CHAPTER I

INTRODUCTION

Ruminant animals can be considered as essentially two systems, the microbial ecosystem of the rumen and the tissue metabolism within the animal. The rumen microbes enable ruminants to utilize fibrous materials in which the carbohydrates have β 1, 4 linkages; these linkages are relatively indigestible in animals. The rumen microorganisms provide the host with protein synthesized from non-protein nitrogen and so that the animal is at times independent of a dietary source of amino acids.

The rumen ecosystem has been studied intensively in the past 25 years (see for instance Baldwin and Allison, 1983). However, knowledge of rumen fermentation is still limited due to diversities and complexities of the rumen ecosystem (Owens and Bergen, 1983). The microbial ecosystem of the rumen was believed to be composed mostly of bacteria and protozoa. However, studies of Clarke and Dimenna (1961), Lund (1974), Orpin (1975, 1977a,b,c), Bauchop (1979a), Ogimoto and Imai (1981) have shown that, besides bacteria and protozoa, yeasts, anaerobic fungi, bacteriophages and mycoplasmas are also rumen inhabitants.

The relatively recent discovery of rumen-anaerobic fungi by Orpin (1975, 1977a,b,c) and Bauchop (1979a) and their suggested apparent importance in fibre breakdown in the rumen (Bauchop, 1979a; Akin *et al.*, 1983) has emphasized an important area for research, that is to determine the quantitative role of these microorganisms in the rumen of animals on high-fibre feeds and the factors that influence their activity. The fungi appear to be highly cellulolytic and therefore this knowledge may assist in manipulation of their activity to increase fibre digestion substantially.

Large numbers of fungi are found in the rumen of animals fed high-fibre diets; their population has been estimated to constitute up to 8% of the total microbial biomass (Orpin, 1981). As a result research on their role in fibre digestion has increased (see for example, Orpin and Letcher, 1979; Bauchop and Mountfort, 1981).

From recent *in vitro* studies, there is no doubt that rumen fungi have a high capacity to digest the structural components of plant cell walls. Orpin and Hart (1980) reported that cellulose, hemicellulose, and lignin of wheat straw were digested by pure cultures of rumen fungi to as much as 58%, 52% and 22%, respectively under *in vitro* conditions. However, there is no information available at present on the role of rumen fungi in feed digestion *in vivo*.

There is no doubt that a number of factors affect the density of microbes in the rumen; among these is the presence of a substantial population of rumen protozoa. The engulfment of bacteria by protozoa in the rumen has been studied extensively by Coleman (1964; 1975) and Coleman and Laurie (1974a,b). Because of predation on bacteria by protozoa, the bacterial population density is generally lower in faunated than in defaunated animals. A large population of protozoa in the rumen reduces the amount of protein available for digestion by the host animal because of this predation, and moreover protozoa appear to be preferentially retained in the rumen (Weller and Pilgrim, 1974; Leng, 1982). Recently, Orpin (1975) observed in *in vitro* incubations that the protozoon *Entodinium spp.* engulfed the spores of rumen fungi indicating an important interaction between fungi and protozoa.

Experiments with protozoa-free ruminants generally indicate that overall digestibility is depressed by the elimination of protozoa, although some contradictary results have appeared (see Demeyer, 1981 for review). However, in the studies reviewed by Demeyer (1981) the animals were fed substantial amounts of concentrates. Over the last seven years, Bird and Leng have published results from a research program indicating the beneficial effect of the absence of protozoa from the rumen of sheep and cattle fed high-fibre diets. Growth rate and wool production of defaunated animals increased substantially when animals were fed high energy, low protein diets and under grazing conditions (Bird and Leng, 1978; 1983).

The studies presented in this thesis are part of a continuing research program to examine the effects of defaunation on ruminants given low quality, forage-based diets. In the studies reported here

the effect of defaunation on rumen anaerobic fungi and on fibre digestion have been examined in sheep given high-fibre diets. The research program was a comparative study on the fungal population and related metabolic parameters in groups of faunated and defaunated sheep.

CHAPTER II

REVIEW OF LITERATURE

2.1 Scope of Review

The utilization of feed by ruminants involves complex relationships among plant components, microorganisms in the rumen, and the animal. Among the three major groups of rumen microbes: bacteria, protozoa and fungi, only the first two have been studied extensively in relation to their role in the digestion of various kinds of feed in the alimentary tract of ruminants. However, with respect to the role of protozoa in fibre digestion, equivocal conclusions have been drawn from a number of studies carried out in the last 10 years. This is due, at least to three possible reasons: variation in the basal diets used in the experiments (Demeyer, 1981), the relatively small number of cellulolytic protozoa in comparison with bacteria (Hungate, 1975), and lack of strong evidence for cellulase secretion by protozoa (Delfosse-Debusscher *et al.*, 1979). Furthermore, since the discover of rumen fungi by Orpin (1975) and a report of Bauchop (1979a) which suggests that high numbers of rumen fungi are found in animals fed on high-fibre diets, these microorganisms have opened a new area for further studies on their role in fibre digestion.

This review will, therefore, be directed on those aspects of rumen fibre digestion and metabolism in which protozoa and fungi are directly involved. Several recent reviews (e.g. Demeyer, 1981; Russell and Hespell, 1981; Leng, 1981; Mertens and Ely, 1982; Orpin, 1981; Ørskov, 1982; Van Soest, 1982) discuss the more general aspects of rumen microbiology. The role of protozoa in rumen digestion has been reviewed by Burggraaf (1980) and Bird (1982). Emphasis in this review is given to the involvement of rumen fungi in rumen digestion.

2.2 The Kinetics of Fibre Digestion in the Rumen

2.2.1 General

Digestion in the rumen is a dynamic process involving the inflow of feed to the rumen through ingestion, and the outflow of fluid, microbes and undigested feed residues through the omasum to the lower tract.

There is ample evidence to show that with diets consisting mainly of roughages, voluntary feed intake is limited by the capacity of the reticulo-rumen and by the rate of disappearance of digesta from this organ and passage to the lower digestive tract (Balch and Campling, 1962; Freer and Campling, 1963; Doyle, 1981). The rate of breakdown of digesta in the reticulo-rumen, in which microbial and mechanical processes are involved, largely determines the rate of disappearance of digesta from this organ. The soluble products of digestion are absorbed, gaseous products are eructated, and the remainders, undigested food particles, are transferred to the abomasum and intestines (Campling, 1970).

Ruminal digestion can be divided into four components (Mertens, 1977): digestion lag, digestion rate, potential digestibility and passage rate of particles. Digestion rate is believed to be a very important factor in affecting digestibility (Ørskov, 1982) and intake, although other authors suggest that the size of the potentially digestible fraction is probably more important than other components of digestion (Mertens and Ely, 1982).

It is generally believed that digesta particles do not pass through the reticulo-omasal orifice until sufficiently reduced in size. In this regard, chewing during eating and rumination can be considered as the main factor responsible for size reduction of food particles (Balch and Campling, 1962; Ulyatt, 1982). The digestion of such roughages as cereal straw in the rumen requires the attachment of the microorganisms and the penetration of their enzymes, since the major component of roughage organic matter is insoluble in water (Weston, 1984).

Leng (1982) stated that the digestibility of straw is probably limited to the retention time of feed particles in the rumen which in turn is limited by its rate of comminution to sizes small enough to move out of the rumen.

2.2.2 Potential extent of digestion

Several factors such as chemical composition, plant morphology and crystallinity are known to affect the potential extent of digestion (see Smith *et al.*, 1971; Waldo and Smith, 1972; Mertens, 1977; Mertens and Ely, 1982). Of the chemical components, lignin has been shown by a number of authors to play an important role in limiting biodegradability of cell wall materials. Waldo and Smith (1972) reported that the extent of ruminal digestion in vivo is influenced by the lignin concentration in fibre, with correlations of 0.78 and higher being obtained in the study of Smith *et al.* (1971). Delignification of forages such as lucerne has been shown to increase the potential digestibility of cellulose from 14% to 72% (Belyea *et al.*, 1983). Similarly, Van Soest (1975) stated that hemicellulose which is also responsible for variations in digestibility, can be altered to become more soluble in water and ultimately digestible by exhaustive non-hydrolytic oxidative delignification. In contrast, the effect of silica on the extent of forage digestion is still uncertain (see Minson, 1971; Hartley, 1981; Roxas *et al.*, 1984), even though this fraction is often associated with low digestibility of straws.

Recently, Akin (1982) has shown that certain tissues in plants are virtually indigestible, indicating that plant morphology also influences the potential extent of digestion. With regard to morphological effects on digestion Mertens and Ely (1982) postulated that the lignin content of those indigestible tissues may be a major determinant since these tissues are known to contain lignin in high proportions. However, this opinion may be misleading since Orpin and Hart (1980) have shown that the lignin fraction of wheat straw leaves can be digested to the extent of 20% by rumen anaerobic fungi even though other workers (Gordon and Ashes, 1984) were unable to demonstrate the digestion of lignin of wheat straw by rumen fungi.

Another factor which seems likely to influence the extent of forage digestion is the presence of phenolic compounds such as cinnamic and vanilin (Varel and Jung, 1984). Certain phenolic compounds apparently depress dry matter disappearance *in vitro*. Addition of these compounds to the medium has been reported to reduce dry matter disappearance of cellulose *in vitro* 10% to 50% when compared with controls. This may be associated with toxicity of various phenolic acids to rumen bacteria and protozoa (Chesson *et al.*, 1982; Akin, 1982).

2.2.3 Rate of Digestion

Rate of digestion is the quantity of feed that is digested per unit of time (Ørskov, 1982; Van Soest, 1982). It is influenced by plant, microbial, and animal factors (Mertens and Ely, 1982).

Digestible fibrous feed can be divided into two fractions in terms of its rate of digestion: fast-digesting and slow-digesting (Mertens, 1973 cited by Mertens and Ely, 1979). These fractions are largely composed of mesophyll and phloem tissues (fast-digesting fraction), and bundle sheaths and epidermal cells (slow-digesting fraction), while the indigestible fibrous feed is mainly composed of vascular bundles and schlerenchyma tissues (Akin *et al.*, 1984; Akin and Amos, 1975). Each tissue contains cellulose, hemicellulose pectins and lignin in various proportions. Among the substances mentioned, cellulose is the major constituent of plant cell walls (Morrison, 1981). However, cellulose can vary considerably in the amount present in the plant cell walls and the difference in rate of degradation due, primarily, to differences in the overall structure of the basic composition of cellulose (Morrison, 1981).

Mesophyll cell wall is relatively easily digested because its cellulose has a low degree of order (amorphous) (Wood, 1981). Furthermore, Gordon (1977) as cited by Wood (1981) reported that mesophyll cell walls isolated from grasses showed a low crystalinity of cellulose. Therefore, it seems likely that the degradation of mesophyll and phloem tissues occurs without the direct adherence

of rumen bacteria or enzymes freed from the bacteria, although bacteria are always found near the degraded zones (Akin, 1981).

