry weight,[127] although more usually it is 10 to 30%, so it may be necessary to take this into account in growth yield calculations, to avoid distortion of Y_{ATP}^{obs} by the low energy cost of polysaccharide synthesis compared with that of other polymers.[243,254,261]

Extrapolated yields (Y^{max}) from continuous cultures should also take into account the different cell composition at different μ, but sometimes a more fundamental problem is that the stoichiometry of the fermentation changes with μ. *Selenomonas ruminantium*

produces only acetate and propionate at low μ, then as μ increases to >0.2/hr, lactate production increases[303,304] until at high μ a homolactic fermentation may occur.[303] *Strep. bovis* shows a similar trend, except that the alternative products to lactate at low μ are acetate and ethanol.[259] Estimation of Y^{max} from these fermentations is therefore made difficult, as Pirt-type double reciprocal plots were nonlinear.[259] The same difficulty did not seem to occur with *Bact. ruminicola, B. fibrisolvens* or *M. elsdenii,* which gave linear graphs.[259] However, despite these problems, the yields actually measured at the high dilution rates are useful in determining the real yield and fermentation products one would expect from these bacteria in the rumen.[80,257,259]

Unless it is certain that the substrate under study is the sole source of energy for growth, it is impossible to evaluate true growth yields with respect to that substrate. Rumen bacteria are awkward in this respect, since few of the common species can grow in a medium containing only a single carbon compound and mineral salts. Vitamins, branched chain fatty acids, CO_2, amino acids, and peptides are essential to various degrees. It is often therefore very difficult to equate substrates and products in a carbon balance. For example, 10 to 12% of the succinate and 17% of the acetate was derived from U-^{14}C-labeled algal peptides used to replace the more usual source of peptides, Trypticase, during growth of *Bact. ruminicola* on glucose.[261,305] Similarly, the CO_2 gas phase or medium CO_2^{2-} required by many rumen bacteria may under some conditions give quantitatively significant incorporation into cells or end products and affect the C balance.[304,306,307] All of these reactions, which are seldom measured in conjunction with growth yield work, would be expected to consume or produce ATP and therefore influence Y_{ATP}^{obs}.

The susceptibility of bacteria to lysis has always been a problem in determining growth yields of bacteria.[245] Among the bacteria commonly isolated from the rumen, *Bact. amylophilus* is probably the most fragile, so that although it would seem ideal for growth yield work because of its ability to use NH_3 as sole N source,[308] it is awkward to use as rapid lysis occurs at low μ, causing a decline in yield.[258,309] The decline in cell density in stationary phase with *R. albus,*[254] *Bact. succinogenes,*[127] *Bact. ruminicola,*[261] and others may be due at least partly to lysis, and may prevent yield determination by the end-point method.[242] Clumping or granular growth may also present problems in some instances.[80,261]

These practical difficulties obviously create problems in the measurement and interpretation of growth yields of individual species of rumen bacteria. Nevertheless, they are common to growth yield work with all bacteria, and should not be overemphasized. It is the theoretical problems which are of the utmost importance with rumen bacteria, not only from an academic interest in anaerobic mechanisms of energy conservation, but in practical applications as well.

D. Growth Yields In Vivo

Growth yields of mixed rumen microorganisms in vivo have been discussed and reviewed by several authors. Hespell and Bryant[243] explored the various factors which can influence yields and attempted to evaluate their significance in vivo, but their approach was based on Y_{ATP}, which as can be seen from the discussion above, actually creates more problems that it solves. Y_{ATP}^{obs} will be even more difficult to evaluate accurately in a mixed population than in pure cultures. Van Nevel and Demeyer,[70] Harrison & McAllan,[310] Stern and Hoover,[311] and Czerkawski,[312] on the other hand, expressed yields in terms of crude protein (CP) or microbial N formed/kg organic matter (OM) digested and gave comprehensive reviews of the practical literature in these terms. In general, these reviews discussed the difficulties of measuring microbial biomass in the presence of abundant food material (as discussed earlier in the present review) and the

effects of diet, fermentation stoichiometry and dilution rate on yields. The reader is directed to these reviews for a comprehensive account. Only a few points will be discussed here.

A commonly used way of calculating microbial production in vivo or in mixed cultures in vitro, without requiring any of the determinants previously mentioned as used for microbial biomass estimation, is to measure the fermentation products and calculate the ATP produced in the formation of these products, making various assumptions and approximations as to the reactions involved in ATP synthesis. Then, using a Y_{ATP} of, say, 10.5 g/mol, the microbial biomass produced during the fermentation can be calculated. Clearly the theoretical foundation of this type of exercise is extremely shaky, but nevertheless the figures obtained can be valuable, as, by and large, they are used for comparative purposes rather than on their own. We would, however, argue that the use of Y_{ATP} in the in vivo situation is unnecessary and scientifically imprecise. Since the growth yield does not change appreciably within the limited changes in fermentation stoichiometry found in the rumen[313,314] and the main interest lies in the g microorganisms produced/g of food digested, the units of g dry wt/hexose equivalent fermented (the latter can be calculated from the fermentation products, if necessary), or simply g N incorporated/kg OM digested are far more appropriate as well as being simpler.

Czerkawski[312] concluded from his survey of 75 determinations by a variety of methods in 25 papers that a mean of 19.3 g N/kg OM truly digested was suitable for calculations, so long as other factors likely to influence yields were borne in mind. Conversion of this value to the more usual units of growth yields in pure culture is interesting. If microbial cells consist of 8% N[312] and the OM is polyglucose, then the mean yield becomes $\frac{19.3}{0.08} \times \frac{162}{1000}$ g/mol glucose = 39 g/mol, a value in reasonable accord with pure culture work and indicating clearly that energy conservation by other than substrate level phosphorylation must occur in vivo to a considerable degree.

In recent years, one of the most promising topics in the manipulation of the rumen fermentation has been to influence the dilution rate (D) in vivo. According to the Pirt[249] equation, cell yields will be higher at the highest attainable D, where energy expended for maintenance (see below) is minimized. It should be borne in mind, however, that increasing D in a mixed culture can eliminate slower-growing species of bacteria (e.g., Reference 315), and protozoa,[316] which may be useful to the fermentation, and will decrease the time available for microbial degradation of more resistant components of the feed. Furthermore, since much of the microbial biomass is associated with food particles, the nominal D of the liquid portion of rumen contents is likely to be quite different from the actual growth rate of the particle-bound bacteria *in situ*. Nevertheless, evidence from experiments in vivo suggests that increasing D causes marked increases in the microbial yield, whether D is increased by the infusion of artificial saliva,[317,318] or by cold stress.[319,320] At the same time, increasing D tends to increase the molar proportions of acetate and butyrate in the rumen VFA.[321,322] Similar effects have also been found in some mixed continuous cultures in vitro, but not in all. The important influence of retention time of solids was highlighted by Crawford et al.,[323] and it was seen that in vitro increasing D increased yield as predicted when glucose was substrate,[302,324] but when digestion of solid food was involved the effect was variable[135,323] presumably because of effects on digestion rates as well.[316] Similarly, the use of glucose in vitro may explain why the molar proportion of propionate increased with D in continuous cultures[302,324] while in those cultures receiving normal ruminant diets it changed little[135] or fell,[316] as occurs in vivo. Thus, while it is more convenient to use soluble nutrients for yield experiments with a mixed population, the results obtained may not be applicable to the production of bacteria from less easily digested materials in vivo.

E. Endogenous Metabolism, Maintenance Energy, Overflow Metabolism, Uncoupling and Turnover in Rumen Microorganisms

The ATP generated during growth is used partly for purposes of net growth, with the remainder (sometimes a large proportion of the total) used for reactions which do not result in the net synthesis of new cell material. The latter reactions, although nonproductive, may indeed be essential for growth to occur. Over the years, many definitions have been drawn up to describe individual groups of reactions which might dissipate energy nonproductively. Definitions of this sort are almost invariably ambiguous, for one reaction may be described by two or more definitions. For example, intracellular protein turnover occurs in endogenous metabolism and maintenance energy. To minimize confusion, we shall use the most common meanings of the above terms when discussing these processes in rumen microorganisms.

Endogenous metabolism comprises those reactions which occur in nongrowing cells, whether or not they are essential for the survival of the organism. Surprisingly little work has been done on the endogenous metabolism of rumen bacteria, especially in view of the relatively long time intervals some bacteria may be in a state of starvation during a feeding cycle. The only work of which we are aware is that described earlier, in which reserve polysaccharide previously accumulated during nutrient excess is rapidly fermented during starvation. No work on the endogenous metabolism of other cellular components such as cell walls, nucleic acid, or protein has been published to our knowledge.

Maintenance energy as defined by Pirt[249] differs from endogenous metabolism in that it represents energy necessarily expended during growth for purposes which do not result in the net synthesis of new cell material. The biochemical reactions may be involved in osmotic regulation, turnover of cell protein and other macromolecules, motility, supramolecular organization and a variety of reactions which might form futile cycles whereby ATP or other high energy intermediates are synthesized then dissipated nonproductively by a closely related reaction which reforms the original substrates without energy being conserved.[248,250,325-329] Pirt assumed that the maintenance coefficient is a constant for a given set of growth conditions for a given organism: the energy used is a function of time only, and independent of μ. Neijssel and Tempest[250] showed that a linear Pirt-type plot could be obtained even if the maintenance coefficient changed with μ (this would make extrapolated yields invalid), but there is no unequivocal evidence that this latter does occur, although it would be difficult to prove experimentally.

In nearly all of the studies with rumen bacteria, the maintenance coefficient has been found to be small. Isaacson et al.[302] estimated from continuous cultures of mixed rumen microorganisms that m was 1.63 mmol ATP/g dry wt/hr or assuming an ATP yield from the fermentation, 0.047 g glucose/g dry wt/hr. Despite the fact that this value is small compared with those found with other organisms, maintenance can still have a profound effect on yields at the dilution rates found in the rumen. For example, from the data of Isaacson et al. it can be calculated that at a typical rumen dilution rate of 0.08/hr, 22% of hexose fermented would be used for maintenance. Furthermore, the specific growth rate of some bacteria such as those attached to large particles may be considerably less than this, and so maintenance in these organisms may be considerably more significant.

Among individual species of rumen bacteria compared by Russell and Baldwin,[259] *M. elsdenii* had the highest maintenance coefficient, of 0.187 g glucose/g dry wt/hr followed by *Strep. bovis* (0.150), *Bact. ruminicola* (0.135), *B. fibrisolvens* (0.049) and *S. ruminantium* (0.022). *Bact. amylophilus* had a higher maintenance coefficient of 0.253 g maltose/g dry wt/hr,[258] possibly reflecting a high turnover of cell material due to lysis.

'Uncoupled growth' is a term used by Senez[238] to describe growth in which the energy source is not the limiting nutrient and is metabolized less efficiently than when it is growth-limiting. This results in a decreased growth yield expressed per unit of energy source. The most common form of uncoupled growth is probably nitrogen limitation, illustrated by an example used already in this review, that of the high apparent maintenance coefficient under uncoupling conditions of tryptophan limitation of *K. aerogenes*[248] and the lower value under glucose limitation.[253] The usually complex nature of the nitrogenous nutrients of rumen bacteria make nitrogen limitation in continuous cultures difficult, and only a little work has been done on this aspect of their growth. *Bacteroides amylophilus* grows on a medium containing only NH_3 as a source of nitrogen,[308] and so has been suitable for NH_3 limitation experiments. The same is true of some strains of *S. ruminantium*, provided that small quantities of vitamins are included in the medium.[304] Both of these organisms have been grown in NH_3-limited chemostats, giving quite different results in each case. A plot of Q (specific rate of substrate utilization) against μ should be linear, with the intercept on the ordinate axis equal to the maintenance coefficient and the gradient equal to $(Y^{max})^{-1}$.[248] Henderson et al.[309] found that the plots were linear under both maltose and NH_3 limitation of *Bact. amylophilus*, but the maintenance coefficient was increased and Y^{max} decreased under NH_3 limitation. Jenkinson and Woodbine,[258] on the other hand, found the intercepts to be similar, although Y^{max} was again depressed under NH_3 limitation. Wallace (unpublished) found the plots for *S. ruminantium* apparently similar in both respects. Further interpretation of this data was made difficult by the changing fermentation pattern of *S. ruminantium* with μ and according to the limited nutrient,[303,304] the changing polysaccharide composition under different conditions,[293] and difficulties in achieving a carbon balance.[304] Thus, few conclusions regarding uncoupling in rumen bacteria can be drawn from the little work which has been published. The significance of uncoupling caused by nitrogen limitation in vivo would appear to be minor, however, since only very rarely does the prevailing NH_3 concentration fall to the level of the saturation constants of 50 μm and less for NH_3 exhibited by pure cultures of rumen bacteria,[330] and so NH_3-limited growth would seldom occur. The possibility of amino acid-limited growth is discussed elsewhere in this review. *Bacteroides amylophilus* has also been grown under phosphate limitation,[258] which again might cause uncoupling not only in pure cultures, but sometimes also in vivo.[331]

Another form of uncoupling has been described by Neijssel and Tempest[250,266] as "slip" reactions, "energy spillage", or "overflow metabolism". This differs from uncoupling in that it occurs during sufficiency of all nutrients. It is apparently caused by imbalances between the rates of energy generation and utilization and occurs particularly during transitory excesses of energy source.[266] Thus cells can be carbon-limited but not energy-limited, even though carbon and energy are derived from a single compound. "Energy spillage" will then occur. The full extent of this phenomenon has not so far been investigated, and its significance to rumen bacteria is not known. It might be expected to occur immediately following the ingestion of a meal by the ruminant, when all nutrients are likely to be in excess in the rumen.

Most of the cell turnover which occurs in the rumen is probably not of the type measured as part of the maintenance coefficient or as endogenous metabolism in pure cultures. The large quantities of bacterial cell debris seen in the electron microscope[332] are most likely the result of factors absent from laboratory cultures, such as physical damage, predation, and infection or other antagonistic effects, which give rise to a more rapid turnover of cell material. Radioactivity from ^{14}C-labeled *Escherichia coli* and *Bacillus subtilis* was rapidly converted to ^{14}C-VFA,[333] showing that the digestion of dead bacteria could be very rapid. Van Nevel and Demeyer[70] developed a method for estimating the degree of turnover in mixed rumen microorganisms, in which rumen fluid

was incubated in vitro with ^{32}P-phosphate and soluble sugars. 'Total' growth was estimated by ^{32}P uptake into microorganisms, and 'net' cell synthesis by the amount of nonprotein N incorporated. Since the latter was 50% of the former, considerable turnover was indicated. Although this type of experiment is open to criticism on a number of grounds, such as the use of soluble sugars rather than polysaccharides of plant fibers, the possibility of changing N:P ratios (essential for calculations) during the fermentation, and turnover of nonlabeled cells, it nevertheless illustrates the relative importance of turnover in decreasing the growth yield under batch-culture conditions. At these growth rates, the true maintenance energy requirement would be expected to be small.[243]

This high degree of turnover in rumen microorganisms is due partly to interspecies predation. Jarvis[334] showed that *Strep. bovis* was destroyed much less rapidly when protozoa were removed from rumen fluid, and it has often been implied that predation by protozoa increases the rate of turnover of bacteria in the rumen.[313,324,334] Some experimental work in vivo has tended to support this idea,[335] and numerous observations on protozoal-bacterial interactions in vitro indicate that this is likely.[169] Demeyer and Van Nevel,[336] using the method outlined above, tested the effect of defaunation on yields directly. As expected, the net yield (i.e., the real increase in microbial dry matter per substrate fermented) was more than doubled by defaunation. However, the gross or total yield (i.e., the total growth, including material broken down again) was also increased, by approximately one third, for reasons which were not apparent. Since little is known of growth yields, maintenance energy, or intracellular turnover in protozoa, their contribution to the overall fermentation efficiency is difficult to predict. Jarvis[334] also found that the degradation of *B. fibrisolvens* was enhanced by protozoa, but that soluble lytic factors were of greater importance with this organism. These factors were destroyed by autoclaving, and Jarvis suggested that bacteriophages or even soluble hydrolytic enzymes might be involved. In similar experiments, it was found that *E. coli* was subject to degradation by whole rumen contents, but not clarified liquor, whereas *Bacillus subtilis* was lysed by soluble factors.[332] Again, the nature of the soluble factors was not established.

The existence of bacteriophages in the rumen has been known for some time. Many bacteriophage particles are present, a large number of which are associated with bacterial cell walls.[332,338-341] Many of these phages have exceptionally long tails,[332,342,343] perhaps indicating that the accessibility of bacterial cell surfaces in the rumen may be limited by the thick capsules.[301] A good example of this type is E241, a virulent phage which infects the large rumen bacterium Eadie's Oval.[341] Phage particles have also been found to be associated with *Strep. bovis*[338] and W461,[341] a Gram negative bacillus isolated for its ability to hydrogenate unsaturated fatty acids.[342] The latter were of special interest as they appeared to be temperate phages, and could not be induced to lyse cultures of Eadie's Oval by exposure to UV light, hydrogen peroxide, or mitomycin C.[341] Thus both virulent and temperate phages exist in the rumen, and might be expected to cause some fluctuation in the numbers of individual species of bacteria, perhaps increasing the extent of turnover of these particular cells and decreasing the net growth yield.

Mycoplasma are another class of organism which may influence the rates of death and resynthesis of rumen bacteria. An obligately anaerobic mycoplasma has been isolated on several occasions from dilutions of rumen fluid.[343,344] It is apparently free-living, and digests both live and autoclaved *Butyrivibrio, Ruminococcus albus* and *Escherichia coli,* and cell walls of *Butyrivibrio.*[344] *Strep. bovis* was not affected. The soluble lytic enzyme produced may be concerned with the protozoa-free lytic activity seen by Jarvis[334] and Hoogenraad and Hird.[337]

It may be concluded, then, that there are many factors which can potentially influence the microbial growth yield in the rumen. The problems involved in identifying and

evaluating these factors make growth-yield work complex and difficult, but conversely, some offer scope for modulation of the fermentation by altering growth yields in vivo, and hope for improving feed efficiency in ruminants.

VII. NITROGEN METABOLISM OF RUMEN MICROORGANISMS

The metabolism of nitrogenous compounds in the rumen is very complex owing to the number of compounds and the number of microbial species involved. Ammonia is the principal source of N for microbial growth, and most nitrogenous compounds entering the rumen, such as protein and urea, are degraded to NH_3 before their nitrogen is assimilated by the microorganisms. In the case of protein, free amino acids are intermediates in this process, but as they are rapidly deaminated their concentration in rumen fluid is usually low.

This overall process is clearly inefficient insofar as the utilization of a good dietary protein is concerned. Energy will be required for the resynthesis of protein by microbial cells; resynthesis of protein from the NH_3 released may be incomplete so that nitrogen is lost to the host as excreted urea; part of the protein N will form bacterial cell walls, which are largely unavailable to the host; and microbial protein may sometimes contain smaller amounts of essential (to the host) amino acids than the original protein. Hence the emphasis in feeding ruminants concentrates containing a high proportion of good quality protein has been to 'protect' the protein from degradation in the rumen and allow it to reach the abomasum intact.

In contrast, the ability of the rumen microorganisms to convert ammonia N into protein is a valuable property. Animals on a low protein diet may conserve nitrogen by recycling urea to the rumen in saliva and by diffusion from blood across the rumen wall, followed by hydrolysis of the urea to ammonia, thereby making N available for microbial protein synthesis. Furthermore, the ability of the microorganisms to convert ammonia N to protein N has enabled urea and other forms of nonprotein N to be used in place of or as a supplement to dietary protein.

Numerous reviews on nitrogen metabolism in the rumen and in the ruminant have appeared in the literature.[345-351] More recent aspects of microbial nitrogen metabolism will be described here.

A. Protein Digestion

Here we use the true meaning of the word 'protein', as distinct from 'protein supplement' such as fishmeal, etc., which contain materials other than protein which may affect degradation of the true protein. However, much of the work is applicable to both proteins and protein supplements.

It has been known for many years that proteolytic activity in the rumen is associated mainly with the particulate fraction of rumen fluid.[352,353] Activity was found in protozoa, and both large and small bacteria.[352] Furthermore, recent evidence using isotope-labeled protein suggests that even soluble proteins are adsorbed on to microbial surfaces while they undergo hydrolysis.[354] Protein solubility has usually been cited as the main determining factor in the rate of degradation of a protein by rumen microorganisms, with less soluble proteins being degraded more slowly than those which are highly soluble.[355] In practical terms, this is a useful rule of thumb, provided that it is combined with a knowledge of outflow rates from the rumen.[356] There have always been doubts about its validity, however, since, for example, bovine albumin and ovalbumin are both water soluble yet are degraded in the rumen much more slowly than casein or other soluble proteins,[357-359] although it has been argued that since albumin is not very soluble in synthetic rumen fluid the relative degradations might again be explained by solubility.[345]

However, comparisons between the rates of degradation of the soluble diazo-casein and diazo-albumin,[360] azo-casein, azo-albumin, and diazo-ovalbumin[361] by mixed rumen microorganisms in vitro suggest that this is unlikely. Treatment of bovine albumin with dithiothreitol broke some of the disulfide bridges crosslinking the protein, and at the same time markedly increased its rate of degradation.[359] Similarly, the rate of degradation of the insoluble fraction of diazo-fishmeal was increased nearly tenfold by treatment with mercaptoethanol.[362] Thus, the number of disulfide bridges and the tertiary structure of a protein in general are obviously also very important factors in determining its degradability. An additional question which has not been investigated is: what influence does the availability of end groups on the polypeptide chain have on its degradability? The amino-terminal glycine of ovalbumin is acetylated, and the carboxy-terminus is proline,[363] so presumably ovalbumin is not susceptible to exopeptidase activity until the chain is nicked internally. The relative contributions of endo- and exopeptidases in the rumen are, as far as we are aware, unknown, although the lag periods seen with some proteins prior to degradation[362] might be indicative of a need for endopeptidase activity. Thus, although solubility is an important factor, the degree of tertiary structure and the availability of end groups may be almost as crucial in determining the susceptibility of a protein to degradation.

It is interesting to speculate how much the protein, which is an integral part of the primary plant cell wall, might be a barrier to digestion of the cellulose, hemicellulose, and other components of the wall. It is clear that this type of protein, of which extensin is the best known example,[364] is quite different from the easily degraded fraction I protein isolated from the soluble material.[359] The availability of end groups seems likely to be restricted, and the high hydroxyproline content[365,366] suggests a fibrous, cross-linked tertiary structure, similar to that of elastin,[367,368] which is highly resistant to degradation by rumen microorganisms.[361] Extensin is known to be closely associated with cellulose[364] and release of hydroxyproline by pronase enhanced cell-wall digestion by a crude cellulase preparation,[369] so the digestion of this type of protein as a rate limiting step in fiber digestion should be investigated. Pronase treatment increased the degradability of ground sorghum almost to the level of starch,[370] indicating a possible limitation of this type in grain as well.

More practical aspects of protein degradation in the rumen have been reviewed frequently. The review by Tamminga[371] lists these references, and some of the most important original contributions to this field. So far, the only way in which proteolysis in vivo has been effectively modified has been by processing the protein substrate in some way, such as by heating, or formaldehyde, or other chemical treatments, so that the protein is not hydrolyzed at neutral pH by rumen microorganisms but is still hydrolyzed at the lower pH prevailing in the abomasum. Attempts to influence proteolysis using drugs have generally been unsuccessful, except in affecting deamination of the amino acids once they are released from the protein.[372,373] Van Nevel and Demeyer[374] found that the hydrolysis of casein was inhibited by monensin, but later work suggests that this may have resulted from an inhibition of deamination rather than of proteolysis.[375,376] Although dietary urea did not spare protein from degradation[377] and the degradation of maize protein was unaffected by rumen NH_3 concentration,[378] it still seems possible that proteolysis might be controlled by other factors, such as associate effects between feeds (as with cellulolysis), since diet can affect proteolytic activity.[36]

One of the most actively proteolytic populations of bacteria in the rumen is that associated with the rumen epithelium.[379] These bacteria are tightly bound to the epithelial tissue and can be seen to invade epithelial cells.[380] When sheep are maintained entirely by infusion of VFA and bicarbonate buffer into the rumen and casein into the abomasum, the epithelial bacteria survive while the rumen fluid population disappears,[381] and

the former confer a considerable proteolytic activity on the rumen fluid from the sloughing of epithelial cells and their adherent bacteria into the fluid.[382] The adherent bacteria actively digest epithelial cells,[382] so evidently may have a role in the entry of endogenous N into the alimentary tract. Protease activity in rumen fluid of infused steers increased as the level of nitrogen nutrition increased,[361] but it was unclear whether this was due to heavier colonization of the rumen wall or to an increased sloughing of epithelial tissue.

Initially, the proteolytic bacteria isolated from the rumen fluid were facultative anaerobes present in low numbers,[379-381] and were therefore not regarded as being the 'true' digesters of protein in the rumen. In view of the proteolytic properties of the wall population, which may contain a high proportion of facultative bacteria,[382] the isolation of these bacteria in the early work may not be as accidental as was first thought. Indeed, in a recent experiment at the Rowett Research Institute with gnotobiotic lambs inoculated with a defined flora of many typical rumen species, the dominant proteolytic bacteria subsequently retrieved were in fact the staphylococci which had been isolated from the rumen wall of normal sheep and included in the inoculum for their ureolytic properties.[361]

When greater attention was paid to strictly anaerobic methods of media preparation, a wide range of proteolytic bacteria was isolated, representing most of the types commonly found in the rumen, but especially species of *Bacteroides, Selenomonas* and *Butyrivibrio*.[345,386-390] Up to 38% of the viable bacteria isolated from the bovine rumen were found to be proteolytic.[388] As *Bacteroides* has been the predominant proteolytic genus among the strict anaerobes, it has been studied in greatest detail. *Bacteroides amylophilus* H18 produces two forms of protease, one of which is liberated into the growth medium and represents a maximum of 20% of the total activity, and the other of which remains cell-bound.[391] The cell-bound activity was apparently located on the cell surface, as ultrasonic disintegration and toluene treatment did not increase activity[391] and most activity remained associated with lysozyme spheroplasts, from which the enzyme could be liberated by butanol treatment.[392] For an unknown reason, the purified preparation of cell bound protease contained 23% RNA,[392] so it could not be proved to be the same as the soluble enzyme, which occurred in 60,000 and 30,000 mol wt forms.[393] Other evidence, however, such as the double pH optima, at pH 6.0 and 11.0, of both enzymes,[392] their sensitivity to serine protease inhibitors,[393,394] and their ability to hydrolyze N-α-benzoyl-L-arginine[393,394] is strongly indicative that these two activities are produced by different forms of the same enzyme. The soluble enzyme has trypsin-like specificity, although it is unaffected by soybean trypsin inhibitor.[394]

Hazlewood and Nugent[389] pointed out that casein might be an inappropriate substrate for the isolation of proteolytic bacteria from the rumen, and that leaf fraction 1 protein (ribulose bis-phosphate decarboxylase EC 4.1.1.39) might be a better material, since it comprises a large proportion of the soluble protein of many plant species. Again, a proteolytic *Bacteroides* (R8/4) was isolated which was highly active against fraction 1 protein.[389] On the basis of its apparent requirement for peptides and its alkaline phosphatase activity, it seems likely that this isolate was *Bact. ruminicola*.[390] Since leaf fraction 1 protein presents little greater resistance to rumen proteolytic bacteria than does casein,[359] it is perhaps not surprising that the isolate was familiar. One might speculate as to whether isolates would be similar if the substrate were less easily degraded. An interesting feature of *Bacteroides* R8/4 is that it shows Michaelis-Menten kinetics consistent with substrate inhibition.[390]

Bacteroides amylophilus protease has been assumed to be of major importance in the rumen in protein digestion and, for instance, it has been used in applied studies to investigate effects of chemical modification of substrates.[362] Two factors which suggest that the assumption may not be valid are (1) that the pH optimum of rumen protease is broad

and between pH 6.0 and 7.0[352] whereas *Bact. amylophilus* protease has two peaks at pH 6.0 and 11.0, and, (2) that the *Bact. amylophilus* protease is sensitive to diisopropylphosphofluoridate whereas the rumen fluid activity is little affected by the similar inhibitor dimethylsulfonylfluoride.[361] It therefore remains to be established whether *Bact. amylophilus* protease is of major importance in the rumen and therefore valid in simplified in vitro studies. Furthermore, *Bact. amylophilus* has low exopeptidase activity, whereas *Bact. ruminicola* has higher exopeptidase activity.[387] A mixture of proteolytic enzymes might therefore have greater relevance to practical studies.

Bacteria would be expected to hydrolyze not only dietary protein but their own proteins, especially under starvation.[395,396] Van Nevel and Demeyer[70] introduced the valuable concept of "gross" and "net" growth to demonstrate that turnover of bacterial cell material in the rumen represents a major energy requirement. Surprisingly, predation by protozoa did not seem to be the most important factor, as the difference between net and gross yields was greater when animals were defaunated.[336] It is therefore unclear how much turnover of bacterial protein in the rumen is a function of classical Pirt-like maintenance[249,329] involving specific intracellular proteolytic enzymes,[396] and how much it is due to bacterial death and recycling of dead cells, which presumably would be catalyzed by the same enzymes used in the digestion of dietary protein.

Many species of rumen protozoa can assimilate amino acids from the extracellular medium into protein, although usually protozoal N is more efficiently obtained by the engulfment of particulate forms of protein, either as bacteria or of dietary origin.[169,200,216,218,221,222,397,398] Amino acids are formed as a result of proteolysis by *Entodinium caudatum*[200,212] and *Eudiplodinium* (or *Metadinium) medium.*[399] Other protozoa which would be expected to be proteolytic, judged by their incorporation of bacterial amino acids, are *Entodinium longinucleatum,*[398] *Ophryoscolex* spp.,[400,401] *Epidinium ecaudatum caudatum,*[206,229] *Eudiplodinium maggii,*[216] and *Isotricha* spp.[397,402] Little is known of the proteolytic enzymes of the ciliates, and it is unclear whether they might complement or only duplicate the activity of the bacteria.

B. Peptide and Amino Acid Metabolism

Research in this area in recent years has concentrated on inhibiting the breakdown of these compounds in the rumen, either by chemical or physical protection or by chemically decreasing the hydrolytic activity of rumen contents, and on studying amino acid biosynthetic reactions. Much of the fundamental work has already been reviewed several times[3,345-347,403] so only a brief account will be given here.