Both lignin and silica appear to be inversely related to the digestibility of cell wall polysaccharides in ruminants (Hartley, 1981). However, the differences in rates of digestion between fibrous feed are not correlated with lignin content since it does not directly affect digestion rate (see Lechtenberg *et al.*, 1974; Mertens, 1977). In addition, Thiago *et al.*, (1979) have also shown that the contents of cellulose, hemicellulose, and lignin in forages do not correlate directly with fractional digestion rate from cell wall. Thus, it appears that there are some factors affecting digestion rate which are not detected by present chemical analyses. This opinion is supported by evidence provided by Akin and Amos (1975) who showed that detergent analyses and even cellulase analyses do not isolate fibre components having a relationship to the morphological structure of plant tissue. Moreover, by means of Scanning Electron Microscopy (SEM), it is clear, that bacteria (Akin, 1973; 1974), protozoa and anaerobic fungi (Bauchop, 1980) attack different morphological structures in plant at different rates.

Cheng *et al.* (1984) showed that in the rumen, the surfaces of easily digested cell walls are heavily colonized and digested rapidly by a wide range of bacterial species, whereas the slow and indigestible fractions (e.g. vascular and sclerenchymal tissues) are sparsely colonized and no pitting is observed. Protozoa have been reported by Bauchop (1980) to attach to damaged regions of Lucerne stem (*Medicago sativa*. L) forming a complete ring between the epidermis and the vascular cylinder. However, significant degradation was only detected in phloem and cortex tissues, but did not appear in the epidermis. In addition, despite a complex protozoal fauna present in the rumen, only a single protozoan genus, *Epidinium*, was found attached to and degrading the tissues (Bauchop, 1980). Fungi, on the other hand, have been shown to be associated with more slowly digested plant materials in the rumen (Bauchop, 1979a). The digestion of thin-walled tissues of stem such as mesophyll and epidermal cells by these fungi has been observed by Bauchop (1980), whereas silicified short cells of epidermis were

resistant to digestion (Bauchop, 1979b). The factors which can be related to the control of the rate of fibre digestion are (1) the fragility of plant tissue structures and (2) the areas exposed by particle-size reduction (Mertens, 1977). Consequently, grinding or alkali treatment or swelling via hydration that improve the accessibility of microbes to the cell wall increase the rate of digestion (Hogan and Leech, 1981; Mertens and Ely, 1982).

Laredo and Minson (1973; 1975a, b) demonstrated that at the same digestibility the voluntary feed intake of leaves of grasses was higher than stems. The main reason for this appeared to be the lower resistance of leaf to physical breakdown and therefore the retention time of the leaf fraction in the rumen was shorter than that of stem.

2.2.4 Digestion lag-phase

The slow rate of fermentation of cell wall constituents is shown by the extent of the lag phase, which occurs when the fibrous materials are suspended in nylon bags in the rumen. Regardless of the rumen conditions and the method for comparing different forages, there appears to be no doubt that forages differ in the lag time of commencement of solubilisation. This may be due to difference in rate of hydration of the forage or rate of chemical or physical alteration before enzymic degradation occurs (Mertens and Ely, 1982). Brazle and Harbers (1977) showed that penetration of the epidermal layer may provide an initial barrier to digestion, although Akin (1979) supported the opinion of Brazle and Harbers (1977) that microbial attack of fibres is enhanced when the epidermal layer is fractured even though the tissues are not ground to small particle size.

The fermentative environment and the presence of non-fibre components of the diets such as starch have been shown to increase the lag time of fibre digestion (Mertens and Loften, 1980). They postulated that this was due to preferential digestion of starch by rumen bacteria before cellulose was attacked. Orpin and Letcher (1979) have also shown that in the presence of glucose in the medium the digestion of

cellulose by rumen-anaerobic fungi was delayed until the glucose was exhausted. More recently Van Gylswyk and Schwartz (1984) have shown that although cellulolysis, in general, is delayed when starch is in the medium, there are considerable differences in susceptability among rumen bacterial species. The lag phenomena could also be prolonged due to low numbers of fibre-digesting microorganisms and fibre-digesting enzymes not reaching high enough levels (Mertens and Ely, 1982).

2.2.5 Rate of passage

There is no doubt that the volume of digesta in the rumen and its rate of removal from the rumen are very important to the nutrition of ruminants, particularly when they are fed on high-fibre diets of low digestibility (Dixon *et al.*,1981; Ørskov, 1982).

Although studies in this area have been conducted extensively in the last two decades (Balch and Campling, 1962; Weston, 1983) factors controlling the rate at which long particles are broken down of different forages are largely unknown (Ørskov, 1982). The firmest conclusion drawn and apparently accepted among scientists is confined to the area of the effect on digestibility of reducing feed particles either naturally or artificially. The digestibility of these materials generally decreases as the rate of passage increases (see Ørskov, 1982).

Several factors known to be associated with the removal of organic matter from the rumen have been described by Weston (1984) and presented in Figure 2.1.

Two major factors altering the rate of passage are chewing during both eating and rumination, and microbial digestion. Recently Ullyatt (1982) concluded that the rate of particle size reduction is a dominant factor regulating fibre digestion in the rumen. Further Ullyatt (1982) concluded that to determine the feeding value of forages, attention should be paid to the inherent factors which determine resistance to particle-size breakdown such as tensile strength, shear strength, elasticity, brittleness, anatomy and morphology.

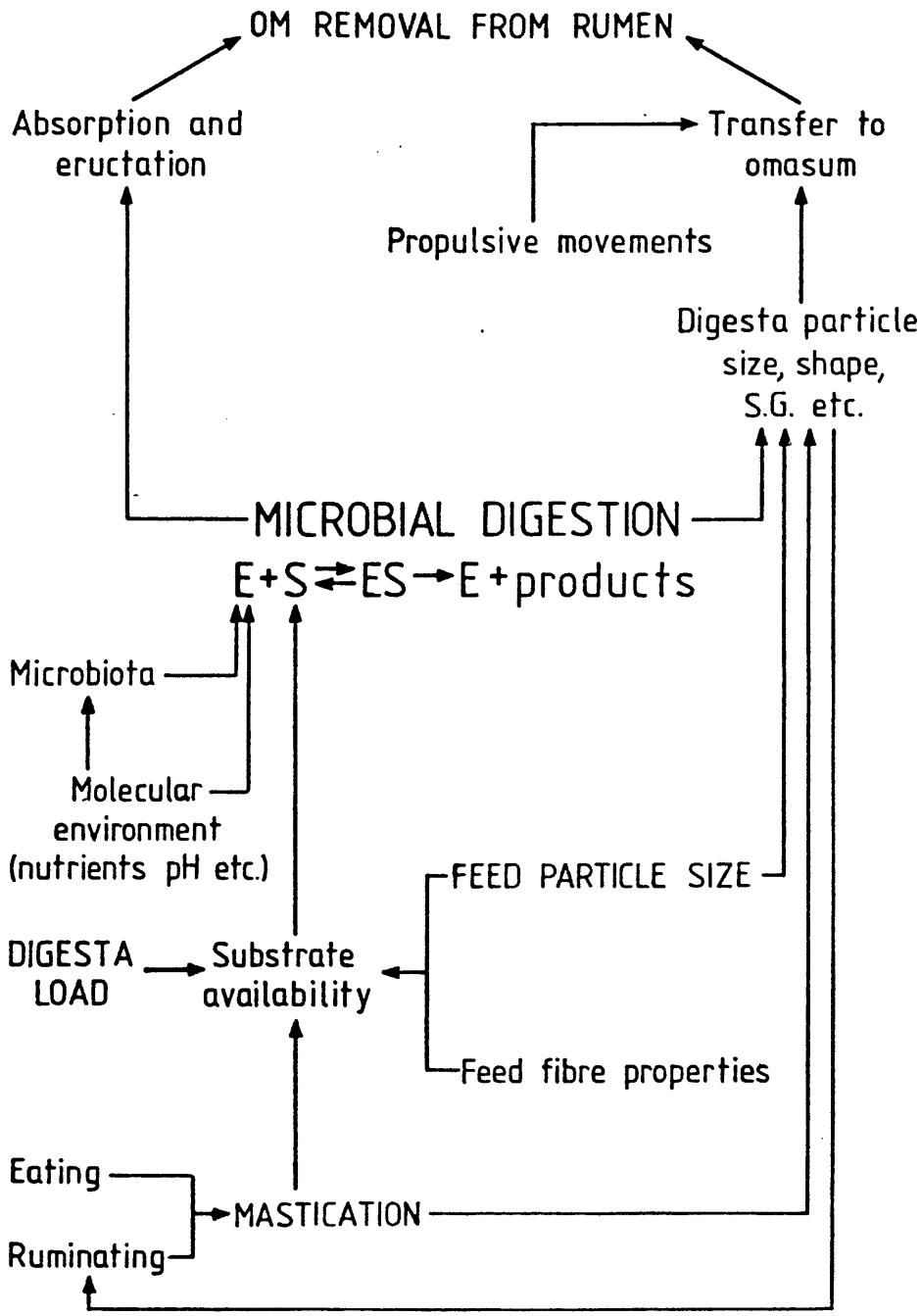


Figure 2.1 **Factors associated with the removal of organic matter (OM) from the rumen.** S.G. is specific gravity, E is enzyme and S is substrate (Weston,1984)

Leng (1982) and Weston (1984) have stated that the availability of substrates for microbial growth in the rumen influences the breakdown of fibrous materials. This is supported by evidence that sulphur fertilization (+S) of the grass *Digitaria pentzii* its retention time and increases voluntary intake and digestibility compared with unfertilized (-S) *Digitaria spp.* (Rees *et al.*, 1974; Rees and Minson, 1978). A further study carried out by Akin *et al.* (1983) concluded that the greater intake of sheep eating sulphur-fertilized compared with unfertilized *D. pentzii* forage is due to the heavily colonization by rumen fungi on +S forage increasing the rate of particle breakdown. Moreover, sheep fed +S *D. pentzii* digested about four times more dry matter in the rumen in 24 h than did sheep eating -S forage. Thus, it seems that the established conclusion on the relationship between digestibility and rate of passage (see for example, Mertens, 1977) cannot be generalised to fibrous feeds, since higher voluntary intake, which is correlated with rate of passage, is not always accompanied by decreasing digestibility of fibre. An increase in fibre digestion in the rumen through dietary manipulation may result in higher voluntary feed intake as has been shown by Akin *et al.* (1983). Furthermore, the estimate of the size of particulate material passing through the reticulo-omasal orifice (Ullyatt, 1982) requires a review, since the more recent work carried out by McBride *et al.* (1984) has clearly shown that large particles (10mm in length) could pass through the reticulo-omasal orifice as against the size usually passed. This probably can be used to explain why such discrepancies occur in the relationship of voluntary intake, digestibility and rate of passage of highly fibrous feeds.

2.3 Determinants of Rumen Ecology

2.3.1 Substrate affinities and preferences

Since the work of Monod in the 1940's on the relationship between bacterial growth and substrate concentrations, it is generally agreed that the bacterial growth rate follows a Michelis-Menten relationship (Russell, 1984):

$$K = \frac{K_{max}\ S}{K_s + S}$$

where K is the specific growth rate, K_{max} is the maximum growth rate, S is the substrate concentration, and K_s is the substrate concentration that will allow one-half maximum growth rate. K_s is also termed the affinity constant (Russell and Hespell, 1981) and it is inversely related to the organism's affinity for the substrate and the capacity to grow rapidly in an environment with no limiting substrates (see Russell & Baldwin, 1979). Recent information suggests that affinity for the same substrate can differ greatly among species and that a species can have higher affinities for some substrates than others (Russell and Baldwin, 1979).