1. Peptides

The metabolism of oligopeptides by rumen microorganisms has received little attention, except for the unusual finding that *Bact. ruminicola* can grow in a medium containing Trypticase, comprising small peptides and free amino acids, but not with free amino acids alone.[404] As the related *Bact. melaninogenicus* also has a nutritional requirement for peptides[405] and experiments in vitro with rumen contents showed that peptide carbon was used more efficiently than that of free amino acids[353] for microbial growth, this preference for oligopeptides may be a more general property of rumen microorganisms. In *Bact. ruminicola,* specific transport systems for oligopeptides seem to exist, and the peptides are rapidly broken down to amino acids on entering the cell, to be subsequently assimilated into cell material or excreted.[406] It was initially reported that transport systems for amino acids did not occur in *Bact. ruminicola,*[406] but more recently it has been shown that inhibitory factors can occur in the growth medium, and that specific systems for the transport of amino acids analogous to those of other bacteria, do exist in *Bact. ruminicola,* presumably to supplement the amino acids obtained from pep-

tides.[407] Biosynthesis of isoleucine by *Bact. ruminicola* was repressed by peptides, consistent with peptides being the preferred form of nitrogen.[408] It is perhaps surprising in view of recent widespread interest in transport of both small and macromolecules across biological membranes that molecular biologists seem to have largely ignored the complexities of amino acid and peptide transport in *Bact. ruminicola* and related bacteria.

2. Deamination

The fate of amino acids in the rumen is predominantly to be broken down rather than to be incorporated intact into cell material,[409,410] although some direct incorporation does occur.[411,412] Numerous experiments in the 1950s and 1960s described the fate of individual amino acids and amino acid mixtures in the rumen and in pure cultures of rumen bacteria. Different amino acids are degraded at quite different rates by mixed rumen microorganisms,[410,413] and a wide range of rumen bacteria are capable of deamination of at least some amino acids. Of these, *M. elsdenii* is probably the most active of those commonly isolated,[414-416] although *Bacteroides* spp. are likely to be of greater importance as they generally occur in higher numbers.[415]

Of special interest is that the volatile fatty acids produced as a result of deamination are essential for the cellulolytic bacteria *R. albus, R. flavefaciens, Bact. succinogenes* and *B. fibrisolvens,* and also for some strains of others, including *E. ruminantium* and *S. ruminantium.*[3,417-420] The acids themselves are formed by microbial attack on the analogous amino acid, e.g., isobutyric acid is produced from valine and isovaleric acid is produced from leucine.[421] Others include 2-methyl butyric and valeric acids. The discovery of these compounds in rumen fluid and realization of their biological significance led to the development of media for the culture of rumen bacteria which did not require the addition of ill-defined factors such as the clarified rumen fluid used in Hungate's original media (e.g., Reference 422). Branched chain VFA production by *M. elsdenii* has been shown to be enhanced by deprivation of glucose[423] and so perhaps the higher branched chain VFA concentrations seen in the rumen after 36 hr starvation[424] may be caused by similar factors.

The rate of deamination of amino acids in the rumen seems to be generally greater than the rate of proteolysis, for amino acids seldom accumulate in rumen fluid. However, this does depend on the properties of the protein. With casein, which is rapidly hydrolyzed, deamination of the amino acids released is too slow to metabolize all of the acids, and they can accumulate.[36,357] Digestion of gelatin, on the other hand, produced ammonia with only a transient appearance of free amino nitrogen.[425] Many reports have indicated low levels of free amino N in rumen fluid of animals receiving a variety of diets,[346] and much of the free amino N which does occur in the rumen is intracellular in any case,[426,427] and so is not relevant to deamination of dietary amino acids.

Clearly there must be many enzymes involved in the deamination of amino acids in rumen fluid, leading to the different rates of loss of different amino acids observed in incubation in vitro.[410,413,428] Sodium arsenite, an inhibitor of the reduction step of the Stickland reaction,[429] produced substantial inhibition of deamination in vitro,[413] indicating that much of the deaminative activity is derived from this type of coupled oxidation-reduction reaction. This conclusion resolves the doubt there has been in the past regarding the relative importance of the Stickland reaction in deamination in the rumen.[428,430,431] Protozoa also participate in deamination, judging from the production of NH_3 from feedstuff protein in several species (reviewed by Coleman, Reference 169), and again their contribution to overall deamination activity will depend on the size of the population. The ciliates are also responsible for some deamination of amino acids derived from microbial protein.[169]

The degradation of amino acids in the rumen is undoubtedly sometimes grossly inefficient for the nutrition of the host animal, as amino acids would be better utilized if they reached the abomasum without degradation. Control of the enzymic activity by the administration of antibiotics and other chemicals is a promising area, and will be discussed in Section XIV. The other methods of decreasing deamination use some modification or protection of the amino acid, for example by acetylation or encapsulation (reviewed by Ferguson, Reference 432), so that amino acids only become available postruminally. Results in terms of weight-gain performance have been mixed, however, as when the growth limitation by one amino acid is relieved, limitation by another is soon imposed.[371,433] Thus while protection by modification improves performance with a protein possessing a full complement of amino acids, it does not work as well for single amino acids and this is why a broad inhibition of deamination has been sought rather than a more specific protection of single acids.

C. Amino Acids as Growth-Limiting Nutrients

It is well known that amino acids, or peptides, or branched chain volatile fatty acids are essential or important nutrients for most species of rumen bacteria.[3,434,435] Even *Bact. amylophilus,* which grows using NH_3 as sole source of N,[308] can incorporate amino acids from the medium,[436] as can *Bact. ruminicola,* despite its requirement for oligopeptides.[407] It is less clear, however, whether growth of the rumen microbial population as a whole might be limited by one or more amino acids. For example, Hume[437] found that the yield of microbes in vivo was increased by addition of higher volatile fatty acids to a diet high in urea but very low in protein. Furthermore, replacement of the urea by gelatin had little effect, whereas casein and zein further increased the yield.[438] There are other papers (e.g., References 439 to 442) which might also be interpreted to show an amino acid limitation of the rumen microorganisms as a mixed population, since the addition of protein improved the microbial yield. The variable effects on milk yield, in which some experiments show an improvement with supplementary protein rather than urea[443-446] while others do not[447-449] may be due in part to factors other than microbial yield in the rumen. The increase in microbial yield sometimes seen when protein is replaced by nonprotein nitrogen[450,451] is more difficult to understand. Thus although growth of the mixed microbial population in the rumen might be limited by the availability of amino acids under some circumstances, this is by no means a general phenomenon, and the addition of single amino acids to ruminant diets is unlikely to be productive.

D. Amino Acid Biosynthesis

The rumen microbial population usually forms much of its amino acids from ammonia. If necessary, it can even function entirely free from dietary amino acids, as amino acids essential for some organisms can be produced from the breakdown of other organisms. This extremely valuable property of the ruminant was emphatically demonstrated by Virtanen[452] with dairy cows reared on diets containing only urea and ammonium salts as sources of nitrogen. These animals gave good milk yields, and the milk differed from the normal product only in its higher fat content. The protein was of normal composition, and protein content of the milk increased when the urea content of the feed was increased. Even with normal diets, 30 to 80% of bacterial N and 25 to 64% of protozoal N may be derived from ammonia.[349] Indeed many rumen bacteria have an absolute requirement for NH_3 even in the presence of amino acids.[3,434,435] Amino acid biosynthesis *de novo* is therefore of great importance to the rumen microbial population and so to the ruminant itself.

The first stage in the synthesis of microbial amino acids from ammonia is the uptake

of NH_3 from the extracellular fluid. So far, methods which have been used with other microorganisms[453,454] have not been applied to NH_3 transport across the cell membranes of rumen microorganisms. Whether the transport of NH_3 is active or passive, or specific or not, has important implications in interpreting kinetic data from the study of NH_3-assimilating enzymes and determining minimum NH_3 concentrations for microbial growth, as will be seen below. If active transport occurs, as it does in *E. coli*,[453] then intracellular NH_3 concentrations may considerably exceed the extracellular values.

Following uptake into the cell, there are several ways in which NH_3 might be assimilated into amino acids. Measurements of enzyme activities have shown that carbamylphosphokinase has very low activity in the rumen[455] although it is involved in arginine biosynthesis by *Strep. bovis*.[456] Another mechanism of NH_3 uptake in many nonrumen microorganisms is the glutamine synthetase-glutamate synthase couple.[457,458] Here, ammonia first forms the amide group of glutamine, then is transferred to α-ketoglutarate by glutamate synthase. This is a mechanism which is useful for the scavenging of low concentrations of NH_3, because of the low Km for NH_3 of glutamine synthetase.[459] It is also energetically expensive, however, as ATP is required for the synthesis of glutamine from glutamic acid. Glutamine synthetase has been shown to occur in mixed rumen microorganisms[427,455,460] and as would be expected, to be activated by very low NH_3 concentrations.[460] However, the activity is low at NH_3 concentrations usually found in the rumen[427,460] and the activity of glutamate synthase is very low as well[427,460,461] so it is unlikely that this mechanism is of major importance in the rumen under normal circumstances.

The very high activity of glutamate dehydrogenase in the rumen has implied that this enzyme is the most important method of ammonia uptake.[427,455,460,462-464] Both NAD- and NADP-linked activities occur, with the former usually being much higher although having a lower affinity for NH_3.[427,460] Aspartate dehydrogenase also had a high activity, but its affinity for NH_3 was very low and it almost certainly is of little importance in ammonia assimilation in the rumen.[427] The mechanism of NH_3 assimilation therefore seemed quite straightforward, that NH_3 was assimilated first into glutamic acid by glutamate dehydrogenase, then transferred to other carbon skeletons by transaminases, which have been shown to exist in rumen microorganisms.[427,455,464,465] That this might not be true was hinted at by measurements of the amino acid pool sizes in rumen microorganisms, for the concentration of alanine often exceeded that of glutamate, especially at high NH_3 concentrations.[426,427,460,466] Studies on deamination also showed that alanine behaved differently from other amino acids.[413] Nevertheless, alanine dehydrogenase activity linked to the oxidation of NADH or NADPH is low,[427] so it was thought unlikely that alanine was the immediate product of NH_3 assimilation — its accumulation was probably the result of an imbalance between its rate of synthesis and utilization. Recently, however, earlier work by Shimbayashi et al.,[467] which suggested that more ^{15}N-urea-nitrogen was assimilated into alanine than other amino acids in vitro has been confirmed by Blake et al.[468] who found that in only 2 min following the administration of ^{15}N-ammonium chloride to the rumen, alanine was enriched more than glutamate or any other amino acid. In view of the high Km of alanine dehydrogenases for NH_3,[427,457] NH_3 would have to be accumulated by an active transport system before assimilation by this enzyme could occur. Further developments in this area will be of great interest.

In pure cultures of rumen bacteria, glutamate dehydrogenase-linked either to NAD or NADP, again predominates,[154,469-474] Glutamine synthetase does occur in *Strep. bovis*,[471] *Bact. amylophilus*,[473] and *S. ruminantium*,[474] but only in *S. ruminantium* was glutamate synthase found as well.[474] Whether this is due to unusual properties of glutamate synthase in anaerobes[474] is not clear. Asparagine synthetase also occurs in *Strep. bovis*,[475] but it is not known if an enzyme is present which could transfer the amide N to α-amino

N of amino acids, in the manner of glutamate synthase. Interestingly, alanine was again the predominant component of the intracellular free amino acid pool of *Bact. amylophilus,* indicating a need for further investigations of alanine biosynthesis in pure cultures as well as in mixed rumen microorganisms.

If the utilization of nitrogen within the rumen is to be made maximally efficient, then as much as possible of the NH_3 that is produced in the rumen should be assimilated by rumen microorganisms, and the quantity absorbed through the rumen wall and excreted, minimized. Ideally, then, the concentration of NH_3, which represents the excess of NH_3 production over utilization, should approach zero provided that the rumen microorganisms do not become nitrogen-limited.

These rather obvious principles have formed the basis of estimating whether nitrogen needs to be added to ruminant diets in North America, but although the practice has been found to be sound in many cases, it is not universally applicable. There is not space enough here to discuss this matter fully, so only a brief sketch will be given. Basically, it was not known how close to zero the concentration of NH_3 could be without affecting microbial growth rates. Satter and Slyter[476] performed continuous culture experiments with mixed rumen bacteria in vitro which showed that increasing the NH_3 concentration did not increase production of acid-precipitable nitrogen from purified and maize-based feeds. Ammonia concentrations are well known to fluctuate widely in the rumen, so in order to ensure that NH_3 never became limiting, a fairly arbitrary, but low, excess of 50 mg NH_3-N/ℓ rumen fluid was selected as a target figure. This is quite different to the 'optimal NH_3 concentration' quoted by Henderickx[477] which refers to the initial concentration in growth medium, and so if the energy source was doubled, so too would the NH_3 concentration have to be doubled. The 50 mg NH_3-N/ℓ *net* figure resulting from optimally minimal overflow was therefore used to predict nitrogen requirements in vivo.[478-480] In fact, it has long been known that rumen microorganisms can take up NH_3 to very much lower NH_3 concentrations (e.g., see Reference 460), and recent very careful work by Schaefer et al.[330] has established that the ammonia activation constants for the rumen bacteria *Bact. amylophilus, Bact. ruminicola, S. ruminantium* and *R. flavefaciens* are less than 50 μM, or approximately one hundredth of the value recommended by Satter and Slyter. Thus a low concentration of NH_3 in the rumen, provided that it is $>50\ \mu M$ is unlikely to affect NH_3 assimilation by rumen bacteria. However, this does not mean that higher concentrations are not beneficial under some circumstances.

Many experiments and feeding trials have been performed to determine if an optimum rumen NH_3 concentration does exist for parameters other than simply nitrogen retention, but the results have been mixed.[34,378,481-484] There is no space here to analyze each experiment, so only two examples will be discussed. Mehrez et al.[482] found that the rate of degradation of barley was increased by adding urea to a whole barley diet, so that the rate of degradation increased with rumen NH_3 concentration to a concentration of 235 mg/ℓ. No important bacteriological changes were noticed, although the bacterial population increased and the free alanine pool was increased relative to glutamate when the NH_3 concentration was increased.[427] In contrast, Slyter et al.[483] found no significant increase in dry matter digestibility or N retention over 45 mg NH_3-N/ℓ using a corn-based diet, and again no significant bacteriological differences were noted at higher NH_3 concentrations. There seems to be no real explanation for these differences apart from diet, and it may be significant that when the barley experiment was repeated using pelleted barley, ammonia concentration had no effect on the rate of degradation (Ørskov, E. R., personal communication). We now suspect that NH_3 concentration in strained rumen liquor is possibly less important than that in the micro environment in particles of partly degraded food. It may be that in the micro environment of partly digested particles from whole barley, rich in starch, the microorganisms are NH_3-limited,

whereas they may be no such limitation in corn or in the smaller, more accessible particles of pelleted barley.

E. Biosynthesis of Carbon Skeletons for Amino Acid Synthesis

Many species of rumen bacteria have a requirement for amino acids or branched chain VFA in addition to NH_3,[434,435] and these must be included in growth media for the culture of many pure strains. In vivo, preformed amino acids or branched chain VFA are not essential (although they may be beneficial, as discussed above) as it was demonstrated that cows could be maintained with urea as the sole source of nitrogen.[452] In this case, branched chain VFA-requiring bacteria were isolated from the rumen,[485] indicating that these carbon skeletons could be obtained from excretion or breakdown of other organisms and that total amino acid synthesis *de novo* did not prevent the growth of individual bacteria with specific nutritional requirements. A neat demonstration of this type of nutritional interdependence was recently made by Miura et al.[486] In a medium containing starch, glucose and cellulose, and NH_3 as sole nitrogen source, successive growth of *Bact. amylophilus, M. elsdenii* and *R. albus* occurred, despite the requirements of the last two for amino acids and branched chain VFA respectively. It was shown that *Bact. amylophilus* produced amino acids which *M. elsdenii* used for growth and incidentally produced the branched chain VFA essential for *R. albus*. Similar nutritional interdependence between *Bact. ruminicola* and *R. albus* was shown by Bryant and Wolin.[487]

More usually, food protein is available to provide amino acids for the growth of rumen bacteria, and at least some of the amino acid carbon is incorporated into microbial protein.[411,412] The majority of the carbon in microbial protein is derived from the general metabolic pool however, and so the biosynthetic routes are extremely complex. Allison[346,403] has reviewed this area most thoroughly, and basically the two areas of most interest have been the biosynthesis of carbon skeletons in the main metabolic pathways and the modification of precursors such as the branched chain VFA to produce amino acids.

There is little doubt that glutamate is one of the central metabolites in microbial nitrogen metabolism, yet it was found that *Bact. ruminicola* lacked citrate synthase and isocitrate dehydrogenase,[470,488] two enzymes essential for the synthesis by the normal Krebs tricarboxylic cycle of α-ketoglutarate, the immediate precursor in glutamate synthesis by glutamate dehydrogenase. Labeling studies showed that α-ketoglutarate is formed by the carboxylation of succinate in *Bact. ruminicola,*[488,489] *S. ruminantium,*[489] and *V. alcalescens,*[489] and it was shown that in vivo 28% of the glutamate of mixed rumen microorganisms was formed in this way.[490] Examination of cell-free extracts from mixed rumen microorganisms[491] indicated that the series of reactions used reduced ferredoxin (FDH) as electron donor:

$$\text{succinate} \xrightarrow{FDH_2,\ FD} \text{succinic semialdehyde} \xrightarrow{CO_2\ FDH_2,\ FD} \alpha\text{-hydroxyglutarate}$$

α-Hydroxyglutarate could then be aminated to glutamate or oxidized to α-ketoglutarate. The other central amino acids, aspartate and alanine, probably derive their carbon skeletons directly from oxalacetate and pyruvate. The pathways of biosynthesis of other amino acids *de novo* in rumen bacteria have not been studied in detail, but no doubt most of the pathways will be similar to those found in other microorganisms. Thus ^{14}C label in a number of nonprotein precursors will be distributed among many of the amino acids (e.g., References 492 to 494).

Several rumen bacteria can synthesize amino acids from exogenous precursors, such as the branched chain VFA already mentioned, phenylacetic acid (giving phenylalanine)[495]

and indoleacetic acid (giving tryptophan).[496] The first step in the synthesis of amino acids from branched chain VFA is again a reductive carboxylation, which requires ATP, CoA and thiamine pyrophosphate, and so the acyl phosphate and acyl CoA are likely to be intermediates.[497] In this way, valine can be formed from isobutyrate in pure and mixed cultures,[498] and isoleucine and leucine can be similarly formed from α-methylbutyrate and isovalerate.[492,494,499]

F. Breakdown of Urea by Rumen Microorganisms

Urea enters the rumen in several ways. Endogenous urea produced by the host animal passes through the rumen wall by diffusion[500] and also is contained in the copious salivary flow characteristic of ruminants.[3] This is an important feature of nitrogen metabolism in ruminants, because it allows urea nitrogen to be recycled to the rumen, where it is hydrolyzed to ammonia which can then be re-assimilated into microbial protein. Thus ruminants can more efficiently utilize feeds low in protein, illustrated by experiments where sheep, whose only nitrogen input was urea administered intravenously, were maintained for 3 months without protein.[501] This aspect of ruminant nitrogen metabolism has been reviewed regularly (e.g., References 502 to 506). Ureolytic activity in the rumen, and the consequent formation of microbial protein, also allows nonprotein forms of nitrogen (NPN) to be used in ruminant diets. Urea is principal among NPN sources, but others such as biuret and glycosyl ureas have been used in efforts to slow the release of NH_3 so that release is balanced to rate of assimilation by the rumen microorganisms and therefore loss by excretion minimized (reviewed by Ferguson, Reference 432, Chalupa, Reference 347, and many others). It has even been reported that urea might enter the rumen ecosystem by excretion from some protozoa.[507] Ureolysis is therefore of major importance to the rumen fermentation.

Although it was suggested that some urea may be metabolized nonhydrolytically,[508] inhibition of urea digestion by acetohydroxamic acid,[509-511] and the rapid conversion of ^{14}C-urea to ^{14}C-carbon dioxide[512] suggest that, in fact, urea is hydrolyzed in the rumen entirely by urease (EC 3.5.1.5). The activity has been partially purified[511,513,514] and there appears, from electrophoretic mobility, to be only one enzyme species present, of smaller molecular weight than jack bean urease.[513] The effects of divalent ions on enzyme activity have been variable,[513,515] but it now seems likely that nickel is present in rumen urease as it is in the enzyme from jack beans.[516] Sheep fed a diet containing 5 ppm Ni had greatly enhanced urease activity,[517] whereas urease activity in vitro was not increased by addition of Ni to the assay,[518] indicating that the stimulation in activity was due to greater synthesis of urease at higher Ni concentrations in vivo. Many other properties of the enzyme have been investigated, including its kinetics,[511,513,514] sensitivity to chemical,[510,511,513,519] and naturally occurring[514] urease inhibitors, and changes with diet, which have given variable results.[455,520,521] In many of these properties it has proved to be different from the jack bean enzyme.[510,513]

While the enzyme itself is easy to measure and purify, the isolation from the rumen of bacteria possessing urease activity has been a most difficult and controversial subject. It is known that urease is found mainly in the bacterial fraction from rumen fluid.[513,522,523] Early isolations of proteolytic bacteria from the rumen gave some bacteria which were also ureolytic, but were not obligately anaerobic.[36,379] Similarly, ureolytic facultative bacteria have frequently been isolated from the rumen in the search for ureolytic organisms. Ureolytic staphylococci and micrococci have been isolated on several occasions[524-527] and other facultative bacteria with urease activity have also been isolated from rumen fluid.[509,523] Indeed, it was calculated from their numbers and activity that *Streptococcus faecium*[509] could account for all of the urease activity found in the rumen, even though it was present in low numbers, and could not be said to be a 'typical' rumen

bacterium. The urease of *Strep. faecium*, incidentally, was shown to be plasmid coded.[528] More recently, it was found that the flora adhering to the rumen wall, which was already known to be highly ureolytic,[500,502] contained a large proportion of facultative, ureolytic bacteria including species of *Staphylococcus, Micrococcus, Propionibacterium, Corynebacterium* and *Streptococcus*.[301,382-384,529] A very recent paper[530] has reported different results, however, and the importance and number of facultative bacteria on the rumen wall remains to be confirmed. It was furthermore suggested that the sloughing of dead epithelial cells into rumen fluid accounted to some extent for the facultative ureolytic isolates commonly found in the fluid. This would provide for a continuous, steady inoculation of rumen fluid with facultative ureolytic bacteria, and obviously for at least some of the ureolytic activity of the fluid.[384] The isolation[527] of ureolytic strains of *Staphylococcus saprophyticus* from the fluid is clearly consistent with the dual roles of ureolysis and tissue digestion by the wall population.[385] Ammonia apparently regulated the urease activity of these adherent bacteria in a manner consistent with many studies of the flow of endogenous urea into the rumen, and it was suggested that the adherent flora might participate in regulation of urea flux across the rumen wall.[384,531] This has proved difficult to confirm directly, however.[529]

Many strictly anaerobic ureolytic bacteria have also been isolated from rumen fluid. The first of these was *Lactobacillus bifidus*,[532] but as it was present at only 10^5/mℓ and has not been found in later work, it is probably of little importance. Ureolytic *Peptostreptococcus* spp. were isolated from cattle fed on a urea-molasses diet,[533] and ureolytic species of *Propionibacterium, Bacteroides, Ruminococcus* and *Lactobacillus* and a ureolytic *Streptococcus bovis* were isolated from cattle fed semisynthetic purified diets.[534] The numbers in which these bacteria might occur in conventional diets is not known, and whether the enzyme activity present in them was sufficient to produce the observed urease activity in vivo is not clear. These isolations have not been confirmed. A ureolytic strain of *S. ruminantium* was isolated from the rumen of a cow,[535] then, using media in which the concentration of ammonia and other small nitrogenous compounds was much reduced, Wozny et al.[536] found that, on average, 5.8% of all isolates from the rumen could be shown to be ureolytic. Isolates included *Succinivibrio dextrinosolvens, Bact. ruminicola, Ruminococcus bromii, Peptostreptococcus productus* and species of *Butyrivibrio, Treponema* and *Bifidobacterium*, which are obviously typical of the largest proportion of rumen bacteria.

At first sight it is perhaps more satisfactory to have isolated ureolytic 'typical' rumen bacteria from the highest dilutions of rumen fluid than to believe that small numbers of atypical bacteria provide such an important metabolic activity. It also corresponds better with the estimate of Jones et al.,[523] using a dilution technique that 35% of viable rumen bacteria were ureolytic. Nevertheless it is by no means clear which of the two types of ureolytic population is of primary importance. Although present in highest numbers, the strict anaerobes have very low activity compared with the facultative anaerobes. For example, *S. ruminantium* had a specific activity of 0.05 μmol/NH_3 produced/min/mg protein[535] whereas *Strep. faecium* had an activity of 70 μmol/min/mg protein.[509] Thus the choice seems to be between populations of high numbers, each of low urease activity, or low numbers of high activity. A factor against the strict anaerobes is that their urease is strongly repressed by NH_3[509,536] possibly via glutamine synthetase.[461] For example, under NH_3-limitation the urease activity of *S. ruminantium* was repressed to 1.44 μmol/min/mg protein.[461] However, at NH_3 concentrations normally prevailing in the rumen, urease activity is low in the anaerobes, while in the facultative bacteria urease is not usually repressed by NH_3.[527,537] Furthermore, the rumen enzyme has a very high optimum temperature (68° C), identical to that derived from the facultative ureolytic organisms from the epithelium.[361] Inoculation of gnotobiotic lambs with a mixed, otherwise

nonureolytic, mixture of organisms and *Strep. faecium* gave fairly high urease activity in rumen fluid[509] and inoculation solely with ureolytic staphylococci also conferred activity on rumen fluid and the rumen wall.[538] However, the numerical argument favors the strict anaerobes and there is at present no definitive evidence that one or other of the ureolytic population types is actually the main urease producer in the rumen. It may indeed be found that both are of similar importance, which would satisfy the arguments in favor of each, but it is improbable that the problem will be solved easily.

G. Digestion of Other Nitrogenous Compounds

As well as protein and urea, other nitrogenous compounds enter the rumen in the food. Nucleic acids are quantitatively the most important of these, with samples of fresh grass, hay, and dried grass containing 5.2 to 9.5% of their total N in DNA and RNA.[47,539] Pure RNA and DNA are rapidly hydrolyzed in the rumen,[47] as are nucleic acids in plants,[540] forming transient amounts of nucleotides, nucleosides, and bases.[540] McAllan and Smith,[541] in a detailed study in vitro, concluded that, as might be expected, the general pathway of nucleic acid degradation was initiated by nucleases to yield the mononucleotides, then nucleosides, then the purine and pyrimidine bases. At some stage during the breakdown, the side groups of the bases were deaminated, so that, for example, no free cytosine accumulated. Instead, the bases formed as products were uracil (from the uracil and cytosine nucleotides in the original nucleic acid), hypoxanthine (from adenine nucleotides), xanthine (from guanine nucleotides) and thymine. These products were resistant to breakdown in vitro, but did not accumulate in vivo. In Coleman's review of rumen protozoa,[169] details of the metabolism of nucleic acid bases, nucleosides, and nucleotides by the ciliates may be found. In general, these may be incorporated into protozoal nucleic acids or catabolized to a limited extent, and nucleotides are incorporated more quickly than the nucleosides or the bases. Our knowledge of bacterial metabolism of the nucleic acids is fairly rudimentary, and it is not known, for example, whether plant nucleic acids are broken down by plant or microbial nucleases. If, as seems likely, the enzymes are of microbial origin, the species involved are not known.

It is sometimes overlooked that nitrate can be present in fairly large amounts in feedstuffs. Nitrate is rapidly reduced to nitrite in the rumen and can cause nitrite poisoning if nitrite is not reduced quickly enough to ammonia.[543,546] Little is known about nitrate reduction at the level of the microorganisms, except that a number of bacterial species can reduce nitrate,[3] and that some strains such as *S. ruminantium* and probably other bacteria can use nitrate as a source of nitrogen for growth.[535] In mixed rumen microorganisms, nitrate reduction was stimulated by the addition of electron donors such as H_2 and glucose,[547] and nitrite accumulated. Only formate was a sufficiently good electron donor to prevent the buildup of nitrite. Denitrification by rumen bacteria is possible, but thought to be unlikely to occur in any quantity.[547]

Biuret and glycosyl ureas are examples already mentioned of a growing list of nonprotein nitrogenous compounds which can be used to replace or supplement protein in ruminant feeds. The practical significance of these compounds has led to many reviews on the subject,[346,347,477,548] and a series of symposia organized by the International Atomic Energy Agency, and their value and properties will not be discussed here. In general, detailed microbiology with regard to these compounds has not been done. The obvious exception is urea, and to a lesser degree the related biuret, which is hydrolyzed to ammonia and CO_2 by organisms tightly bound to plant debris.[347] The enzyme is distinct from urease, and must first be induced.[347,549] Various adaptations of the gross properties of the rumen microbial population to different sources of nonprotein nitrogen have been noted.[548]

Volatile nitrogenous bases other than ammonia found in rumen fluid include the N-methylated amines, methylamine[427,550-552] and trimethylamine.[551] These compounds appear on elution profiles from amino acid analysis of rumen fluid,[427,550,552] but are not distinguished from ammonia by other standard analytical methods.[550] Fortunately, the concentration of methylamine is usually 1 m*M* or less in rumen fluid,[550,551] so this analytical discrepancy is unlikely to influence the validity of work based on the assay of ammonia by these methods. Trimethylamine is derived from choline entering the rumen in plant phospholipid.[553] Free choline is rapidly converted to trimethylamine,[551] so that choline concentration in rumen fluid is negligible.[554] The source of methylamine is not known, however. It has been suggested that it may be derived from the amino acids alanine and aspartic acid[552] but the concentration of methylamine was unchanged by the addition of soluble protein to the rumen.[550] Methylamine concentration also varied independently of ammonia concentration in continuously fed sheep.[427] Surprisingly, it did not appear to be derived from trimethylamine either,[551] so the source of methylamine remains unknown, although its low concentration and slow metabolism[550,552] suggest it may be derived from quantitatively minor components of the feed. The fate of plant nucleotide bases in the rumen, for example, is not known in any detail. The methyl groups of trimethylamine and methylamine give rise mainly to methane in the rumen,[551,555] and presumably the amine groups form ammonia. The bacteria responsible for these hydrolyses were found to occur in low numbers in rumen fluid (10^4 to 10^5 bacteria / mℓ) and were found to be methanogenic.[555] The only bacterium isolated from the rumen capable of hydrolysis of these compounds was *Methanosarcina barkeri.*[555] *Methanobacterium ruminantium,* which normally occurs in greater numbers in the rumen, was unable to utilize these compounds, as were 19 other rumen bacteria tested.[555]

One of the more intriguing possibilities about nitrogen metabolism in ruminants was that rumen microorganisms might fix molecular nitrogen in the air swallowed with the feed. Moisio et al.[556] found no incorporation of $^{15}N_2$ into rumen microorganisms from air bubbled into the rumen, but low rates of acetylene reduction were found in rumen contents, equivalent to 1 to 10 mg N/bovine rumen/day[557-559] and 0.4 mg N/sheep rumen/day.[560] On the basis of the variability in activity, it was suggested that rumen nitrogenase was derived from free-living nitrogen-fixing microorganisms entering with the feed.[560] This was confirmed when Jones and Thomas[561] found that rumen contents of sheep fed sterile grass nuts fixed no N_2, whereas sheep on fresh pasture fixed 6 to 8 mg N/rumen/day. Nitrogen-fixing *Bacillus macerans* was isolated from rumen contents, and sheep receiving a diet containing 10% molasses were inoculated daily with a 10 mℓ culture. This increased N_2 fixation to 750 mg N/rumen/day.[561] However, the fixation of molecular N_2 is not usually of quantitative importance in the rumen.