In the rumen, during much of the feeding cycle, soluble substrate concentrations are low (Hungate, 1966). Under these conditions, the microbial growth rate can be increased by increments of substrate concentrations and the pattern follows saturation kinetics typical of enzyme systems (Monod, 1949). However, Russell and Baldwin (1979) showed that the growth of five rumen bacteria used in their study, *Selemonas ruminantium*, *Bacteriodes ruminicola*, *Megasphaera elsdenii*, *Streptococcus bovis*, and *Butyrivibrio fibrisolvens*, did not always follow typical Michaelis-Menten kinetics. This discrepancy is possibly caused by the fact that wide variations in substrate affinities were seen among the substrates utilized by a species and among species for the same substrate. From their study, it can be assumed that substrate affinity may be a significant determinant of bacterial competition in the rumen, especially where the concentrations of soluble substrates are low as found in animals fed on straw-based diets. Unfortunately, similar work on other rumen microbes, protozoa and fungi, has not yet been conducted. Therefore, it is not possible at the present time to elucidate the relative importance of substrate affinity as a possible determinant of the growth and competition of these rumen microbes.

Russell and Baldwin (1978) concluded that substrate preference in rumen bacteria can play an important role in determining competition between bacteria. These authors have clearly demonstrated that, among the five bacteria used in the experiment, different strategies of substrate utilization were seen. In this regard, the utilization of

every substrate was inhibited by one other substrate (Russell and Baldwin, 1978). This may be the reason for the delay in cellulose digestion by rumen bacteria when starch was added to a medium (Mertens and Loften, 1980) and also by the rumen fungi *N. frontalis* in the presence of glucose (Orpin and Letcher, 1979). 'It can be assumed that other species of rumen fungi will preferentially utilize simple carbon compounds before attacking more complex substrates since most Phycomycetes are the first organisms involved in the decomposition of sugar (Burnett, 1976).

2.3.2 Maximum Growth Rate

Maximum growth rate determines the survival of microbes under certain conditions such as an excess of soluble nutrients. In this condition, an organism with a higher maximum growth rate is able to grow faster than an organism with a lower maximum growth rate.

There is no doubt that bacteria grow exponentially; because of this it is impossible for them to maintain high rates of growth for an extended period (Russell and Hespell, 1981). Mandels (1965) stated that exponential growth does not normally occur in fungi, although there appear to be some exceptions (Plomley, 1959 cited by Mandels, 1965). The reason for an exceptional pattern of growth of fungi is provided by Smith and Berry (1974) who stated that fungi differs from bacteria in this manner because growth in fungi is restricted to the apical region of the filament body. Therefore, the cells produced in the previous generation which are now situated in a subapical position do not contribute to growth. With protozoa, information in this area is scarce, although Clarke (1977b) stated that rumen protozoa are highly specialized organisms that compete successfully with the large population of bacteria.

Under favourable conditions, for instance in ruminants fed large quantities of grain, the number of *Entodinium* becomes very high (Hungate, 1966). Clarke (1977b) mentioned that up to 40% of the microbial nitrogen and 60% of the total microbial fermentation products might be through protozoal activity. Whether or not rumen ciliates follow an exponential growth pattern is difficult to assess as a major

difficulty in estimating the microbial biomass in the rumen has been to distinguish it chemically from dietary organic matter (Latham, 1979). In addition, the work of Eadie (1967) has clearly demonstrated that certain species of ciliates, for example *Ophryoscolex spp.* could appear and disappear in the rumen of lambs without any explanation at the present time.

2.3.3 Cell Yields and Maintenance

It is generally agreed that on low protein diets the majority of protein which reaches the small intestine is of microbial origin (Latham, 1979). Thus, the quantitative aspects of microbial growth in the rumen are of considerable nutritional significance to the host.

The productivity of the rumen fermentation can be assessed in terms of microbial cell synthesis, with the possibility of splitting this parameter into subunits according to individual cell types (Hungate, 1966). In general, energy (ATP) is the first limiting factor for microbial growth and therefore energy supply and energetic efficiency of growth become important (Tamminga, 1978) in determining the balance of end products.

The early work of Bauchop and Elsden (1960) concluded that the yield of bacterial cells in grams per mole of ATP produced (defined as Y_{ATP}) was 10.5. However, the value of Y_{ATP} could be as high as 32.5 for growth of an organism in a complex medium, as has been calculated by Stouthamer (1973 and 1979 cited by Russell and Hespell, 1981). Bergen and Yokoyama (1977) also stated that the value of Y_{ATP}^{MAX} was apparently around 25, although this value is largely dependent on the growth rate of the microorganisms. Y_{ATP} as has been reported by those authors, can be lower than 10 in slow-growing cultures but it may approach the Y_{ATP}^{MAX} of 25 at high growth rates. Nevertheless, since the factors that affect the efficiency of microbial growth in the rumen are so numerous and complex, it is not surprising that Y_{ATP} varies considerably.

Hespell and Bryant (1979) stated that at low bacterial growth rates, the majority of the ATP produced by fermentation is used for maintenance resulting in low cell yields. Therefore, such manipulation of rumen fermentation which reduces the maintenance ATP requirements of the population of microbes is advantageous, especially where animals depend largely on microbial protein supply for their essential amino acids for tissue growth and wool production.

Several factors have been recognized to influence the maintenance requirement of microbial cells, including conditions of growth (e.g. excess or limited substrate availability), osmolarity, redox state, the presence of growth inhibiting substances and unfavourable shifts in the ionic composition in the culture media (Stouthamer and Beltenhaussen, 1973 cited by Bergen and Yokoyama, 1977). Leng (1981) has emphasized the effects of protozoa in the rumen on the M_{ATP} requirements and the estimated Y_{ATP}. Protozoa have been shown by a number of authors (Weller and Pilgrim, 1974; Bird *et al.*, 1978; Harrison *et al.*, 1979; Leng, 1982) to be preferentially retained in the rumen, and in the absence of protozoa the microbial protein flow to the duodenum was significantly increased (Lindsay and Hogan, 1972; Veira *et al.*, 1983; 1984). In addition, the engulfment of bacteria (Coleman, 1975) and fungal zoospores (Orpin, 1975) by protozoa suggests a low rate of microbial protein synthesis and flow rate to the small intestine of faunated animals.

From the evidence presented, it seems likely the M_{ATP} of protozoa may be high as they move continually, their apparent retention in the rumen is extended and their numbers are some times very large (Leng, 1981).

Estimates of Y_{ATP} for other rumen microorganisms is lacking. Nuzback and co-workers (1983) have recently reported that protozoa contribute up to 95% of the rumen fluid ATP concentrations of cattle fed 50:50 alfalfa concentrates. It appears that the supply of microbial cells to the host animal is greatly reduced in the faunated compared with the defaunated animals for a number of reasons mentioned above with respect to the common behaviour of protozoa in the rumen.

Wool growth which is very sensitive to the supply of amino acid in the small intestine is highly likely to be affected by the absence or the presence of protozoa. Wool growth increases in defaunated animals fed on high-energy low-protein diets by Bird *et al.* (1979). In the absence of ciliate protozoa, the contribution of flagellates, some of which have been reported by Orpin (1975, 1976a, 1977b) to be the fungal zoospores, to Y_{ATP} may be quite significant as their numbers are generally increased (Orpin, 1984). Although the numbers of these microorganisms were reported to reach up to 1×10^4 per ml of rumen contents (see Warner, 1962), Nuzback *et al.* (1983) have found in the defaunated state of the rumen, their numbers reached 1.3×10^6 per ml of rumen fluid. This number is in agreement with the report of Orpin (1976a) who stated that in the defaunated sheep the number ranged from 3.9×10^5 to 2.2×10^7 per ml, while in the normally faunated animals it ranged from 4.2×10^3 to 6.4×10^5 per ml. Nuzback *et al.* (1983) estimated the contribution of flagellates to the microbial ATP in the defaunated animals was 0.6 µg ATP per ml where the number of flagellates was equal to 47.8×10^4 per ml of rumen contents. However, since this value was obtained on the basis of the ATP concentrations of the rumen fluid, the real value of Y_{ATP} of rumen anaerobic fungi may be difficult to assess. This is mainly due to the fact that the life cycle of rumen fungi is divided into two stages: a motile stage which occurs in the rumen fluid followed by rapid attachment to the feed particles resulting in the growth of sporangia (a vegetative-reproductive stage) (Orpin, 1975, 1977a).

The relative contribution of fungal protein to the microbial protein supply to the host animal may be small as most of the nitrogen is bound in the chitin which is likely to be unavailable to the host animal when the microbial biomass is subjected to digestion in the abomasum or lower alimentary tract (Orpin, 1981a). Thus, it seems likely the major role of rumen fungi is confined in the rumen to breakdown of the fibrous materials rather than providing protein for the host.

2.3.4 Rumen pH

Evidence that ruminants have only a limited ability to control rumen pH has been demonstrated by Schwartz and Gilchrist, (1975);

Russell and Hespell (1981). Many factors influence rumen pH, including availability of food (Hungate, 1966) a low rumen pH is associated with an accumulation of lactic acid in the rumen (Russell and Hespell, 1981). Lactic acid-utilizing bacteria which may be as high as 10^8 per ml or more, cannot metabolize lactate fast enough to prevent an accumulation in the rumen (Schwartz and Gilchrist, 1975). Kaufmann *et al.* (1980) reviewed the changes in the microbial composition dealing with the changes of rumen pH. Reducing rumen pH from 7.0 to 5.5 which is generally associated with the involvement of some grain in the diet has a detrimental effect on cellulolytic bacteria. Under these conditions amylolytic bacteria become a predominant species in the rumen.

The rumen protozoa are generally more sensitive to pH changes than bacteria, and the first organisms to be influenced by an increase (Clarke, 1977b) as well as a decrease (Leng, 1976) in acidity. Hungate *et al.* (1964) reported that at rumen pH 5.3 the numbers of rumen protozoa were 3×10^5 per ml, and on the same diet at pH 5.9 their numbers were increased to 6.2×10^5 per ml. An increase of rumen pH above 7.8 (Myburgh and Quinn, 1943 cited by Clarke, 1977b) and lowering rumen pH below 5.5 (Hume, 1976) had been reported to inhibit and to kill the rumen protozoa.

The recent discovery of rumen fungi by Orpin (1975) has opened the way to more detailed study of these organisms in relation to the microbial environment of the rumen. From a number of studies carried out by Orpin (1975, 1976a, b, 1977b) it was concluded that the activity of rumen fungi is optimized at rumen pH 6.0 - 7.0. There is no evidence to show that rumen fungi are inhibited or killed at rumen pH below 5.5 or above 7.0. However, Orpin (1976b) reported it to drop in *Piromonas communis* as low as 25% when rumen pH was altered to 5.5 or 8.0.

The effect of rumen pH is not only confined to the composition of rumen microbes, but the rate of absorption of the fermentative products (e.g. VFA) is also altered by changes of rumen acidity.