VIII. LIPID METABOLISM IN THE RUMEN

Dietary lipid is extensively metabolized by rumen bacteria and protozoa, and the metabolism of lipids in the rumen has received considerable attention over the years because of the overall dietary importance of lipids. There are many reviews dealing with various aspects of lipid digestion, metabolism, and nutrition in the ruminant,[562,568] so this section will only outline the biochemistry of lipids in the rumen and deal in more detail with lipid metabolism as it applies to individual rumen bacteria.

The predominant type of dietary lipid depends upon the type of feedstuff. Animals grazing pasture will receive mainly mono- and di-galactoglycerides[569] whereas triglycerides predominate in cereal and concentrate diets.[567,570] The esterified long chain fatty acids in feedstuffs contain a large proportion of polyunsaturated linolenic and linoleic acids, with smaller quantities of saturated acids.[571-574] The lipid leaving the rumen,

on the other hand, consists primarily of free C_{16} to C_{18} saturated fatty acids bound to food particles, together with phospholipids and other bacterial lipids.[188,566,570] From this broad picture it can therefore be seen that the major metabolic transformations of lipids in the rumen are the hydrolysis of ester linkages (lipolysis) and the hydrogenation of unsaturated fatty acids.

Lipolysis proceeds rapidly in rumen fluid[553,575,576] and is catalyzed by microbial lipolytic enzymes.[576] Triglycerides are hydrolyzed by esterases to yield glycerol and free fatty acids apparently without the accumulation of intermediate di- and monoglycerides.[577] A lipolytic Gram negative curved rod, *Anaerovibrio lipolytica* was isolated from the rumen using linseed oil roll tubes.[578] Subsequently, other strains of *A. lipolytica* have been isolated and examined.[579,580] Its lipase is entirely extracellular[579,581] and associated with cell-surface or extracellular membranous structures.[157] Like the enzymes in whole rumen contents, it did not form intermediate di- and monoglycerides from triglycerides.[582] Phospholipids and galactolipids were not hydrolyzed,[582] although if they were first converted by esterases to diglycerides, hydrolysis by the *A. lipolytica* enzyme was rapid.[579] From the activity of pure cultures and the numbers found in rumen fluid, it was calculated that *A. lipolytica* would account for all triglyceride hydrolysis occurring in the rumen.[580]

The major lipids of plant chloroplasts are mono- and di-galactosyl di-glycerides, which are also degraded rapidly in the rumen. A lipolytic *Butyrivibrio* sp. (S2) which deacylates galactolipids, as well as phospholipids and sulfolipids, has been isolated from the sheep rumen.[583] Lipolytic Butyrivibrios had been isolated previously,[584,585] but *Butyrivibrio* S2 was unusual in that it was a fatty acid auxotroph, requiring long chain fatty acids for growth.[583] Some of its properties are described below.

The bacteria responsible for the hydrolysis of phospholipids were again Butyrivibrios and one of these, a noncellulolytic *B. fibrisolvens,* was investigated further.[585] Lecithin was attacked firstly by a phospholipase A, yielding a free acid and the lysophospholipid, which was then degraded by a lysophospholipase.[585] Deacylation of this sort was not a property common to many rumen bacteria; from more than 200 isolations, only 3 were very active.[585]

Hydrogenation by rumen microorganisms is the reason why the depot fats of ruminants are rich in saturated fatty acids, and why their composition is less affected by the nature of dietary lipid than in the nonruminant.[571] The saturation of free fatty acids leaving the rumen is not complete, as *trans*-mono-unsaturated acids are present,[568] but hydrogenation of linolenic and linoleic acids is rapid.[565,586] A free carboxyl group is required for hydrogenation[577] so only the free products of lipolysis are attacked. All lipolytic bacteria so far examined have possessed hydrogenation activity, although several bacteria which hydrogenate unsaturated acids are not lipolytic and hydrogenation is not an uncommon activity among rumen bacteria.[587,588] The biochemical mechanism of hydrogenation of polyunsaturated acids involves a number of possible and established routes,[565] too detailed to describe here. It is convenient to divide these reactions into those which hydrogenate polyunsaturated acids and those which hydrogenate mono-unsaturated acids. The first step was found in some butyrivibrios,[583,587,589–591] a strain of *Ruminococcus albus,*[587] two *Eubacterium* spp.,[587] two *Fusocillus* spp.,[587] a Gram negative micrococcus[592] and a *Treponema* sp.[593] Strangely, in the most lipolytic species, α-linolenic acid was hydrogenated only as far as *trans*-11, *cis*-15-octadecanoic acid.[588] The further reduction of mono-unsaturated acids was shown to be catalyzed by the *Fusocillus* spp.,[588] a nonlipolytic Gram negative rod[588] and a Gram negative anaerobic bacillus.[594] On the basis of their hydrogenation and isomerization patterns the hydrogenating bacteria have been divided into three classes, which possess different hydrogenation specificities and different fatty acid isomerases.[588]

The role of protozoa in lipid metabolism in the rumen is less clear. The capacity to hydrolyze triglycerides is found partly in the protozoal fraction from rumen fluid,[584,595] but the contribution to lipolysis by engulfed and attached bacteria is not known.[169] Defaunated animals were found to have a greater proportion of unsaturated fatty acids in their blood plasma,[596] indicating positively a role for protozoa in hydrogenation. Similarly, the digesta of protozoa-free calves contained a higher proportion of saturated fatty acids than did digesta from faunated animals.[597] Hydrogenation of linoleic acid proceeded at a similar rate before and after defaunation[598] but this was possibly due to compensation by an increased bacterial population.[169] Direct evidence has been obtained for hydrogenation in oligotrichs,[203,599,600] but not holotrichs,[599,600] so, while the protozoa are active in lipid metabolism as in the case of other metabolic properties, their quantitative contribution is difficult to determine.

Although the modifications of dietary lipid are restricted mainly to lipolysis and hydrogenation, and incorporation into microbial lipid does occur, there is also considerable *de novo* synthesis of lipids, particularly phospholipids, by rumen microorganisms.[567] As a result of the presence of branched chain VFA and propionyl CoA in rumen fluid, the fatty acids of rumen microorganisms contain an unusually high proportion of branched chain and odd-numbered long chain fatty acids.[563,566,601,602] Furthermore, one of the most interesting recent developments in the biochemistry of rumen microorganisms is the discovery of a new type of long chain fatty acid in the previously mentioned lipolytic *Butyrivibrio* S2.[583] These compounds, the diabolic acids, are so far unique to the rumen and hindgut, and their novel structure has led to great interest from microbial ecologists and membrane biochemists alike, for their synthesis and function are virtually completely unknown.

Butyrivibrio S2 was unusual in that it had a natural auxotrophic requirement for straight-chain saturated (C_{13} to C_{18}) or monoenoic fatty acids.[583] This property enabled the incorporation of exogenous ^{14}C-labeled fatty acids to be traced, and led to the discovery of the diabolic acids. The newly discovered lipid component[583] was identified as a long chain, vicinyl dimethyl-substituted dicarboxylic acid,[603] giving the appearance of two saturated fatty acids linked by their penultimate carbon atoms. Diabolic acids were found in nearly all of the phospholipids of *Butyrivibrio* S2, but in few of the nonphospholipids, and none were in a free, nonesterified form.[604] The two major lipids of *Butyrivibrio* S2 grown in the presence of palmitic acid were found to contain diabolic acid, linked at each carboxyl group to the C2 hydroxyl groups of two molecules of alkenyl glycerol.[605] Diabolic acids have been found in the rumen and hindgut, but appear not be absorbed by the host nor incorporated into animal tissues.[606]

Butyrivibrio S2 also rapidly hydrogenated linoleic and linolenic acids so that unsaturation was absent from hydrophobic chains.[583,604] This led to interest about how membrane fluidity was maintained in this organism,[583] because the degree of unsaturation of membrane fatty acids generally determines the fluidity of that membrane. It was found that the membrane of *Butyrivibrio* S2 became fluid at 34.5° C, thus permitting growth,[607] and it was suggested that the presence of the diabolic acids, and of butyroyl groups in glycolipids and phospholipids,[608] both contributed to this essential membrane fluidity.[607] One therefore might speculate that in any highly reducing environment, such as the rumen, one might expect to see the easily hydrogenated long chain unsaturated fatty acids replaced in their membrane function by diabolic acids, or similar saturated fatty acids, which do not stack as easily as simple straight chain saturated acids and hence lead to membrane fluidity. Use of short chain acids such as butyrate and branched chain acids might also enhance fluidity. It is as yet unclear whether the diabolic acids span the lipid bilayer (they are of suitable dimensions for this) or if both carboxyl groups are attached to groups on the same side of the leaflet.[604]

Although their distribution among other rumen microorganisms is also not yet known, the lipid composition of other butyrivibrios is unusual when compared with nonrumen bacteria.[608] The occurrence of diabolic acids may be quite widespread among anaerobes, depending on their function, and they may be of considerable importance.

Feeding free long chain fatty acids to ruminants, or infusing them into the rumen, inhibits methane production[609] and may also decrease digestibility.[610,611] The effect on methanogenesis seems to be caused partly by a direct effect on *Methanobacterium ruminantium*[612,613] and partly by an increase in the propionate: acetate ratio[614,615] because of the nonsensitivity of the propionate- and succinate-producing bacteria, *Selenomonas ruminantium, Bacteroides ruminicola, Megasphaera elsdenii* and *Anaerovibrio lipolytica,* and the toxic effect on *Ruminococcus* and *Butyrivibrio* spp.[612] Oleic acid is the most toxic of the acids[612] although the VFA can also be inhibitory.[616] Triglycerides were not toxic to *M. ruminantium*[613] and because of their very slow rate of digestion can be used to protect food from degradation in the rumen.

IX. METHANE PRODUCTION

In anaerobic metabolism hydrogen produced during the catabolism of carbohydrates can be removed either through the formation of reduced fermentation products, such as lactate or ethanol, or by the formation of hydrogen gas. Rumen organisms in pure culture may produce either reduced fermentation acids, ethanol, or gaseous hydrogen, or mixtures of these and acetic acid. However, in the mixed culture of the normal rumen gaseous hydrogen is almost undetectable and methane is a large constituent of the gas phase. In addition the balance of the fermentation products lies towards acetic rather than the more reduced acids and ethanol is not detectable. The role of hydrogen-utilizing methanogenic bacteria in keeping the partial pressure of hydrogen low and in controlling the rumen fermentation was realized some years ago,[3,617] and the hydrogen utilizing methanogens, originally named *Methanobacterium ruminantium* and *Methanobacterium mobilis* were amongst the bacteria isolated in the 1950s and 1960s.[618,619]

In the last few years the possibilities of production of 'biogas' as an alternative energy source has renewed interest in the methanogenic bacteria and the reactions concerned in the production of methane from various primary substrates, and many advances have been made by the study of methanogenesis in anaerobic digesters. In particular much of the confusion surrounding the reports of isolations of methanogenic bacteria has been resolved by the demonstration that the methanogenic bacteria are limited in the substrates which they will utilize and that in many cases production of methane is not by one bacterium but by a close association of two bacteria, one of which by utilization of hydrogen enables a primary reaction to proceed.

In this review the more recent work on methane production in the rumen will be discussed and this has involved experimental demonstrations of the role of the methanogens in controlling fermentation of carbohydrates, the possibilities of decreasing rumen methanogenesis, and the generation of methane from fatty acids.

A. Measurement of Hydrogen in the Rumen

McArthur and Miltimore[620] in their analyses of rumen gas detected about 0.2% hydrogen amongst the minor constituents. However, the hydrogen of importance to the rumen organisms, and particularly the methanogenic bacteria, is that dissolved in the rumen fluid, and this is difficult to determine.

Hungate[617] used a method in which the hydrogen in the rumen fluid (in the animal or in an apparatus in vitro) was equilibrated through a dialysis sac with a sterile salt solu-

tion. The dissolved carbon dioxide was absorbed with alkali and the hydrogen finally transferred to a gas chromatograph for analysis.

Czerkawski and Breckenridge[621] used a method similar in principle, but different in apparatus, in which a liquid sample was taken and the hydrogen finally transferred with nitrogen as a carrier gas to a gas chromatograph. More recently another method has been introduced[622] which is again simpler in apparatus and quicker than the Hungate method and has an advantage over the second method in that the hydrogen is concentrated and so the lower limit of detection of dissolved hydrogen is lowered to 10 pmol/mℓ of liquid.

Hungate showed[617] that the rate of methane production in rumen fluid was linearly related to the dissolved hydrogen concentrations with various substrates added to an in vitro fermentation, and concluded that hydrogen was an important, or the most important, intermediate in rumen methanogenesis. Formate was shown not to be an intermediate in hydrogen utilization. However, formate produced by sugar-fermenting bacteria might be the substrate for about 18% of rumen methane formation.[623] The hydrogen-using methanogenic bacteria also use formate.