Kaufmann *et al.* (1980) showed that the rate of absorption of the VFA is increased with a greater proportion of undissociated acid molecules. In general, decreasing rumen pH narrowed, and increasing pH widened the ratios of acetate:propionate (Chalupa, 1977). However, VFA concentrations were not apparently altered at rumen pH between 6.2 and 6.8 but they were influenced at pH between 5.6 and 6.2 (Esdale and Satter, 1972). From evidence presented above, there is no doubt that the environmental pH can affect both the types of microbes and their products in the rumen. Thus, regulation of ruminal pH may be important in manipulation of ruminal fermentation.

2.3.5 Cell lysis

As has been suggested by Hungate (1966), under certain conditions of limited nutrients such as starvation, the rumen microorganisms start to lose their ability to ferment a substrate even after only relatively short periods without food. The potential growth capacity of rumen bacteria decreases and is low when the nutrients are again made available (Russell and Hespell, 1981). In the absence of substrate, it was reported by Hespell (1979) that about 60% of rumen bacteria died and about 30% were lysed shortly. This suggests that adaptation for a certain period of time is important when study of rumen microorganisms requires the starvation of animals for certain reasons such as defaunation.

2.3.6 Predation by protozoa

There appears to be little doubt that the presence of protozoa in the rumen is not essential to ruminants (Hungate, 1966). In the past 50 years, there have been a number of conflicting results on the effect of the absence or presence of rumen protozoa on the growth of ruminants under different feeding regimens as reviewed by Coleman (1979), Demeyer (1981), Bird and Leng (1983) and Leng (1984).

Predation of microbes by protozoa is well documented (see Coleman, 1975; Orpin, 1975). In a recent review Hobson and Wallace

(1982) stated that in very active conditions, the potential increase in bacterial population could be nullified due to predation by protozoa. An example of reducing the number of bacteria in the rumen by engulfment of protozoa is provided by Russell and Hespell (1981); rates of engulfment range from 130 to 21200 bacteria/protozoon/h at bacterial densities of 10^9 cells/ml (see also Coleman, 1975). Intracellular digestion rates of bacteria range from 345 to 1200 bacteria/protozoon/h, and in a sheep's rumen, with high protozoal concentrations (10^6/ml), approximately 2.4 - 45g per day could be digested by protozoa. Moreover, selective engulfment of bacterial species *in vivo* was lead to altered ruminal fermentation patterns. Nevertheless, the effect varies markedly as this is dependent on numbers as well as species of protozoa.

Considering the preferential retention in the rumen in one respect, and the predation on other rumen inhabitants, in other respects, several authors have concluded that a large protozoal biomass reduces bacterial protein leaving the rumen (see Leng, 1976; 1981, Leng, Bird and Burgraff, 1980; Bergen and Yokoyama, 1977; Owens and Isaacson, 1977). This opinion is supported by recent evidence provided by Bird and Leng (1983) who reported their 6-year research programme designed to investigate the effect of defaunation on sheep and cattle production. The absence of protozoa from the rumen, has increased the availability of protein for digestion in and absorption from the intestines. The effect of defaunation on fibre digestion is relatively known (see Demeyer, 1981). Despite the extent of predation on cellulolytic bacteria and fungi by protozoa, Kurihara, *et al.* (1968) have stated that the bacterial metabolism was stimulated due to predatory action resulting in higher digestibility of fibrous materials in the rumen (see Demeyer, 1981). This opinion is also supported by a number of authors (see for example Prins and Van Den Vörstenbosch, 1975; Kurihara *et al.*, 1978; Jouany and Senaud, 1979). Unfortunately, the reason for such stimulation remains unclear as has been stated recently by Hobson and Wallace (1982).

From the evidence presented, it is clear that the major effect of removing protozoa from the rumen should be an increase in bacterial-protein availability as a large-protozoal biomass causes a high M_{ATP}

and a low Y_{ATP} (Leng, 1981). Moreover, with regard to predatory action by protozoa, a high bacterial-cell turnover may occur. Apart from the arguments mentioned, the predation by protozoa on rumen fungi may result in a detrimental effect on fibre digestion, especially when animals are fed on high-fibre diets, as rumen fungi have been shown to be cellulolytic microorganisms (Orpin, 1981a). Thus, there is no doubt the effect of predation by protozoa on other rumen inhabitants is dependent on complex conditions with the major aspect being dietary conditions.

2.3.7 Rumen dilution rates

Rumen fermentation can be likened to a continuous, anaerobic microbial-culture system in which there is a more or less continuous substrate supply, end-product removal and a buffering system to keep the fermentation active (Hungate, 1966; Bergen and Yokoyama, 1977), however Bergen (1979a) argued that ruminal fermentation does not strictly resemble a continuous culture under typical production conditions because the fermentation is not in a steady state and is characterized by wide fluctuations in microbial cell and substrate concentrations. Suporting the latter authors, Russell and Hespell (1981) stressed that in the rumen, most nutrients are supplied in an insoluble form, and nutrient addition is discontinuous with meals, which clearly differ from a continuous culture. Thus, the rumen does not operate as a homogenous system because there are, at least, two major dilution processes occurring in the solids and the liquid phases (Russell and Hespell, 1981).

Several factors have been associated with the changes of liquid dilution rate in the rumen such as inclusion of mineral salts in the diet (Rodgers *et al.*, 1979), and infusion of artificial saliva (Harrison *et al.*, 1975). It is well recognized that alteration of dilution rates causes changes in rumen fermentation patterns (Harrison *et al.*, 1975; Chalupa, 1977) with the most marked change in this fermentation shift appearing to be reduction in the molar proportion of propionic acid (Russell and Hespell, 1981). In addition, the growth rate of rumen bacteria is a function of dilution rate (see Bergen and Yokoyama, 1977; Hespell and Bryant, 1979).

An increase in dulution rates has been associated with an increase of microbial cell yields in the rumen (Harrison and McAllan, 1980) even though the improvement in microbial growth declines rapidly with increasing dilution rate and little or no improvement occurs above a rate of 0.1. Moreover, Leng (1981), who summarized the recent work in this area, suggested that although theoretically dilution rates alter the microbial cell-yields, under practical conditions, there appears to be no measurable increase in cells flowing to duodenum occurs with increasing dilution rate of rumen contents in cattle and sheep. In addition, Russell and Hespell (1981) indicated that it is still difficult to associate an increase of microbial growth rates when dilution rates increase, since a complex of compensated factors is involved. These include the contribution of dead cells of bacteria which wash out the rumen and the possibility of a reduction in protozoal predation on the bacteria. The factors mentioned contribute to the increased availability of microbial protein to the animal.

2.3.8 Availability of other factors

There is evidence that the efficiency of growth of rumen microbes are limited by the availability of certain substances in the rumen such as vitamins, inorganic compounds or toxic compounds. Within the rumen some bacteria are probably growing on a carbohydrate-vitamin-fatty acids-inorganic salts type of medium with ammonia as N source, others,for instance cellulolytic bacteria, are growing on simple media composed of carbohydrates, certain volatile fatty acids, some vitamins and inorganic salts. Maeng *et al.* (1976) emphasized the importance of amino-acid availability for the microbial growth and possibly other unidentified co-factors needed for optimal cell synthesis. This is particularly relevant when low-protein diets incorporating NPN are fed to ruminants. Hume and co-workers (Hume *et al.*, 1970; Hume, 1970a, b; Hume and Bird, 1970) showed that microbial protein synthesis in the rumen of sheep was increased by a number of factors including (1) increasing the level of nitrogen intake, (2) the addition of a mixture of higher VFA (3) the addition of amino acids as casein and zein in the diet, and (4) raising the sulphur intake to 1.95 g per day.

The understanding of sulphur requirements bacterial protein synthesis is greater than for the other rumen microorganisms. Recently, Akin *et al.*(1983) and Gordon *et al.*(1983) have shown that the growth of fungi is low in the rumen of sheep fed low-sulphur diets. Addition of methionine to the diet clearly increased the the number of fungal zoospores in the rumen (Gordon, *et al.* 1983).

With the increased knowledge of fermentation pathways in the rumen, there appears to be considerable scope for the manipulation of fermentation to improve the efficiency of the rumen ecosystem of the animal. Chemical substances commonly used for manipulation can be classified into seven main groups: (i) propionate enhancer (ii) methane inhibitor, (iii) protease/deaminase inhibitor, (iv) urease inhibitor, (v) deaminase inhibitor, (vi) dilution rate enhancer (no pH effect) and (vii) pH regulator (Chalupa, 1977; 1980; Bergen, 1979b). Monensin, a propionate enhancer, has been reported to have no effect on the numbers of protozoa, total bacteria, and cellulolytic bacteria when up to 33 ppm of this material was fed to steers in a forage diet (Dinius, *et al.* 1976). Similarly Leng, *et al.*, (1984) have reported that the protozoa were not significantly affected by the inclusion of monensin in the diet of sheep. Methane inhibitor, on the other hand, was reported to have impaired microbial cell yields (Chalupa, 1980) which is apparently related to the result of Hungate (1970) who reported that in the presence of hydrogen in a batch culture which contained glucose, the growth of rumen cellulolytics was decreased. This evidence suggests that methane formation is a means of hydrogen disposal in the rumen which increases rather than decreases the ATP available (Hungate, 1966). Other rumen-fermentation manipulators have been reviewed extensively by Chalupa (1977; 1870).

More recent information provided by Fonty *et al.*(1983) revealed that apart from the essential requirement for growth, the establishment of cellulolytic bacteria and protozoa in the rumen depends on the presence of other micro-organisms, requires an abundant and diversified surrounding flora and is favoured by early animal innoculations.

2.4 The Rumen Anaerobic Fungi

2.4.1 General

Clarke (1977a) divided the rumen fungi into two major groups, yeasts and moulds. Many of them are present in the rumen only as transients since many yeasts can only be found in low numbers (Lund, 1974); also considerable numbers of moulds are known to enter into the rumen continually but are unable to grow anaerobically (Clarke, 1977a). Therefore, they will not be included in this review.

Other groups of fungi which resemble protozoan flagellates were considered as a rumen phycomycete for the first time by Orpin (1975). However, not all rumen flagellates are rumen phycomycetes; as indicated in a recent publication, some flagellates are still classified as rumen protozoa (Ogimoto and Imai, 1982) and among these microorganisms there are only five species which have been shown to be genuine fungi (Orpin, 1971). In this thesis, the term rumen-anaerobic fungi means the rumen phycomycetes.

The unique characteristics of rumen fungi which differentiate them from all other fungi are, their requirement of strict-anaerobic conditions for growth (Orpin, 1975; 1976a; 1977a, b; Munn, Orpin and Hall, 1981, Heath *et al.*1983). In addition, phycomycetes, unlike other rumen inhabitants, require two stages in their life cycle, that is, a motile zoospore stage and a vegetative-reproductive stage, which occur in liquid medium and plant tissues, respectively (Orpin, 1981) (see Figure 2.2).

The time span of the life cycle of these phycomycetes in the rumen was reported in the order of 24-30 h (Bauchop, 1981; Joblin, 1981). The morphology of the fungi varies in shape both in the generative and vegetative stages. The zoospores of the three-known species, *Neocallimastix frontalis*, *Piromonas communis*, and *Sphaeromonas communis* vary in shape (see Table 2.1).