In both the later experiments a peak in dissolved hydrogen concentration some 1 to 2 hr after feeding was noted, and this was followed by a rapid fall. The phenomenon occurred in animals on different diets. The concentrations of methane in the rumen gas were high at times corresponding to the peak of dissolved hydrogen and then declined,[621,622] so again hydrogen and methane were related. An observation in one of the experiments[622] was that there was a large variability amongst samples in the peak concentrations of hydrogen which the workers believed to be due to 'patchiness' in the rumen hydrogen concentration. If this is so it could have interesting links with the ideas of microhabitats and colonial growth in the rumen microbial system. Later experiments also showed that methane production by mixed rumen bacteria was proportional to hydrogen concentration when hydrogen was added to the gas phase.[624]

B. Hydrogen Utilization in the Rumen

That hydrogen is a principal substrate for rumen methanogenesis was shown by experiments such as those above and the isolation of the two species of hydrogen-utilizing methanogenic bacteria previously referred to in numbers sufficient to account for ruminal methane production. Pure culture studies also showed that a number of the carbohydrate-fermenting bacteria and protozoa produced gaseous hydrogen. The additional theory, that utilization of hydrogen by methanogenesis could, by a form of mass action, increase hydrogen production and change the balance of fermentation, was first demonstrated experimentally by Chung[625] with *Clostridium cellobioparus*. Culture of the clostridium with *M. ruminantium* gave greater growth and production of more acetic acid and less lactic and butyric acids and ethanol than when the clostridium was grown alone.[626] Iannotti et al.[627] also found that growing *Ruminococcus albus* with *Vibrio succinogenes* (which used hydrogen to reduce fumarate to succinate) on glucose plus fumarate changed the *R. albus* fermentation products from ethanol, acetate, and hydrogen to acetate: extra hydrogen was produced at the expense of ethanol and utilized by the vibrio. Utilization of hydrogen from *R. flavefaciens* by *M. ruminantium* with similar effects to those of the previous combination has also been shown.[628] *Selenomonas ruminantium* is usually reported as not producing hydrogen from sugar fermentations. However, some strains produce very small amounts that can be detected only by very sensitive gas chromatography. Although no free hydrogen accumulates, hydrogen production can be increased considerably when the selenomonad, growing by sugar fermentation, is cocultured with a hydrogen-utilizing methanogen. The hydrogen production can be measured by the amount of methane formed.[629]

The effects of hydrogen utilization by methanogens may become apparent in other

ways. The alkaloid heliotrine is toxic to sheep grazing pastures containing *Heliotropium europaeum*. Bacteria detoxifying this compound can be isolated from rumen fluid, but the detoxification involves hydrogen and under normal rumen conditions the detoxifying bacteria cannot compete with the methanogens for hydrogen.[630,631]

It is mentioned in another section that methane may be considered a loss of carbon and energy to the ruminant although the methanogenic bacteria do convert ammonia into microbial protein of use to the animal. If methane production could be stopped and the hydrogen and CO_2 diverted into formation of propionic acid then the useless methane would be converted to a useful product. If this were accompanied by bacterial cell production then this could compensate for loss of methanogenic bacteria. Mere stoppage of methane production would, of course, convey no benefit, and indeed could be a positive disadvantage as accumulation of hydrogen may inhibit growth of hydrogen-producing, cellulolytic bacteria.[626]

In one of the previously mentioned experiments[624] chloroform inhibition of methanogenesis in an artificial rumen increased dissolved hydrogen concentration. Inhibitors of methane production added to the rumen itself lead to increase in hydrogen concentration and increase in the proportion of propionic acid relative to acetic acid.[632-634] The practicability of long term use of methane inhibitors is discussed in another section, but the short term experimental results just mentioned could be explained either as a hydrogen inhibition of bacteria producing acetate and hydrogen or a use of hydrogen by propionate-producing bacteria. That extracellular hydrogen could be used to reduce fumarate in formation of succinate or propionate was shown by experiments with cultures of *Bacteroides ruminicola, Anaerovibrio lipolytica* and *Selenomonas ruminantium* growing on glucose or fructose, with or without a hydrogen atmosphere.[260] *Megasphaera elsdenii* showed a slight increase in propionate production under the same conditions, but this was not large enough to require net uptake of extracellular hydrogen; use could have been made of hydrogen formed by the bacterium in production of acetate. With strains of *Bact. ruminicola* succinate production was increased and lactate production decreased. With *A. lipolytica* propionate and succinate were increased relative to acetate. The two strains of *S. ruminantium* had different fermentation patterns under CO_2 alone, and under H_2/CO_2 propionate production in one was stimulated at the expense of acetate and in the other increase in propionate and succinate decreased lactate production. Only *A. lipolytica* showed an increase in cell yield and this may have been due to slightly faster growth in the hydrogen culture. The K_m for hydrogen uptake by these bacteria ranged from 4.5×10^{-6} to $4.4 \times 10^{-5} M$. These values are considerably higher than that for *M. ruminantium*, $1 \times 10^{-6} M$,[623] and show that the fermentative bacteria could not successfully compete for hydrogen with the methanogenic bacteria in the normal rumen. *Desulfovibrio* are present in the rumen in numbers which could account for ruminal sulfate reduction.[635] In the rumen these Desulfibrios may also be ble to grow on lactate or other substrates without sulfate as a hydrogen acceptor but with *M. ruminantium* removing hydrogen, as this reaction with nonrumen *Desulfovibrio* ssp. has been shown in culture.[636]

C. Production of Methane from Fatty Acids

Acetate is used for cell synthesis by *M. ruminantium*[637] but is not used for methanogenesis. However, although hydrogen and formate have been shown to be the major substrates for methane production in the rumen, acetate or higher fatty acids are major precursors of methane in other anaerobic habitats. The bacteria concerned in these reactions must be present in the air, soils, and waters, and so might be expected to enter the rumen.

Nelson, Opperman, and Brown[638,639] studied enrichment cultures obtained by incu-

bating an inoculum of rumen fluid for some weeks with the appropriate substrate. From these cultures they obtained mixed cultures which produced methane from formic, acetic, butyric, and valeric acids. In the dissimilation of butyric acid, acetic acid built up and then declined. In the dissimilation of valeric acid, propionic acid built up and was not metabolized, and cultures producing methane from propionic acid were not obtained.

The production of methane from acetic acid by a single bacterium has been known for some time, although the number of such species in pure culture is small. Although pure cultures producing methane from higher fatty acids were claimed, recent work has shown that degradation of higher fatty acids by a form of β-oxidation can occur only if a methanogen, or some other hydrogen-utilizing bacterium, is present, and the constituents of such mixtures have been characterized.[640-643] The reactions carried out are as described above, conversion of acids via acetate and hydrogen, with propionate being an end product if an odd-numbered-acid chain is being degraded. In all systems studied propionate degradation seems the most difficult reaction.

A bacterium degrading butyrate or longer chain fatty acids in coculture with a hydrogen using methanogen was isolated by enrichment culture from rumen fluid from a steer fed on corn and corn silage.[642] The methanogenic bacterium was a *Methanosarcina,* which also degrades acetic acid to methane and carbon dioxide, not the usual rumen hydrogen-utilizers. It was suggested that the *Methanosarcina* might utilize hydrogen more efficiently at the low growth rates of the butyrate-degrading bacterium.

The maximum growth rates of bacteria degrading acetic or other fatty acids are low[644] and in the normal rumen one might expect such bacteria to be washed out, except perhaps for a few whose residence time was prolonged by entrapment in particles in the rumen. Opperman et al.[645] concluded from studies with ^{14}C-labeled acetate that a maximum of 5.6% of the methane production in the rumen of a cow fed on alfalfa and concentrates could have come from acetate. Most of the radioactivity of the acetate was found in butyrate, a normal reaction of the *Butyrivibrio*.[306]

In sheep fed on lucerne Rowe et al.[646] found negligible conversion of acetate to carbon dioxide, but in sheep fed on molasses-urea plus oat straw and soyabean meal there was extensive conversion of acetate to carbon dioxide. With this diet there was a very slow rumen turnover rate and the molasses sugars were quickly fermented. It was suggested on the basis of observation of large numbers of sarcina-type bacteria which autofluoresced blue-green in UV light, that acetate was being converted to carbon dioxide and methane by a *Methanosarcina,* although radioactivity in methane from uniformly labeled acetate was not determined.

Czerkawski and Breckenridge[647] found that many simple compounds incubated in an artificial rumen inoculated from a sheep fed on molassed sugar-beet pulp gave rise to methane. However, no methane was produced from acetate. Some compounds were known rumen methanogenic substrates either directly (e.g., formate) or by degradation with production of hydrogen (e.g., lactate, glycerol). But amongst others were the alcohols, methanol to butanol. Methanol is a known substrate for methanogenic bacteria from other sources and can be formed in the rumen from pectin breakdown. It was previously shown to disappear in rumen contents, presumably by conversion to methane.[4] The degradation of ethanol to methane has been shown (with nonrumen bacteria) to be by a coculture of a bacterium producing acetate and hydrogen and a hydrogen-utilizing methanogen.[648] Presumably degradation of higher alcohols could take place by a similar mechanism. Ethanol could be formed in the rumen fermentation and has been occasionally used in ruminant feeds, but the other alcohols are not likely to be found except perhaps in small traces as byproducts of the main fermentation pathways.

What has been shown in these experiments is that although the main precursors of methane in the rumen are hydrogen and carbon dioxide, as might be expected inocula of methanogenic bacteria found in other habitats get into the rumen and may exist there in numbers sufficient to carry out minor reactions, and occasionally, if conditions become right, their activities can increase.

The growth requirements of the methanogens are simple. Most nitrogen for cell synthesis is obtained from ammonia, and *M. ruminantium* can produce much of its cell carbon from acetate.[637] The structure of these bacteria differs from that of others and studies have been made on cell-wall composition and other aspects (e.g., References 649 and 650). The bacteria form a unique group and details of the enzymic activities, cofactors, and ATP generation in the production of methane have only comparatively recently been worked out. Such details are not strictly relevant to the role of the rumen methanogens and will not be considered here. Work on metabolism and structure of the methanogenic bacteria has been reviewed by Balch et al.,[28] and these authors propose a new classification scheme and new nomenclature for the methanogens. The reader is referred to this important review for further information on these bacteria.

Wolfe[651] reviewed the classification of the methanogenic bacteria known at about 1970 and described a method for large scale culture of hydrogen-utilizing methanogens. He also reviewed the biochemistry of methanogenesis.

X. OXALATE DIGESTION IN THE RUMEN

Although it has long been known that oxalate is digested in the rumen,[652-654] it is only recently that obligately anaerobic, oxalate-degrading bacteria have been isolated from the rumen.[655] Other oxalate degraders isolated from rumen contents were not strict anaerobes,[656,657] and therefore appear unlikely to be of importance in the rumen degradation of oxalate, as this is a strictly anaerobic process.[315,658] The predominant carbohydrate fermenters of the rumen are unable to degrade oxalate.[658] The anaerobic oxalate-degrading bacteria were Gram negative, non-motile, non-spore-forming rods of various lengths.[655] They degraded oxalate stoichiometrically to formate and CO_2 but it did not utilize a range of other common substrates for growth. These unique bacteria were isolated from an 'uncoupled' chemostat culture, in which the conversion of oxalate to methane had been interrupted by an increase in dilution rate to $\geqslant 0.078$/hr so that methanogens were washed out and oxalate degradation terminated in formate rather than methane.[315] A coliform-depleted medium was necessary for the final enrichment cultures to avoid overgrowth by *Escherichia coli*.[655] The cell yield was low, 1.1 g dry wt/mol oxalate,[655] corresponding well with yields in mixed bacteria taken from 'uncoupled' chemostats.[315] Owing to their narrow substrate specificity, it seems likely that these bacteria will be present in low numbers in the rumen, unless oxalate-rich food is consumed. Then the absence of other oxalate degraders will enable the bacterium to compete successfully,[655] and the capacity of rumen fluid to detoxify oxalate will increase[658] so that otherwise lethal doses of oxalate may be digested.[659]

XI. CELL SURFACES OF RUMEN MICROORGANISMS

It has been observed since the earliest light microscopy studies of rumen fluid that many rumen microorganisms do not appear singly, free in the fluid, but are associated with each other in clumps of the same species, clearly derived initially from a single cell. Later microscopic work showed bacteria also attached to the rumen wall. In recent years, developments in electron microscopy and in our understanding of the

cell coats of nonrumen bacteria have led to more formalized descriptions of these properties and their relationship with cell surfaces.[301,382]

The rumen ciliates are often found free-swimming in rumen fluid, but they can also bind to food particles during the processes of digestion, as described in the section on fiber digestion. Orpin and Hall[660] found with *Isotricha intestinalis* a ridge on the surface of the protozoon which appeared to be the site of attachment to grass. Electron microscopy revealed at this attachment organelle plasma membrane-bound projections and below the plasma membrane a layer of microtubules not found at other locations.[141,660] Attachment depended on a chemotactic response to soluble carbohydrates which also occurred with *Isotricha prostoma*[141] and some entodiniomorphs.[661,662]

The cell surfaces of many rumen bacteria are covered with a layer of coat material, variously known as slime, capsular material, exopolysaccharide or glycocalyx, which give some roll-tube colonies a very fluid, sticky form. Recent improvements in preparation of thin sections for EM have shown that the coats of rumen bacteria are much more extensive than had previously been realized.[663] These coats tend to give rumen bacteria adhesive properties so that they stick to each other to form microcolonies[663] or to food particles as has already been discussed, or to the rumen wall.[383,664] Cheng and co-workers have done extensive morphological studies on the walls of mixed and pure cultures of rumen bacteria.[296-298,665] The relationship found between extracellular polysaccharides of *S. bovis* and the viscosity of the medium suggested a causative role in feedlot bloat,[295] as will be discussed later. There were some surprising findings with *Butyrivibrio fibrisolvens,* for although *B. fibrisolvens* stains Gram negative, its cell walls contain glycerol teichoic acids[666] and lipoteichoic acids,[667] typical of Gram positive walls. Furthermore, EM showed its cell wall morphology also to be Gram positive.[297] The most detailed analysis of a cell envelope of a rumen bacterium has been done with *Selenomonas ruminantium* var. *lactilytica,*[668-676] which again seems to be atypical compared with other bacteria, because both inner and outer membranes lack phosphatidyl glycerol and cardiolipin[674] and the outer membrane seems to lack the usual Braun type of lipoprotein.[675] Another very unusual property was the covalent attachment of a polyamine, cadaverine, in the peptidoglycan layer.[676] In retrospect, it is perhaps unfortunate that similar studies were not done with a bacterium such as *Bact. succinogenes,* whose highly adaptable morphology is itself of interest,[677] and also because the cell coat plays an important role in anchoring cells during fiber digestion. The attachment of bacteria during fiber digestion has already been discussed, and at this point it need only be emphasized that this attachment can be specific as, for example, cellobiose specifically inhibited the attachment of *Bact. succinogenes* to cellulose[130] and in any case attachment usually occurs mainly at breakages in plant material. Moreover, such specificity, and the cellulase[678] and protease[679] activity associated with bacterial coats, point to a more active role for these structures which are usually envisaged as being rather inert. The recent finding of cellulolytic activity associated with cell surface vesicles in *Bact. succinogenes* is another interesting development in this area[677] and the separation of rumen bacteria using affinity adsorption methods has already been developed by Minato and Suto.[130,680]

XII. WHY ARE MOST OF THE RUMEN MICROORGANISMS STRICTLY ANAEROBIC?

Clearly this is an ambiguous question, and it is intended to be so, because it highlights two important related areas of rumen microbiology, and the microbiology of anaerobes in general. One possible interpretation concerns the mechanism whereby oxygen is toxic to these organisms, and thus makes them strict anaerobes; it demands a mechanistic

answer. The other is more philosophical, and concerns the selective advantage conferred by a strictly anaerobic physiology. It is possible, in fact, that both aspects have a common root, whereby the molecule or reaction which is sensitive to O_2 is the same one which confers the selective advantage, but there is no proof of this.

Morris,[681-683] in his reviews of the physiology of obligate anaerobiosis, emphasized that the strict anaerobes comprise only an extreme end of the wide spectrum of degrees of oxygen toxicity to all organisms. Within the collective term 'obligate anaerobes', wide differences in oxygen tolerance exist, and it is well known among rumen microbiologists that, for example, *S. ruminantium* survives a slightly oxidized medium much better than does *M. ruminantium*. Indeed, Wolfe and Higgins[684] emphasized how manual dexterity possessed only by the most skilled worker can be necessary for the culture of some strict anaerobes but not others.

One possible mechanism put forward by Morris[681-683] was that the damage caused by O_2 in anaerobes is the same as it is in aerobes, and it is only the degree of protection from that damage which is different. It need not be O_2 itself which is toxic, but its peroxide (O_2^{2-}), superoxide (O_2^-) or ozone (O_3) derivatives. The hydroxyl radical produced from these is an exceedingly powerful oxidant. For a time, it was thought that anaerobes lack catalase[685] or superoxide dismutase,[686] and so were more subject to this kind of damage. More recent evidence, in which these enzymes have been found in anaerobes,[681-683,687,688] tends to make this appear unlikely. In rumen bacteria, catalase has been found in only *V. alcalescens* among those commonly isolated,[689] and superoxide dismutase has been found in *S. ruminantium*[690] while others have not yet been examined.

It seems more probable that O_2 itself is the toxic moiety, perhaps by oxidizing labile thiol groups[681] or possibly by behaving as an alternative electron acceptor to fumarate or other normal anaerobic acceptors and thereby disturbing internal redox couples.[682] We would go further, and speculate that O_2 may well itself be an energy poison in anaerobes, by interacting with an essential component of the anaerobic electron transfer chain, in a way similar to the effect of carbon monoxide in aerobes. Certainly O_2 can oxidize components of the anaerobic electron transfer chains of rumen bacteria.