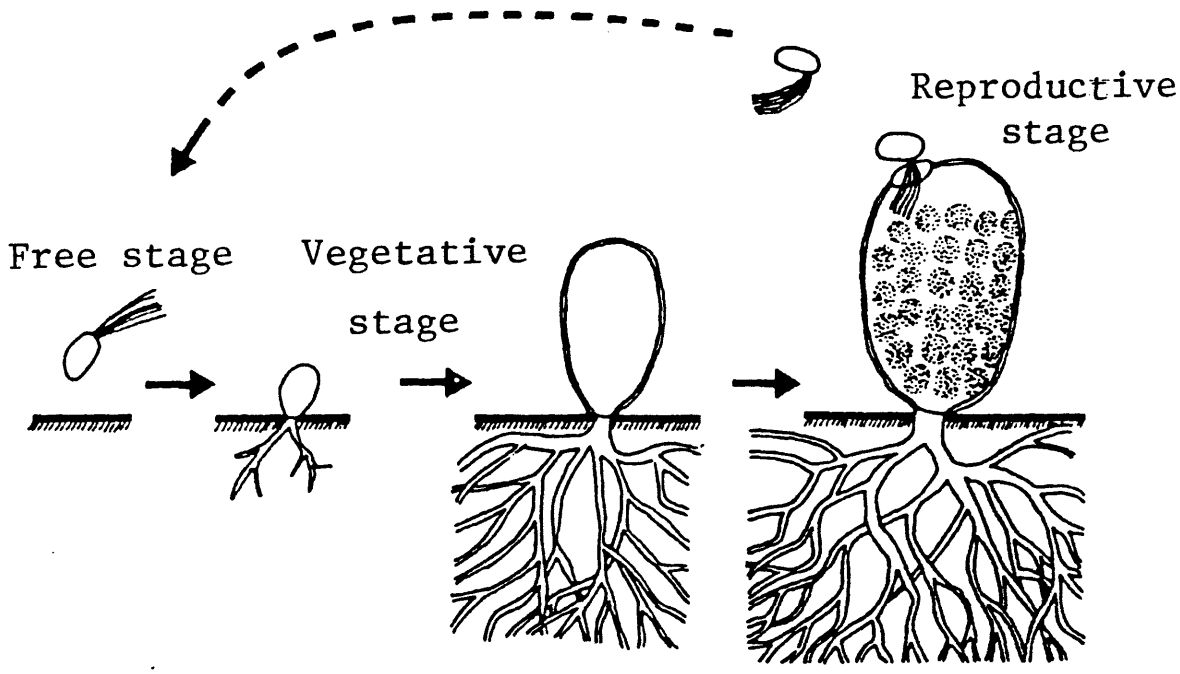


Figure 2.2 Life cycle of rumen-anaerobic fungi (redrawn from Bauchop,1981)

Table 2.1 Morphological variations of rumen-anaerobic fungi commonly found in the rumen (summarized from Orpin, 1975 ; 1976a, c ; 1981a)

Characteristic	Species				
	1	2	3	4	5
a. Zoospores :					
- shape	variable but normally bean shape	pyriform or elongate	spherical to ovoid	n.i	small
- axial dimension (um)	20.6 X 8.7	7.1 X 14.6	9.23 X 24.9	n.i	4.5 X 3.0
- number of flagella	up to 14	single	single	single	single
- position of flagella	anterior region of the straighter of the two sides of the cell	posterior	posterior	n.i	n.i
- motility	erratic and	amoeboid	n.i	n.i	n.i
b. Sporangia :					
- size (um):					
under *in vivo* conditions	varies from 21 X 9 to 74 X 52	n.i	n.i	n.i	n.i
under *in vitro* conditions	up to 155 X 83 with rhizoid up to 1380 in length	up to 89 X 45 with rhizoid up to 365 in length	up to 95 X 64	n.i	up to 45 in diameter.
- shape	variable depending on the carbon source for growth	cylindrical to ovoid	variable but usually ovoid	variable but usually ovoid	spherical
- rhizoid systems	single and branches	single,non-septate highly-branched rhizoid	short-thick single and sometimes branches	thallus commonly bore 2 and up to 4 sporangia	single highly branched-rhizoid

1 = *Neocallimastix frontalis*; 2 = *Piromonas communis*; 3 = *Sphaeromonas communis*; 4&5 = unidentified species

n.i = no information

As reported by Bauchop (1982), these fungi are found in the gut of a wide range of herbivorous animals, being detected in the gut of ruminants, horses, elephants and the kangaroo.

With regard to the ability of rumen-anaerobic fungi to digest structuralplant polysaccharides, several authors have reported that a wide range of plant polysaccharides, including cellulose, hemicellulose, starch, xylan and lignin, are utilized by these organisms for growth (Orpin and Letcher, 1979; Orpin, 1984; Gordon and Ashes, 1984) under *in vitro* conditions. Unfortunately, such information under *in vivo* conditions is scarce.

2.4.2 Classification

The affinity of rumen-anaerobic fungi to certain taxonomic position is still unclear (Heath *et al.*, 1983). From a number of studies, Orpin (1975; 1976a; 1977) has demonstrated that the three species of rumen flagellates: *N. frontalis*, *S. communis* and *P. communis* are the zoospores of a phycomycete fungus. This opinion is supported by evidence provided by a subsequent study which observed that the cell walls of those three species contain a chitin (Orpin, 1977b) which is specific for Chytridiomycetes (Bartnicki-Garcia, 1968). However, despite a close resemblance of these fungi to the characteristics of aquatic Phycomycetes as described by Sparrow (1960), the number of flagella found in the rumen has definitely posed a question on their position in the existing taxonomy. Therefore, it is not surprising that Barr (1980) stated that the present systems of classifying the Chytridiales are still far from satisfactory due to morphological variations.

Earlier authors such as Sparrow (1960) still consider the concept of operculation, methods of development and thallus structure, the number and position of flagella to determine the orders of Phycomycetes. In his classic book, he divided the zoospores of Phycomecetes in terms of the number of flagella into two: uniflagellate and biflagellate zoospores.

Recently, Barr (1980) has revised the concept of classification of the Chytridiales on the basis of zoospores ultrastructure which allows the accommodation of *N. frontalis* which produces polyflagellate zoospores into the family *Neocallimasticaceae* in the order *Spizellomycetales* (Heath *et al.*, 1983).

The other known species of rumen fungi can be tentatively classified into Chytridiales on the basis of their uniflagellate even though their family's names cannot be given at the present time since the species names of *P. communis* and *S. communis* were originally given to the flagellated protozoa found in the rumen by previous authors (see Orpin, 1976a; 1977c).

Table 2.2 A tentative classification of rumen-anaerobic fungi commonly found in the rumen

Kingdom	:	Mycetae	
Division	:	Mastigomycota	
Class	:	Chytridiomycetes	
Order	:	1. Chytridiales	2. Spizellomycetales
Family	:	?	Neocallimasticaceae
Genus	:	a. Sphaeromonas	Neocallimastix
		b. Piromonas	
Species	:	a. Sphaeromonas communis	Neocallimastix frontalis
		b. Piromonas communis	

2.4.3 Factors influencing fungal population densities in the rumen

i) Enumeration of rumen fungi

Although a number of studies have been conducted to elucidate the relative importance of these microorganisms in the digestion of fibre in the rumen, in no single sutdy has the successful quantification of the fungi been accomplished either under *in vitro* or *in vivo* conditions. In early work Orpin (1974; 1975; 1976a; 1977b) used direct microscopic enumeration of the live zoospores and also sporangia on leaf blades. It is difficult to determine accurately the number of

zoospores in the rumen fluid using the Orpin's method since it often depends on the movement of flagella of zoospores which is in some species the flagella become inert very quickly resulting in the chages of morphology (Orpin,1981a). In addition, in faunated animals, the accuracy of this method is largely influenced by the movement of protozoa.

Bauchop (1979a), Joblin (1981) and Akin *et al.* (1983) used a culture technique on the basis of the method developed by Hungate (1969) to enumerate the number of zoospores in the rumen. This method has several advantages over Orpin's method in which the fungal zoospores are enumerated in fresh rumen fluid microscopically, the results are in good agreement with the estimated zoospore population reported in the literature; the presence of protozoa in rumen fluid used as a source of inoculum does not influence the enumeration of fungal zoospores; and the culture can be stored for long periods. However, the accuracy of the culture method is sometimes reduced by overgrowing of the colonies which produce a mycelium; poor results generally appear as the dilution factor of inoculum increased. Determination of fungal biomass in the rumen on the basis of the numbers of live zoospores is not advisable as they cannot serve this purpose due to their rapid attachment to plant fragments (Bauchop, 1979a).

The numbers of sporangia on the feed particles have been enumerated within a defined area of a light microscope by Bauchop (1979a) and Akin *et al.* (1983) after the feed particles have been stained with a particular dye. This method is more meaningful since the sporangia which are found to attach to plant tissues indicate by their activity that digestion is in progress. Unfortunately, the surface area of plant materials used as a medium is not colonized evenly by rumen fungi resulting in a large variation within and between samples.

Recent findings of Orpin (1977c) on the occurrence of chitin in the cell walls of rumen fungi prompted him to use chitin as an indicator to determine the biomass of rumen fungi. This was estimated to be as high as 8% of the total microbial biomass in the rumen (Orpin, 1981a). The accuracy of this method is probably affected

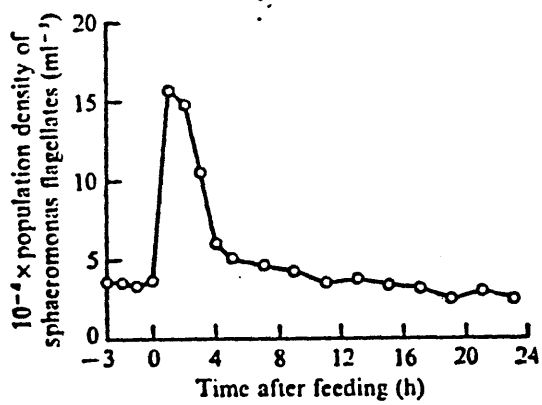
by the bacterial wall components as well as by the amount of chitin in the fungal walls and the amount of fungus in the digesta. More information in this area is obviously awaited with interest.

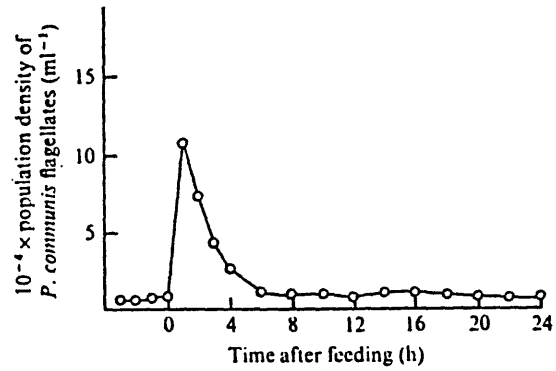
Another possibility for quantifying fungal biomass in the rumen is to determine the concentration of the total rumen cis, 24:1(Δ^{15}) fatty acid and triterpenol, as done by Kemp *et al.* (1984). It was shown by these authors that the rumen fungi contained these chemical substances. Nevertheless, they found some two-fold variability of the C_{24}: content of the fungi even when they were grown under laboratory conditions. The variations might be from dietary sources; if this is so, the problem may be eliminated by the use of specific ion monitoring by mass-spectrometry as suggested by Kemp *et al.* (1984). In conclusion, the importance of the rumen fungi cannot be assessed by enumeration of any stages of their life cycle (Bauchop, 1979a) until an appropriate method is developed to quantify their biomass in the rumen.

ii) Diurnal fluctuations

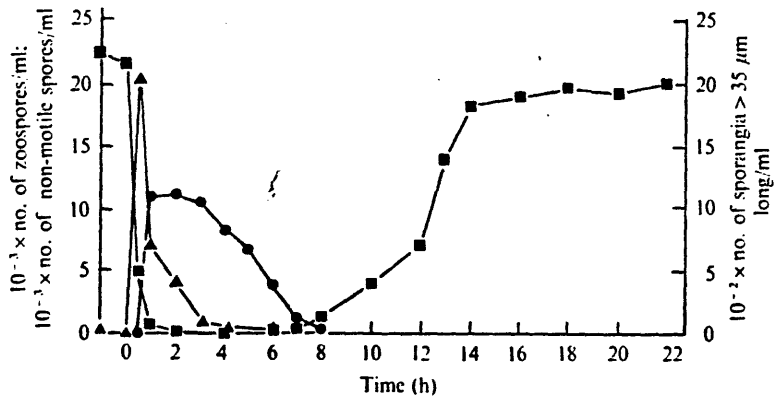
Among the known species of rumen fungi, only the diurnal variations of the three species *N. frontalis, P. communis* and *S. communis* have been studied (Orpin 1975; 1976a; 1977b). Depending on the individual species, the peak production of fungal zoospores occurs between 15 and 60 minutes after feeding. However, there is not doubt that animal variations also determine the fluctuation in the number of fungal zoospores.
Figure 2.3 shows the fluctuations of fungal population density of the three known species in the rumen of sheep (adapted from Orpin, 1975; 1976a and 1977b).

Despite the intimate association of the vegetative stage of rumen fungi with slowly-degraded materials in the rumen, Bauchop (1979a) observed a low number of sporangia attached to the wheat straw used in his experiment after 24 h, and the big sporangia were found after rumen incubation periods of 2 to 4 days. In contrast, large numbers of spherical to ovoid bodies were found attached to exposed vascular cylinders of lucerne stems suspended in the rumen of sheep at 2 h (Bauchop, 1979a).





Neocallimastix frontalis



Typical population density curves for various phases in the life history of *N. frontalis*. ■, Sporangia > 35 μm long; ●, non-motile, non-flagellated cells and sporangia < 35 μm long; ▲, motile neocallimastix cells. Inducer from 300 g crushed oats was added to the rumen at zero time to synchronize the growth of the neocallimastix.

Figure 2.3 Fluctuation of fungal population density of the three-known species in the rumen of sheep (adapted from Orpin, 1975; 1976a and 1977b)

iii) Influence of the diets

As has been suggested by Bauchop (1979a), the rumen fungi are closely associated with the more slowly digested fractions of plant tissues, and together with the results of Orpin's work (1976a,b; 1977a,b) on substrate preferences by the fungi, the population of fungi in the rumen is largely influenced by the nature of the diets consumed by the host animals. Orpin (1977d) found indications that zoosporogenesis in the rumen was induced by the plant components which were, in some plants, principally present in the leaves and aerial tissues with no apparent taxonomic relationship. The inducers were probably simple sugars, common amino acids or fatty acids.

A high population of rumen fungi were found in sheep and cattle when the diets were chaffed lucerne and meadow hay, respectively (Bauchop, 1979b); also a very high number of fungi were found in rumen of animals grazing stalky pastures such as perenial ryegrass (*Lolium perenne*, L.) compared with sheep grazing on soft, leafy diets such as a pure stand of young lucerne, red clover (*Trifolium pratense*, L.) or white clover (*Trifolium repens*, L.).

The influence of diet upon the numbers of rumen fungi is not fully understood under *in vivo* conditions, although under *in vitro* incubations, Orpin (1977d) has demonstrated that the increment of oats from 1.6 mg to 3.1 mg per ml of rumen fluid resulted in the increase of the numbers of *N. frontalis* zoospores.

iv) Rumen pH

The acidity of the rumen is probably the most important factor governing the changes in fungal populations. The absence of rumen fungi in animals fed on high-concentrate diets (Bauchop, 1981) may provide a good example of the relationship between the fall in rumen pH which is generally associated with the inclusion of a high proportion of grain in the diet, and the survival of the fungi in the rumen.

The zoosporogenesis of the three known species *N. frontalis*, *P. communis*, and *S. communis* has been demonstrated by Orpin (1975; 1976a; 1977b) to reach a maximum rate of rumen pH 6.5. However, an increase in rumen pH up to 7.5 had little effect on the rate of zoospore production by these three species of fungi (Orpin, 1975; 1976a). In contrast, *P. communis* was shown to be very sensitive to changes in rumen pH. The rate of production of *P. communis* zoospores diminished rapidly at pH below 6.0 and above 7.0 (Orpin, 1977b).

v) The presence of toxic substances

Some antibiotics and anti-fungal agents have been demonstrated by Orpin (1975; 1976a; 1977b) to inhibit zoospore production by some species of rumen fungi. Polymixin B and cytochalasin B are the antibiotics which were shown by Orpin to impair the production of zoospores of *N. frontalis* and *P. communis*. The effect of such antibiotics as polymixin B on the genesis of rumen fungi is probably through blocking or reducing the cation binding to the fungal cell walls (Burnett, 1976).

Actidione (cyclohexamide), an anti-fungal agent, has also been shown by Orpin (1975; 1977b) to inhibit completely the growth of rumen fungi at a very low concentration. Cyclohexamide (a protein synthesis inhibitor) is known to inhibit the incorporation of L-alanine into protein in Basidiomycetes (Burnett, 1976). However, in other organisms this compound is believed to inhibit the transfer of activated amino acids to ribosomes (Niederpruem, 1964 cited by Burnett, 1976), causing the premature release of polypeptides from ribosomes.

vi) Microbial interactions

Leng (1984) has recently discussed the occurrence of microbial interactions within the rumen. The existing interactions between bacteria and fungi in the rumen apparently vary between competition and synergism. For instance, the number of rumen fungi is increased in the presence of antibiotics in the medium to control the bacteria. In contrast, Orpin (1981b) reported that the activity of zoospore inducer

was rapidly destroyed by the rumen bacteria but relatively little destruction occurred by protozoa. Therefore, it seems reasonable to assume that rumen bacteria and fungi compete for substrate and/or essential nutrients. Another kind of relationship between these microorganisms is synergism which has recently been demonstrated by Bauchop and Mountfort (1981) and Mountfort, Asher and Bauchop (1982). It was shown by these authors that the intermicrobial hydrogen transfer occurred between rumen-anaerobic fungi and rumen-methanogenic bacteria. A synergistic relationship between bacteria and fungi in fibre digestion is also postulated by a number of authors; rumen fungi penetrate deeply into plant tissues, enabling the extracellular enzymes of cellulolytic bacteria to contact plant polysaccharides prior to digestion (Bauchop, 1982).

With protozoa such intermicrobial relationships as reported between bacteria and fungi are not clear. Orpin (1975) reported that predation by the protozoon *Entodinium spp.* of the zoospores of *N. frontalis* occurred *in vitro*; this seems likely to also occur *in vivo*. There is ample evidence to show that fungal zoospores (some of which had been believed to falgellated protozoa) increase in numbers in the rumen of defaunated animals (Eadie and Gill, 1971; Orpin, 1976a). Under these conditions, Leng (1984) suggested that protozoa either compete for essential nutrients or prey upon fungi.

2.5 Recapitulation

Fibre digestion in the rumen involves complex relationships between host animals and the microorganisms which ferment fibrous materials into products which are available for further digestion and absorption by the host animal. Although rumen-anaerobic fungi have only recently come to our attention since their discovery by Orpin (1975), there is no doubt that these organisms are highly cellulolytic and able to digest a wide range of plant polysaccharides. The quantitative significance of these microorganisms is still unclear because no suitable method has been established to quantify their biomass in the rumen. However, in the absence of rumen ciliates the

numbers of fungi zoospores increase markedly (Orpin, 1976a) and from the report of Orpin (1975) who showed the rumen protozoa preyed on the fungal zoospores, it seems likely that the role of rumen fungi in fibre digestion is reduced in faunated animals because of predation by protozoa. On the other hand, there is no strong evidence for a significant in fibre digestion (see for example Delfosse-Debusscher *et al.*, 1979) even though some authors (see for example Demeyer, 1981, Kayouli *et al.*, 1984) have reported that, in general, overall fibre digestibility is impaired in the absence of protozoa. However, the earlier work reviewed by Demeyer (1981) and the recent work of Kayouli *et al.* (1984) was based on high-concentrate diets. There are no reports of the effects of defaunation of sheep fed high-fibre diets on the rumen anaerobic fungi. The project described in this thesis is therefore based on this aspect of rumen microbiology with the view to enhancing our ability to effectively use high-fibre materials in ruminant diets.

CHAPTER III

GENERAL MATERIALS AND METHODS

3.1 Management of experiment animals

The design of each experiment, diets and feeding procedures are described in the relevant experimental sections. Sheep used in all experiments had been in the animal house for at least a month before the experiments were begun. In the experiment reported in Chapter VI; both defaunated and faunated animals were held in the same animal house, but the defaunated sheep were in a separate room. All animals were held in individual pens and had access to water at all times.

The defaunated animals were always handled first to minimize the risk of re-infection with protozoa.

3.2 Parasite control

The animals were subjected to a drenching programme which had previously been proved to be effective in eliminating internal parasites. The programmes used were the following drenches: Ranizole (Merck, Sharp & Dohne, Australia Pty. Ltd.), Ranide (Merck, Sharp & Dohne) and Nilverm (ICI, Australia, Ltd.) given at 4d intervals. Animals were dosed according to the manufacturers recommendation.

3.3 Surgical methods

Rumen cannulae were inserted into sheep using the method of Hecker (1969) at least five months before the experiments were commenced.

3.4 Experimental methods

3.4.1 Defaunation

An anionic detergent, Alkanate 3SL3 (ICI, Australia, Ltd., active ingredient: Sodium Lauryl Diethoxy Sulphate) was used to defaunate the rumen of the sheep. Animals were given 125 ml of a

10% solution of Alkanate 3SL3 intraruminally via the cannula on each of three consecutive days. Feed was withdrawn one day before and on the day of treatment.

3.4.2 Refaunation

Animals were refaunated by inoculating with rumen digesta from sheep with a high population density of protozoa. Approximately 100 ml of rumen digesta was given on each of three consecutive days. Data collection commenced only when the number of protozoa had reached between 10^4 and 10^5/ml rumen fluid. The time required for complete refaunation was usually 7-10 days.