NADH oxidase may be a general mechanism for O_2 detoxification in rumen microorganisms, leading to the very low E_h -250 to -450 mV[7,691] of rumen fluid. There is considerable NADH oxidase activity in mixed rumen bacteria, and it has been suggested for nonrumen anaerobes[687,692] and for *S. ruminantium*[690] that NADH oxidase plays an important protective role in removing low O_2 concentrations. However, the mechanism whereby O_2 is toxic remains unresolved.

The other way of looking at this question raises a number of interesting points. For example, it is easy to understand why rumen bacteria would lack the ability to respire using O_2 — the synthesis of cytochrome oxidase, etc., would be energetically wasteful for the limited amount of O_2 available — but why O_2 should be positively toxic is more difficult to explain. Also why do facultative organisms not predominate in the rumen? The normal situation in the rumen must be assumed to be one of energy limitation. Only rarely does NH_3 limit growth, and conditions in which all nutrients are in excess are limited to the period immediately following ingestion of a meal. Otherwise a lactate fermentation will occur, leading to acidosis and possibly to death of the animal. Thus the maximum μ of bacteria under nutrient excess will not be a determinant of whether a species will survive in the rumen.

Some other factors are obvious, and are discussed elsewhere. The ability to adhere to and digest plant fiber is clearly of great importance, as is the ability to use a variety of substrates for growth. However, for those bacteria which use soluble sugars or starch, under energy limitation, there must be some other reason why strict anaerobes predominate. Continuous cultures have shown that different species of rumen bacteria have

different substrate affinities for different sugars,[693] yet these substrate affinities are no higher than those of facultative anaerobes, and the specific growth rates of these bacteria suggest that the rate of assimilation of substrate would not limit growth.[694] It must be concluded, therefore, that strict anaerobes predominate over facultative anaerobes in the rumen because they utilize the energy substrate more efficiently. Facultative bacteria have anaerobic growth yields roughly half as great as those of strict anaerobes. Therefore for equal substrate uptake they can grow only half as efficiently, and will quickly be supplanted. The exception is at the rumen wall, where O_2 diffuses into the rumen and facultative anaerobes may prosper.[381,382] One can only speculate that in order to achieve this improved growth efficiency, a mechanism was evolved which we now find causes O_2 toxicity in these organisms. Since electron transfer-linked reactions are both sensitive to O_2 and probably also responsible for the improved growth efficiency, this is in all probability the answer to both meanings of the original question as to why rumen micro-organisms are strictly anaerobic.

XIII. 'MODEL' RUMINANTS, IN VITRO AND IN VIVO

While germ-free small animals have become a common laboratory 'tool' for the assessment of physiological actions uncomplicated by animal-associated bacteria, the use of large animals, because of technical difficulties, is much less common. The animals used in such experiments normally contain, as do all animals, a gut microflora, but they are not dependent on this flora and can, perhaps with some modification of the normal diet, live out their whole lives in a germ-free condition. The ruminant, however, can live only for a limited period as a germ-free young animal fed on milk. Even when reared germ-free, the ruminant is adapted to utilization of microbial fermentation products, and as it grows older it can make little use of diets which can support a germ-free non-ruminant, and, of course, is unable to digest fibrous, ruminant, feeds. The development of the rumen itself is dependent on the ingestion of solid feeds and their fermentation by the bacteria. In the young, milk-fed ruminant, the rumen is smaller than the abomasum and milk is digested as in other young animals by gastric and intestinal processes. The young ruminant normally ingests more and more solid feed as it grows, and, finally, weaning completes the development of the rumen and its flora, and the animal is then a true 'ruminant'.

It is obvious, then, that a germ-free ruminant is an impossibility, but a ruminant with a defined rumen flora might be possible, and the production of such an animal has been the object of experiments; mainly in the authors' laboratories.

The gnotobiotic ruminant is an extension of the in vitro mixed culture or artificial rumen systems to include 'animal factors': for instance — rumination (as an aid to microbial digestion); removal of acids and ammonia through a living membrane (the rumen wall) rather than a dialysis membrane; intermittent mixing differing from that provided by the mechanical systems; passage of rumen contents by a selective mechanism rather than by a nonselective mechanical system. But the gnotobiotic ruminant is also an extension of the in vitro defined mixed culture experiments, in that it does not illustrate just one possible interaction of the rumen organisms with two species of bacteria, as with examples given in this paper. The gnotobiotic rumen must carry out all the reactions of the natural rumen and must carry them out at natural rates so that the animal itself can thrive and grow.

The natural rumen contains many species and genera of bacteria of different metabolic functions. It would seem, however, from our knowledge of the rumen that there is a relatively small number of metabolic pathways leading to the desired end products and that it is possible to isolate a small number of bacterial species which in culture apparently carry out all the stages in these pathways. Acting together, these bacteria

should then reproduce the whole rumen function. On this basis the rest of the rumen bacteria are 'passengers' or 'contaminants', perhaps metabolizing but not contributing to rumen function.

The gnotobiotic ruminant is essentially a 'model', depending for its success upon the completeness of our understanding of the rumen metabolic pathways and the truth of the assumption that rates, and extents, of attack on substrates in vitro are repeated in vivo. The mathematical model of the rumen also depends on understanding of the metabolic pathways and the roles of the different organisms and on the assumption that the kinetics of pure culture growth of bacteria can be used in the mixed culture model to describe substrate use and product formation. The mathematical model also assumes that only a few bacteria can reproduce the rumen function; those whose reactions are sufficiently defined to be used in the model.

There are, obviously, differences between the two 'models'. The mathematical model can be defined on the behavior of part or the whole of the rumen system, and its success is determined by its ability to predict the outcome of the reactions originally defined. If the prediction is not successful the incorrect part of the model may be found and altered. The gnotobiote model must successfully reproduce the behavior of the whole system, or the animal dies, and if it is not successful it may be very difficult, or impossible, to determine in what part of the system the fault lies. (And the fault may not be in the rumen system but in the development of the animal's absorptive or metabolic system). Another difference between the models lies in the fact that there are 'minor' (but important) rumen reactions, such as vitamin synthesis, details of which are still unclear. In the mathematical model it is really assumed, even if not so stated, that if the major reactions are predicted correctly, the minor reactions will also be correct: if the model makes sufficient volatile fatty acids and microbial cells then the animal lives. With the gnotobiotic animal the same basic assumption is made, but the minor reactions are tested: if the rumen flora makes sufficient volatile fatty acids and microbial cells the animal may still die of vitamin deficiency.

The mathematical model deals with only one stage in the life of the ruminant, usually the adult. The rumen flora is then defined in terms of reactions, is fully functional, and will digest feed components to a defined pattern. Changes in feed composition will then bring about changes in digestion products according to this pattern, and these will always occur. The gnotobiote model starts with no rumen flora and makes the assumption that if suitable bacteria are inoculated at times commensurate with age and changes in feeding pattern from milk to the adult solid diet, then the flora will develop into one able to carry out the defined adult rumen fermentation. It is also assumed that this flora will produce the adult rumen tissue structure.

The constraints governing the behavior of the gnotobiotic rumen populations which can be 'programmed' into the model are very few. On the other hand the constraints on the mathematical model are many and are limited only by knowledge of the bacterial reactions and the computer time required to work out the program. This latter does, indeed, set a practical limit to the complexity of the model that can be developed, even if the theoretical basis is available.

The gnotobiote model is, in some sense, much more rigorously tested than the mathematical model. The models differ, but both define the limits of our knowledge of the rumen system, and both will be briefly discussed here. Baldwin et al.[695,696] have reviewed mathematical modeling of the rumen in more depth and has given more details of actual computer modeling programs.

A. Mathematical Models

The mathematical model described in a few words above is actually extremely complicated and is the 'ultimate' model in one sense. In fact such a model has not been

made and cannot be made solely from our knowledge of the behavior of rumen bacteria, or protozoa, in culture. It will be evident from previous sections of this paper that, for instance, the rate and extent of breakdown of an isolated polysaccharide in a culture may not be relevant to the breakdown of that polysaccharide in a natural fiber, and the effects of rumination cannot be defined from culture experiments. The modelmaker may, then, in a sense 'cheat' by putting in kinetic constants from animal tests of digestion. On the other hand fermentation products of the different fiber polysaccharides may be defined from pure cultures of different cellulolytic and hemicellulolytic bacteria, as may growth yield and other things.

However, the model based on individual microorganisms and their experimentally determined characteristics is not the only type of model and may not be needed. There are basically two types of model which may be made which are intended really for two different jobs. The model referred to in the introductory paragraphs was said to be the 'ultimate' model because all the equations used are based on actual organisms and metabolic pathways and kinetics experimentally determined for these organisms. Inhibitions or stimulations of reactions and interreactions must be related to known metabolic functions. The model, of course, to predict rumen digestion in the animal must bring in factors other than purely microbiological ones such as rumen flow rates, but its ultimate success depends on knowledge of the rumen biochemistry and microbiology. So while it is validated by prediction of actual rumen reactions under various feeding conditions, its use is not just that of prediction. The model's behavior reflects not only our overall microbiological knowledge, but investigation of reasons for its failure in part or in total can show where we are lacking in knowledge and so where more investigation would be profitable. The model, then, can be used not only to measure the results of research, but also to define pathways for research. Although we have been discussing a model of the complete rumen function, simpler models defining only a part of this function, if based on microbiological facts, can be equally useful in defining our knowledge.

On the other hand there is a type of model the only, or main, use of which is to make predictions of practical value. This model is based on observations that a process follows certain mathematical rules. The underlying biochemistry is not known and the factors in the model may have no known counterpart in the living system, but the behavior of this system can be described by certain types of mathematical equations. If such a relationship between two parameters, say feed intake and faecal output, can be determined then changes in the output consequent on changes in the input can be described without reference to the reactions in between. Such a model cannot show in detail where more investigation is needed, but by its failure to predict under certain circumstances it can show that reactions are not always the same.

A small selection of recent models will be used to illustrate these points.

Ørskov and McDonald[356] produced a model for estimating the extent of protein degradation in the rumen. The degradation of protein or any other material is, in a batch process, limited by factors affecting the rate at which microbial degradation takes place and the maximum possible degradation. In a continuous process such as the rumen there is the further factor of flow out of the rumen which means that protein may be washed out of the rumen before it is completely degraded. The rate of breakdown of soyabean meal protein was determined from loss of protein from nylon bags suspended in the rumen (the batch process). It was found that percentage degradation could be expressed in an equation involving the time of incubation (t) of the form $p = a + b(1 - e^{-ct})$. The rate of loss of protein from the rumen was found by measuring the amount of chromic oxide in rumen contents at different times after a feed of sodium dichromate-treated soyabean meal. This treatment marks the particles and also renders them undegradable by rumen organisms. The loss of particles was exponential, as might be expected, so an exponential

function could be used to modify the degradation rate equation for passage of particles from the rumen. So the effective degradation (p) was shown to be $p = a + [bc/(c+k)](1 - e^{-(c+k)t})$ at time t after feeding.

The model showed that when dried grass was given at a restricted level with one protein supplement, then protein digestion was higher than when dried grass was fed *ad lib.* and the rate of passage of digesta from the rumen was greater.

This model allows of no deductions about the microorganisms involved in the process and it is limited in what it can predict. It combines the kinetics of two factors involved in protein degradation in the rumen system to describe the degradation obtained under specified feeding conditions.

In this model the rate of loss of the undegradable protein was exponential (i.e., it washed out with the rumen fluid) and it was assumed that the rate of loss of the degradable protein was the same. This is most likely correct because the particle size of protein supplements is such that they can leave the rumen without having to undergo any reduction in size by rumination and microbial degradation. Long fibers must be reduced in size before they can pass from the rumen and involvement of particle size and flow of digested fibers from the rumen was one of the aspects of a computer model of fiber digestion proposed by Mertens and Ely.[697] The fibers were divided into fast digesting, slow digesting, and indigestible fractions, as would seem reasonable from studies of microbial fiber digestion. Kinetics of post rumen digestion were also included in the model.

While this model was more complicated than the previous one it was essentially a kinetic model with data for the equations or for testing derived from various in vivo or in vitro tests. Like the previous model it gave overall mathematical form to a complex biological process but did not consider the biological processes in detail. For instance, it assumed that fiber digestion was limited only by certain fiber characteristics such as particle size, as in the last model. Decreasing particle size of the feed allowed of faster passage from the rumen, as in the model three particle-size compartments were used and passage from the rumen was mainly from the small-size compartment which small particle feed would enter directly (large and medium particles entered the appropriate compartments and were reduced in size with first-order kinetics).

Models involving more of the actual microbial activities are too complicated to describe here. However, Reichl and Baldwin[698] produced a linear programming model which used eight groups made up from known species of rumen bacteria. The data included substrate specificities, amounts of fermentation products from various substrates, and nutrient requirements (e.g., amino acids or ammonia). This model then begins to define interrelationships between bacteria and can show effects on, say, fiber digestion, of nitrogen or other limitation; not just the physical parameter of particle size as in the previous models. Bacterial growth and ATP production and utilization were also put into the model, as were protozoal growth and other concepts. The model, although successful in many ways, did not adequately cover competition amongst bacteria, for instance, and showed that, as the authors put it, 'additional data and concepts are required'.

Baldwin et al.[696] described a further model for rumen cellulose digestion incorporating such concepts as bacterial colonization of fibers, and provision of energy for bacterial maintenance as well as growth, amongst other inputs. But again the model did not completely represent rumen digestion.

The models show that data on various reactions may be lacking or that, since the data have in some cases to be simplified to permit computer manipulation in a reasonable time, the fine changes continuously going on in the normal rumen cannot be reproduced. The rumen of an animal fed a few times a day is a very complex system. As pointed out in

previous reviews, microorganisms may be using different fermentable substrates and have different limiting substrates as time after feeding changes. Growth rates, and so fermentation products, may vary, and so on. The system can be simplified by feeding the animal continuously or nearly so, when the rumen attains more to a steady state, and can be further simplified by feeding a synthetic diet.

As an example of this, Hume and co-workers[34,437] fed sheep at 2-hr intervals on equal amounts of a diet containing cellulose as a wood pulp preparation, corn flour starch, sucrose, molasses, urea, minerals, and polythene chips for bulk. This diet contained virtually no protein, the cellulose was in an easily degradable form, starch was readily degradable, the sugars would quickly dissolve in the rumen fluid and the nitrogen source was soluble. The constituents, except for the plastic, were virtually completely digestible. The feed was chosen for the study of factors influencing microbial protein production as, obviously, a measurement of protein in or leaving the rumen would be microbial protein. Changes in nitrogen content of this diet, and some additions, were made in different experiments, and casein was also added to the abomasum. So the feed simplifies experimental results but it also simplifies modeling. The production of protein in the rumen and some other aspects can be quite well reproduced by treating the fermentation of the different carbohydrates as separate single-step reactions with bacteria growing at different rates according to substrate availabilities, and using data on bacterial growth and yield factors, etc., from in vitro pure batch or continuous cultures of representative bacteria, and some data from the papers on rumen flow rates, etc. Such a model could be further refined (Hobson, unpublished).

The use of purified and easily degradable substrates limits the amount of microbial interactions necessary to break down the feed and also limits the number of hydrolytic enzymes necessary for polymer degradation to molecules assimilable by the microbes. It was suggested earlier that digestion of starchy feeds (particularly where starch granules are liberated from processed grains) is a comparatively simple process and this is borne out not only by consideration of mathematical models but by the results of gnotobiotic lamb experiments.

B. Gnotobiotic Lambs

Although some others (see Reference 699 for list) have reared milk-fed germ free lambs or calves for short periods, most work on gnotobiotic ruminants (i.e., post weaning and with defined rumen flora) has been done by the authors and colleagues initially in Cambridge and then in Aberdeen. (The microbiological work is described in a number of papers and these give references to papers on the techniques employed in the 'birth' and rearing of lambs under sterile conditions).

The most successful experiments in reproducing rumen function with a defined flora of about ten bacteria have been the ones where the lambs were milk-fed and then changed to a commercial ruminant diet of pelleted barley plus protein and mineral supplements.[700] The pelleting cracks the barley grains and so starch granules are released in the rumen; the protein is soluble. The fermentation on these diets fed ad lib to the normal animal is such that the rumen pH is lowered and cellulolysis and growth of cellulolytic bacteria is inhibited and the fermentation products come from the starch. The low pH also prevents growth of ciliate protozoa, which for various reasons[701] were left out of the defined rumen populations.

The lambs grew much as normal lambs for 21 days on a milk feed and without rumen bacteria. These and other experiments[699,701,702] showed that a digestive tract flora is not required by young lambs on a milk feed. The lambs were then weaned onto the barley and given inoculations of eleven strains of bacteria. Inoculations in all experiments were given at intervals, starting with a lactobacillary flora typical of the preweaning ruminant and proceeding to adult rumen bacteria. The bacteria stabilized to a predominantly Gram

negative flora as in the normal rumen, and the viable count was a high proportion of the total (the viable bacteria are always higher in normal grain-fed animals than in fiber-fed ones). Concentrations of bacteria, volatile fatty acid totals and proportions, weight gains, and feed conversion efficiencies were similar to normal lambs for about 120 days. At this time, and the phenomenon has been observed in other gnotobiotic lambs, appetite and the rumen flora failed. Three of the four lambs died at 140+ days. The fourth did not fail quite as dramatically as the others and moving it to a pen with normal lambs saved it. It was finally killed at a weight of 59.2 kg.

These, and other unpublished experiments showed that the 'model' bacteria for starch digestion can provide a near normal rumen function for a time. But in other experiments, as in these, the digestive functions have suddenly (over a week or so) begun to fail. The suggestion is that the rumen flora fails, as the lambs if caught before they are too weak can be saved by exposing them to inoculation from normal animals. After exposure, the rumen flora of the lamb above remained the same in predominant types, but whether they were the same strains could not be said. The principal types were in similar numbers to the gnotobiotic rumen, but were quickly joined by the usually mixed rumen flora of facultative and other bacteria.

The results suggest that the rumen flora can only be maintained by continued inoculation from outside sources. This may account for the large number of varieties of rumen species normally found, and, for instance, changes in serological type of a numerically constant population of one species.[703] The other fact, shown in a number of these experiments, is that although it is difficult or impossible to introduce 'foreign' bacteria (even rumen species) into a normal rumen,[703,704] the gnotobiotic flora, even if in numbers similar to the normal total counts, cannot exclude the many types of bacteria found in the normal rumen and which have no obvious function therein.

Attempts to produce an adequate fiber-digesting defined flora, including cellulolytic, hemicellulolytic, pectinolytic, and other bacteria have been little if any more successful than those described in published experiments,[702] although many variations have been tried. As said before, the problem of fiber degradation is complex. (Vitamin and other deficiencies have been excluded in these experiments). However, defined floras in milk-fed animals, or ones on starch-plus-fiber diets, have proved useful in 'modeling' the ureolytic flora in nitrogen metabolism,[531,538] interactions of lactobacilli and pathogenic *Escherichia coli,*[705] the formation of antibodies to the commensal flora,[706] the role of various bacteria in producing kale anaemia,[707] and cerebro-cortical necrosis,[708] and hydrogenation of dietary fats.[709] The role of the rumen bacteria in physical development of the rumen and in populating the intestines has also been demonstrated.[710,701]

'Modeling' of the rumen population whether by mathematics or by gnotobiotic animals has shown that while we have an overall knowledge of the rumen, and detailed knowledge of many aspects, there still remain gaps in our knowledge. Both types of experiment also emphasize the difficulties in deducing from experiments with laboratory cultures which may have changed on isolation and subculture and which may be growing on 'unnatural' substrates, and the rates and extents of reactions in vivo.

XIV. MANIPULATION OF THE RUMEN FERMENTATION

Much of the scientific interest in rumen microorganisms has had an objective not only on understanding of the rumen fermentation but an improvement in the nutrition of ruminant animals. Throughout this review, possible areas of inefficiency have been pointed out. It is now appropriate to assess how the research findings have contributed to improving these areas, and whether improvements have been achieved as a direct result of research effort in rumen microbiology.

One of the areas which can be readily identified is the use of nonprotein nitrogen

products in diets, especially those based on urea, as has already been discussed. It is quite straightforward to compare the rate of hydrolysis in vitro of, say, a new glycosyl urea with urea and then predict with some confidence how quickly NH_3 will be released and how efficiently it will be utilized in vivo. An alternative approach with this and many other aspects of rumen metabolism has been the search for an inhibitor of urease activity which could be of practical value in slowing hydrolysis by rumen microorganisms. Many inhibitors have been found in vitro, including hydroxyurea,[513] phenylurea,[513] hydroxylamine,[513] hydroxamates,[509-511,513,519] and melon seed inhibitor,[514] and some have been tested in vivo,[711,712] but so far none seem to be used for nutritional purposes. This is perhaps not surprising, since it is much easier to balance the energy and nitrogen content of feeds to reduce NH_3 overflow[478,479] than to screen and test inhibitors.

Also in the discussion of nitrogen metabolism it was shown how the rumen fermentation could cause inefficiency in the use of dietary protein and amino acids. Protection of proteins by physical or chemical treatments has been fairly successful while similar protection of single amino acids, although successful in preventing degradation, has not been productive simply because there is no individual amino acid or group of amino acids which severely limits ruminant production.[432] Hence the aim of those interested in chemical manipulation of protein and amino acid metabolism has tended to concentrate on finding an inhibitor of amino acid deamination with a broad spectrum of activity. Diaryliodonium compounds were shown to achieve this in vitro[372,713] and to be of value in vivo.[714,715] The biochemical and bacteriological effects of diaryliodonium chemicals are not known, although it has been suggested that they interfere with the transport of amino acids into microorganisms.[716] Inhibition of Stickland-type deamination has already been mentioned, but the practicability of these inhibitors is not known.

It might be supposed that one of the most fertile areas of research in rumen microbiology would be in the prevention and control of digestive disorders. Bloat is a frequently fatal condition that occurs as a result of the fermentation gases produced in the rumen forming a stable foam, which does not allow the gases to be freed and eructation to occur. Unless relieved, pressure within the rumen increases until pressure on other organs causes death. The importance of bloat is such that much research effort has been put into the problem, and there are many reviews in the literature dealing with the subject (e.g., References 3, 717, and 718). For the most part the work has taken a chemical direction, aiming to identify the factors causing stabilization of the foam and to eliminate these chemically. In the case of pasture or legume bloat, it is thought that the foam-stabilizing agents are proteins and lipids derived from the feedstuff,[717,719] whereas the agents in feedlot or grain bloat appear to be of microbial origin. These include slimes and other polysaccharides and proteins released by lysis.[295,718,720] One suggested microbiological factor in the etiology of feedlot and legume bloats is that in bloating animals, bacteria might digest mucins in the saliva which can disperse foam.[721,722] Mucinolytic bacteria have been isolated, including *Streptococcus bovis, Megasphaera elsdenii, Butyrivibrio fibrisolvens* and *Selenomonas ruminantium*,[723,724] and it was found that inoculation of bloating animals with mucinolytic bacteria aggravated the bloat.[718] In feedlot bloat, the numbers of starch-fermenting *S. bovis* and lactate-fermenting *M. elsdenii* were found to increase,[719] and it was thought that the extracellular dextran produced by *S. bovis* was a major cause of the stable foam.[725] Later work seemed to refute this as the numbers of *S. bovis* did not correlate with the incidence of bloat,[726] but it has since been shown that the production of slime by *S. bovis* depends on the availability of sucrose[295] or sodium bicarbonate,[263] and so numbers in vivo need not relate to the quantity of slime produced. Slime is also produced by many other rumen bacteria and they may also be involved in feedlot bloat. Relief of bloat has so far involved treatment of

the foam rather than the microbial population, using detergents or antifoam agents.[717,718,727] Oxytetracyline,[718] penicillin,[728,729] and other antibiotics[729] have had little if any beneficial effect.

The other digestive disorder commonly seen is the overfeeding of a high starch diet such as barley, or the too rapid transition from a roughage to a concentrate diet.[3,730,731] When easily degraded starch and soluble sugars are present in excess, rumen microorganisms produce fermentation acids very rapidly, causing the pH to fall. Lactic acid is produced proportionally much more than normal, as a result of various regulatory mechanisms which rumen microorganisms possess.[304,732,733] The low pH prevents fiber digestion by inhibiting the growth of cellulolytic bacteria,[156,734] and inhibits the growth of *Veillonella,* the major lactate fermenter in the normal rumen.[735] Lactate concentrations then increase so that eventually only lactic acid bacteria can survive, and the host often dies of lactic acidosis. If the transition to a concentrate diet is made gradually, *Megasphaera elsdenii,* which is also a lactate fermenter but more tolerant of low pH,[734] will be capable of dissipating the lactate.[735] Again the microbiologist has played mainly a descriptive role. Avoidance of acidosis can be achieved simply by proper management of feeding regimes. Gradual introduction of grain diets, the use of whole rather than pelleted grain, and a frequent, restricted intake all increase stability.[730]

Associative effects between feeds, whereby the mixing of one feedstuff with another need not give additive nutritional effects can also be partly explained microbiologically. This has already been discussed in the section on fiber digestion. Again, the effect is mainly dependent on pH. Since cellulolytic rumen bacteria do not grow below a pH of 6.0[156,734] fiber digestion in one component of the feed may be inhibited if another causes the pH to fall below this value. A qualitative assessment can be made for predictive purposes, but only feeding trials can supply quantitative data.

Improvement of microbial productivity in the rumen is another area in which various kinds of treatment have been attempted. Some, such as increasing dilution rate to increase the growth yield, as already discussed, have little practical relevance as yet — in this case the ruminant would have to increase its rate of saliva production or intake of water quite dramatically to cause a similar effect. Others, including the removal of protozoa by chemical defaunating agents, have not yet shown definite benefits. There is one area, however, where there is no doubt that the feed efficiency of ruminants can be increased by manipulation, and that is where chemicals have been used to alter the stoichiometry of the rumen fermentation.[373]

The stoichiometry of fermentation depends on diet, but broadly speaking it can be stated that the fermentation products are in decreasing molar proportions: acetate, carbon dioxide, methane, propionate and butyrate.[3] Clearly the loss of carbon dioxide and methane by eructation represents a loss of carbon to the rumen. It also represents an energetic inefficiency, because theoretically if methane production is decreased, an increased production of propionate should occur and the feed efficiency should then increase.[3,313,736] Conversely, any chemical which increases propionate production will automatically decrease methanogenesis, and increase efficiency. Many chemicals have now been shown to achieve both of these, including ionophores (monensin, lasalocid[373]), various halogenated compounds[313,372,373,737] and some antibiotics.[373] Monensin is by far the most researched compound, and indeed is the only one in widespread use.

Monensin is an ion-translocating ionophore which specifically transports Na^+ ions across biological membranes.[738] It can be a highly toxic compound, because uncontrolled ion translocation collapses the electrical potential across membranes, and monensin is, for example, toxic to horses[739] and turkeys.[740] It is not usually toxic to ruminants, presumably because of detoxification by the rumen microorganisms. When incorporated into a ruminant diet, it is not toxic to the whole flora, but mainly to Gram positive

organisms and ruminococci[741,742] suggesting that the cell coats of resistant, mainly Gram negative, bacteria afford a measure of protection from monensin. Thus propionate and succinate producers would be expected to predominate in the rumen of animals receiving monensin, and it has been found in many experiments that the molar proportion of propionate in the VFA does increase with monensin. Monensin also inhibits methanogenesis, but this occurs indirectly by the above mentioned consequences of a change in the stoichiometry of the fermentation. Methane production from H_2 and CO_2 was not affected by monensin,[374] and *Methanobacterium ruminantium* was resistant to the drug.[742]

There are many other effects of monensin, however, which are not so easily explained in bacteriological terms. Monensin has also been claimed to be beneficial by inhibiting proteolysis,[374] deamination,[373,374,376,379,743] increasing rumen dilution rate,[744,745] and decreasing coccidial infection in the hindgut.[746] Furthermore, it prevents acute pulmonary edema and emphysema (fog fever) by preventing the breakdown of tryptophan to 3-methylindole (skatole) in the rumen.[747] Since a *Lactobacillus* seems to be responsible for the second step in this reaction, the decarboxylation of indole acetic acid,[748,749] presumably it is sensitive to monensin. Thus, the possible effects of monensin extend far beyond simply changing the fermentation stoichiometry, and this is probably quite a general phemonenon. Diphenyliodonium chloride decreases methane production and increases propionate production as well as performing its intended purpose of the inhibition of ruminal degradation of amino acids.[373] Similarly, as was pointed out earlier, the effects that antiprotozoal compounds cause in the rumen ecosystem are at least partly due to their effect on the bacteria. Thus it is not clear whether the changes in stoichiometry which the antiprotozoals produce is due to the decrease in numbers of protozoa or to a changed flora. Similar problems are caused by other kinds of chemical manipulation.[373]

In conclusion, therefore, it is fair to say that rumen microbiology has been reasonably successful in a descriptive capacity, but is less successful in a predictive role, as judged by the use of gnotobiotic lambs and computer modelling. Chemical manipulation is as yet mainly empirical, and we do not understand many of the side effects of manipulative treatments.

XV. CONCLUSION

It is impossible to cover every aspect of rumen function in a relatively short paper. This review has been selective — intentionally in topics, to some extent intentionally in work cited. We would hope that the topics show the most active areas of rumen microbiology at the present time and that the papers quoted are representative of the results being obtained, even if some papers have been overlooked.

The work reviewed here has been published mainly during the last ten years, although in some cases much earlier work has been quoted to show how the subject has developed or how, in many cases, we are adding the detail obtainable by new methods and apparatus to old concepts. As an exercise in microbial ecology the microbiology of the rumen is extremely complex. Although we have a great deal of information on how the rumen microorganisms interact, it must be obvious from this review that the details, for the most part, do not combine to enable us to look at the functioning of a rumen in a predictive manner. We cannot yet in theory or practice create a rumen that is fully functional. We cannot yet predict exactly what will happen when we try to modify the natural rumen.

Whether knowledge of rumen microbiology will lead to increases in man's food production is a debatable point. We are dealing with animals and farms, and what the

microbiologist would like to see done to improve the growth or the fermentation of his microbes may be in large-scale practice impossible or uneconomic — many factors influence animal production. What, perhaps, has happened is that the animal nutritionist has been made aware that he must think first of the microbial community and then of the animal. However, the importance of rumen microbiology lies not just in animal nutrition but in the lead it has given to investigation of other microbial ecosystems and to the realization that the rumen anaerobes and similar microorganisms are involved in many aspects of the life of man and all animals. These organisms have some properties quite different from aerobic or facultative organisms or even anaerobes of other habitats, so they are of great academic interest as well. Indeed there are areas such as their genetics which should prove fruitful but which have so far hardly been touched. It will be interesting to survey the field of the rumen and its anaerobes in another ten years.

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