3.4.3 Collection of samples *in vivo*

a. Rumen fluid

Rumen fluid samples were collected *per fistulum* using a sampling probe positioned in the dorsal sac of the rumen. The probe consisted of a metal cage covered with nylon gauze and was connected to the cannula plug by a length of plastic or metal tubing. Subsamples of rumen fluid were taken for the following determinations:

i) Rumen pH: The pH of a 20 ml sample of rumen fluid in a McCartney bottle was measured with a pH meter.

ii) Rumen ammonia and VFA concentrations: A 10 ml sample of rumen fluid in a McCartney bottle was acidified with approximately 5 drops of concentrated H_2SO_4 and stored at -20°C until required for analysis. These were thawed and and centrifuged at 3000 rpm for 15 min. The supernatants were analysed for NH_3 concentration and for total VFA and individual acid proportions.

iii) Number of protozoa: Five ml of rumen fluid was added to 20 ml of formol saline 0.9% (W/V) NaCl in a 10% (V/V) solution of 40% formaldehyde in a McCartney bottle. Subsamples for protozoa counts were taken while mixing using a pasteur pipette and placed in a 0.2mm deep counting chamber (Hawksley, Sussex, England). The counting procedure has been described by Warner (1962).

b. Faeces

In digestibility experiments faeces were collected daily, weighed, sampled and stored at -20°C until required for analyses. Samples were thawed and subsamples were taken for dry matter (DM) and organic matter (OM) determinations.

3.4.4 Measurement of the digestibility of feedstuffs

Three different methods were employed in the present studies to determine the rate of disappearance of feedstuffs (using a nylon bag technique, Ørskov *et al.*, 1980) and the digestibility of feedstuffs (*in vivo* and *in vitro*).

a. Nylon bag technique

The nylon bag technique involves the suspension of several bags containing a known weight of sample (3-5g of air-dried material), which are then removed at timed intervals, dried and weighed. The nylon bags used in all experiments were 7 x 15cm in size, pore size 25μm unless specified otherwise. Nylon bags were weighted with a marble to prevent them floating on top of the digesta in the rumen. After removal of the rumen,the bags were washed (with gentle squeezing) under running tap water until the washings were clear. The bags were then oven dried (70°C for 24 h). The percentage of dry matter which had disappeared at the end of 6, 12, 24, 48 and 72 h was termed dry matter disappearance. In addition the undigested materials in the bags were sometimes analysed for acid-detergent fibre (ADF), based on the method described by Goering and Van Soest (1970). The calculations of ADF disappearance were similar to dry matter disappearance.

b. Digestibility *in vivo*

In the sheep in Experiment 3 (Chapter V) daily feed intake and faecal output were recorded for the last 6 consecutive days at the end of each period. The feed and feed residues were sampled for dry matter and organic matter determinations. On each morning of the collection period the faeces were weighed and about 10% of the total output was stored at -20°C. When the collection period was finished the faeces

samples were thawed and mixed in a plastic bucket and subsampled for a partial dry matter analysis. The dried faeces were then ground through a 1mm screen and oven dried (70°C for 24 h) for dry matter, followed by ashing those samples in a muffle furnace (500-600°C for 4 h) for organic matter determinations.

Apparent dry matter digestibility (DMD) and apparent organic matter digestibility (OMD) were calculated based on the formula:

$$\text{Apparent Digestibility \%} = \frac{\text{nutrient in feed (g/d)} - \text{nutrient in faeces (g/d)}}{\text{nutrient in feed (g/d)}} \times 100$$

(Harris, 1970).

c. Digestibility *in vitro*

In the experiment reported in Chapter VI, a one step digestion *in vitro* (Tilley and Terry, 1963) as used by Graham and Amon (1984) was employed followed by determination of cell wall constitutants (neutral-detergent fibre = NDF) in the undigested residue as outlined by Goering and Van Soest (1970). Feed digestibility and rate of cell wall digestion *in vitro* were determined as described by Smith *et al.* (1971).

3.4.5 Enumeration of fungal sporangia and zoospores

An estimate of fungal biomass was obtained in two ways.

a. Fungal sporangia counts by light microscopy

Leaves and stems of the feed were suspended in nylon bags in the rumen and bags were removed at intervals. Following removal from the rumen the bags were rinsed under running tap water for a few seconds to remove the digesta on the outside of the bags. The bags were then opened and plant materials were transferred to a small bottle and rinsed twice with normal saline solution (0.9% NaCl) and then fixed with 4% (Wt/vol) unbuffered formal dehyde (Bauchop, 1979a) and stored at room temperature until required for enumeration. The plant materials were then randomly subsampled and placed in a glass tube, rinsed twice with distilled water, and then stained using

lactophenol cotton blue (Curr, 1963) for approximately 60 seconds. The stain was then sucked out and plant materials were rinsed with distilled water until the washings were colourless. Three plant blades were placed on a glass slide with water before being covered with a cover slip. The materials were then observed for sporangial colonies by means of a light microscope. The sporangial colonies were counted within the defined area of the 20 x objective lens (area = 0.64 mm^2).

b. Anaerobic culturing of rumen fungi

This measurement was only done in Experiments 3 (Chapter V) and 4 (Chapter VI). The procedures, medium and antibiotics (Benzyl penicillin, 2 x 10^4 IU/ml; and streptomycin sulphate, 2 mg/ml) for culturing rumen fungi were as described by Joblin (1981) and used recently by Akin *et al.* (1983) using Hungate roll tubes.

1. Methods

1.1 Medium 10-XN + AB (Caldwell and Bryant, 1966; Joblin, 1981; Akin, 1980; Akin *et al.*, 1983).

- Composition (g/l except VFA):

Minerals:	K_2HPO_4	0.45
	KH_2PO_4	0.45
	NaCl	0.9
	$(NH_4)_2SO_4$	0.9
	$CaCl_2.2H_2O$	0.12
	$MgSO_4.7\ H_2O$	0.2
Buffer	Na_2CO_3	5.6
Redox indicator:	Resazurin	0.001
Vit., a.a. and growth factors:	haemin	0.001
	yeast extract	0.5
	trypticase	2.0

Reducing agent:		
	Cysteine-HCl	0.52
	$Na_2S.9H_2O$	0.52
Carbon and energy:		
	Glucose	0.33
	Cellobiose	0.33
	Starch (corn)	0.67
	Xylose	0.67
VFA(ml/l):	Acetic acid	1.7 ml
	propionic "	0.6 ml
	n - butyric "	0.4 ml
	i - butyric "	0.1 ml
	n - valeric "	0.1 ml
	i - valeric "	0.1 ml
	Dl-2-metyl butyric "	0.1 ml
Selective:	benzyl penicillin	1.2
	streptomycin sulphate	1.2

1.2 Preparation of medium

All ingredients except reducing and selective agents were dissolved in distilled water and boiled for 5 min. A reducing agent was added to the boiling solution and bubbled with CO_2 for 15 min. on ice. The solution was then dispensed under CO_2 to roll tubes, sealed, capped and autoclaved (120°C for 15 min.). An oxygen-free solution of selective agent was added to the cooled medium just prior to use. Individual roll tubes of medium 10-XN contain 1.8 ml medium and 0.04 agar.

1.3 Anaerobic Dilution Solution (ADS)

This was prepared (9 ml per tube) in the same way as medium 10-XN. The composition was similar with the deletions of growth factors, antibiotics, carbon sources and VFA.

Inoculum was 0.2 ml rumen fluid of each dilution per tube. The dilution factors were 10^{0}, 10^{-1}, 10^{-2} and 10^{-3} and all tubes were incubated at 38° C for 4 days. Enumeration of fungal colonies (on the assumptionthat each colony was formed from one zoospore) was done under a dissecting microscope.

3.5 Analytical Methods

3.5.1 Dry matter and Organic matter

Air-dried feed and faeces were finely ground (1 mm screen), weighed into a crucible and dried to a constant weight in a force-draught oven at 70°C to determine DM. Dried samples were subsequently ashed in an oven (500-600°C for 4 h) to determine OM.

3.5.2 Neutral-Detergent Fibre (NDF)

Approximately 1.0g ground material was placed in a round-bottomed flask. The following reagents were added, in order, 100 ml neutral detergent solution (see next for the composition) (Goering and Van Soest, 1970), 2 ml decahydronaphthalene and 0.5 g sodium sulphide, with a calibrated scoop. The composition of neutral detergent solution (per litre) was as follows:

30 g	sodium lauryl sulphate, USP grade
18.61 g	disodium dihydrogen ethylene diaminotetraacetate (EDTA) dihydrate crystal, reagent grade
4.56 g	disodium hydrogen phosphate, reagent grade
6.81 g	sodium borate decahydrate, reagent grade
10 ml	2-ethoxyethanol (ethylene glycol, monoethyl ether), purified grade.

Samples were refluxed for 60 min., timed from onset of boiling and then filtered through a sintered glass crucible. Samples were rinsed with a hot water (80° C) water, then washed twice with acetone in the same manner and sucked dry. Crucibles were dried at 80°C overnight and weighed.

3.5.3 Acid-Detergent Fibre (ADF)

The procedure for analysing ADF was similar to the NDF determination except for the reagents used in this analysis. After placing approximately 1.0 g of sample in a round-bottomed flask the following reagents were added: 100 ml acid-detergent solution (Goering and Van Soest, 1970) and 2 ml decahydro-naphthalene. The composition of acid-detergent solution (per litre) was as follows:

49.04 g	sulfuric acid, reagent grade standardized to 1N
20.00 g	Cetyl trimethylammonium bromide (CTAB), technical grade.

Samples were refluxed, filtered, washed twice with hot water, washed with acetone, and dried at 80°C overnight.

3.5.4 Nitrogen analysis

a. Total N

The total N content of feedstuffs was converted to NH_4^+ by kjeldahl oxidation and then made alkaline with NaOH. The distilled ammonia was collected into boric acid (2% W/V) and titrated against 0.0075 N H_2SO_4.

b. NH_3-N

Ammonia concentrations in Experiment 1 (Chapter V) were determined by the steam-distillation technique described by Breemer and Keeney (1965) and modified by Nolan (1971), while in Experiments 2, 3 (Chapter V) and 4 (Chapter VI) the ammonia concentrations were determined by an autoanalyser (Technicon). Rumen-fluid supernatants of Experiment 1 were made alkaline with sodium tetraborate (5% W/V). The distilled ammonia was then treated as for total-N using 0.03 N H_2SO_4 for titrations.

In Experiments 2, 3 and 4, samples (0.5 ml) were diluted with 4.5 ml H_2O. If the ammonia concentration of samples was more than 200 mg N/l, the dilution factor was 0.25 ml sample plus 4.75 ml H_2O.

$(NH_4)_2SO_4$ was used as a standard at 10, 20, 30, 50, 70, 90, 110, 130, 150, and 200 mg N/l. The reagents used were 2% H_2SO_4, 0.75% NaOH, sodium salicylate, and sodium cyanurate.

3.5.5 VFA

VFA concentrations of rumen fluid supernatant were determined by the method of Geissler *et al.* (1976) using isocaproic acid as an internal standard in a dual-column gas chromatograph (Model 427, Packard Instrument Company, Illinois, USA).

3.5.6 Chromium

The concentration of chromium in rumen fluid supernatant was determined with an atomic-absorption spectrophotometer (Perkins-Elmer, Model 360, Norwalk, Connecticut, USA) which had been calibrated with a set of potassium chromate standards made up in rumen fluid.

3.6 Estimation of Rumen Fluid Kinetics

Rumen liquid volume and outflow rate were estimated with the Cr-EDTA complex as a soluble marker (Downes and McDonald, 1964) and given as a single injection just prior to the morning feeding. Animals were dosed at 0.5 ml Cr-EDTA per kg body weight injected into the rumen via the cannula; the first sample of rumen fluid was sampled at 3 h post injection. Samples of rumen fluid were again taken at 20, 24 and 26 h post-injection. The concentrations of chromium in rumen fluid was related to time on a lag-linear basis according to first order kinetics. The regression of the line was represented by the equation : $Conc_t = Conc_o e^{-mt}$ where $Conc_o$ and $Conc_t$ = tracer concentrations at zero time and at time t, respectively, m = the rate-constant described by the slope of line; t = hours after injection.

Rumen volume and outflow route were calculated as follows:

a) Rumen volume (ml) (V) = $\frac{\text{Dose (mg)}}{\text{Conc}_o \text{ (mg pl)}}$

b) Outflow rate (l/day) = V x m x 1440.

CHAPTER IV

PRELIMINARY STUDY ON METHODS FOR THE ENUMERATION OF FUNGAL SPORANGIA

4.1 Introduction

As mentioned in Chapter 2, there is no satisfactory method available at present to quantify the fungal population in the rumen. Direct enumeration of fungal zoospores in fresh rumen fluid under a light microscope gives poor results, particularly when rumen protozoa are present. On the other hand, when the enumeration is done with preserved rumen fluid, the morphology of fungal zoospores is often difficult to recognize due to disintegration of the flagellum and the presence of flagellated protozoa. The use of roll tubes as proposed by Joblin (1981) to estimate the number of zoospores in the rumen, quantifying the concentration of chitin in the rumen digesta to determine the pool size of rumen fungi (Orpin, 1981a), and estimating the pool size of rumen fungi by analysing the concentration of total cis,24:1 (Δ^{15}) fatty acids and triterpenol (Kemp *et al.*, 1984) are all demanding in terms of equipment and labour.

This preliminary study was designed to evaluate the method of sporangial enumeration of Bauchop (1979a) and Akin *et al.* (1983) to estimate the fungal biomass in the rumen of sheep on different feeding regimens by counting the sporangia on forage samples suspended in nylon bags in the rumen for up to 48 h. The main objectives of this study were to establish the validity of the method to screen forages as possible suitable substrates. Two experiments were carried out.

4.2 Experimental

4.2.1 Animals and diets

Four mature merino wethers, each fitted with ruminal and abomasal cannulae, were used in Experiment 1. They were fed with the following diets:

Sheep no. 9288 : 60% lucerne chaff + 40% oat grain given at 800 g/d
Sheep nos. 8259
and 8246 : 60% wheaten chaff + 40% oat grain given at 800 g/d
Sheep no. 2277 : 200 g lucerne chaff + 400 g oat grain.

All rations were given once a day at 0900 h and water was available *ad libitum*.

In Experiment 2 twelve mature crossbred wethers, each fitted with a ruminal cannula, were fed a basal diet of rice straw which had been ensiled with 4% urea for four weeks, and supplemented with either lucerne chaff or fish meal or both:

Diet A : Urea-treated rice straw (UTRS) *ad libitum*
Diet B : UTRS *ad lib.* + 75 g lucerne
Diet C : UTRS *ad lib.* + 75 g fish meal
Diet D : UTRS *ad lib.* + 75 g lucerne + 75 g fish meal

All diets were fed once a day at 0900 h; water was freely available.

4.2.2 Experimental procedures

In Experiment 1 leaf blades of three forages (white clover, kikuyu, and grazed oats) and the flower heads of meadow grass (probably barley grass, *Hordeum leporinum*) were incubated in the rumen of sheep in nylon bags with an aperture size of 45 µm. The leaf blades were cut into 2-3 cm lengths with a razor blade. The bags were weighted with a marble and anchored with 75 g iron weights and suspended in the rumen at timed intervals.

In Experiment 2 leaf blades of *Phalaris aquatica*, and untreated and treated straw particles were cut into 2-3 cm lengths and incubated in the rumen of sheep in nylon bags (see Section 3.4.4) at timed intervals.

All bags were suspended in the rumen immediately prior to the morning feeding. The staining and counting methods were as already outlined in Section 3.4.5.a, unless specified otherwise.

4.3 Results and Discussion

The numbers of sporangia appearing on plant particles in Experiments 1 and 2 are presented in Tables 4.1 and 4.2 respectively. The results of sporangial enumeration from these studies suggest that rumen fungi are highly selective microorganisms in colonizing the plant tissues. As reported by Bauchop (1980), the sites of fungal colonization on leaves observed from these studies were principally near the stomata and in areas close to leaf ribs (Plates 4.1 and 4.2). With straw particles, sporangial colonization was prominent on inner surfaces of rice straw stems (Plate 4.3). This selective colonization by fungal sporangia is the main reason for the large coefficient of variation in sporangial counts (mean value 70%) regardless of diet and substrate used for counting the sporangia.

The use of leguminous leaves such as white clover and graminaceous leaves such as oats and phalaris as substrates appears to give a poor result when the incubation of these materials in the rumen exceeds 12 h. This is due to the fact that all these materials were substantially degraded by 24 h, and no intact plant material was found at 48 g for most of the diets (Tables 4.1 and 4.2), resulting in a coefficient of variation higher than 70%. Straw materials, however, gave better results, mainly because they were still intact at 72 h. The number of sporangia on phalaris grass and untreated rice straw after 12 h incubation, and on untreated and treated straw after 48 h and 72 h incubation were similar (Table 4.2), suggesting that the counting method of Bauchop (1979a) and Akin *et al.* (1983) can be used to compare the number of anaerobic fungi present in the rumen on different substrates at the same sampling time.

The finding that rates of fungal colonization on urea-treated straw were slower than those on other substrates was unexpected (Table 4.2). There is no obvious explanation for these findings. Orpin and Bountiff (1978) stated that rumen fungi showed chemotaxis to a range of carbohydrates, but not to the common amino acids, purines, pyrimidines or vitamins, suggesting that rumen fungi are highly selective colonizers. It is possible that ammoniated substrates are not attractive to rumen fungi.

If this is so, the lower numbers of fungal sporangia found at 6 and 12 h of incubation do not necessarily mean that rumen fungi were not present or that they were present at low population densities, since higher counts of sporangia were obtained on non-ammoniated substrates suspended in the same rumen. For this reason, it is suggested that the substrate used for sporangial enumeration should be relatively resistant to microbial degradation in the rumen and free from chemical treatment.

In conclusion, regardless of diet and animal variations, the results of sporangial counts obtained from small numbers of plant particles in these studies are not likely to be meaningful estimates of the rumen fungal population; estimates of sporangial population densities in these studies can only be used as approximate values.

Table 4.1. Sporangial appearance on plant particles suspended in the rumen of sheep at different time intervals (Experiment 1)

Sheep/Diet	Substrate	Observation 1				Observation 2					Remarks
		No.of sporangia(colonies/mm^2) ± SD				No.of sporangia(colonies/mm^2) ± SD					
		6h	16h	24h	48h	2h	4h	6h	8h	10h	
9288											
60%lucerne + 40%oat grain	white clover	39 ± 36 (n=50) cv=92%	27 ± 13* (n=8) cv=48%	***	***	-	-	-	-	-	*heavily degraded **too heavily degraded
	grazed oats	-	-	-	-	39±35 (n=43) cv=90%	68±82 (n=44) cv=120%	79±50 (n=20) cv=63%	39±31 (n=20) cv=79%	12±12 (n=18) cv=100%	***plant materials completely disappeared
8259											n=number of field area used for counting
60%wheaten chaff + 40%oat grain	kikuyu grass	1 ± 3 (n=40) cv=300%	21 ± 18 (n=10) cv=86%	10 ± 10* (n=10) cv=100%	6 ± 9* (n=10) cv=150%	-	-	-	-	-	nd=not determined cv=coefficient of variation
8246											
Same as above	grazed oats	36 ± 31 (n=40) cv=86%	43 ± 23* (n=8) cv=53%	7 ± 4** (n=5) cv=57%	***	18±23 (n=40) cv=128%	52±49 (n=40) cv=94%	69±53 (n=20) cv=77%	28±16* (n=20) cv=57%	74±37* (n=20) cv=50%	
2277											
200 g lucerne chaff + 400 g oat grain	flower heads of meadow grass+)	nd	79 ± 30 (n=8) cv=38%	nd	**	-	-	-	-	-	+)probably barley grass
	grazed oats	-	-	-	-	16±20 (n=40) cv=125%	48±47 (n=40) cv=98%	11±12* (n=20) cv=109%	23±41 (n=20) cv=178%	***	

Table 4.2 Sporangial appearance on plant particles suspended in the rumen of sheep given a basal diet of urea-treatedstraw(A), supplemented with 75 g lucerne(B), 75 g fish meal(C) and 75 g lucerne + 75 g fish meal(D).(Experiment 2). Values are means of three sheep per treatment;45 field areas used for counting sporangia in each case.

Substrate	Diet	Numbers of sporangia (colonies/mm^2.) ± SD					Remarks
		6h	12h	24h	48h	72h	
-Phalaris grass	A	38 ± 23 cv=61%	52 ± 28 cv=54%	16 ± 13* cv=81%	**	**	*heavily degraded
	B	8 ± 6 cv=75%	9 ± 1 cv=11%	13 ± 12* cv=92%	**	**	**too heavily degraded
	C	19 ± 3 cv=16%	30 ± 30 cv=100%	7 ± 6* cv=86%	**	**	cv=coefficient of variation
	D	60 ± 25 cv=42%	24 ± 11 cv=46%	17 ± 15* cv=88%	**	**	
-Untreated rice straw	A	71 ± 63 cv=89%	47 ± 23 cv=49%	38 ± 22 cv=58%	38 ± 11 cv=29%	19 ± 6 cv=32%	
	B	4 ± 4 cv=100%	11 ± 9 cv=82%	33 ± 8 cv=24%	28 ± 9 cv=32%	11 ± 5 cv=45%	
	C	14 ± 12 cv=86%	24 ± 19 cv=79%	21 ± 6 cv=29%	32 ± 12 cv=38%	22 ± 5 cv =23%	
	D	56 ± 51 cv=91%	22 ± 12 cv=55%	44 ± 19 cv=43%	25 ± 8 cv=32%	21 ± 5 cv=24%	
-Urea treated rice straw	A	0	1 ± 1 cv=100%	14 ± 6 cv=43%	32 ± 6 cv=19%	20 ± 6 cv=30%	
	B	0	1 ± 1 cv=100%	11 ± 6 cv=55%	27 ± 5 cv=19%	13 ± 8 cv=62%	
	C	0	4 ± 1 cv=25%	18 ± 8 cv=44%	53 ± 14 cv=26%	33 ± 23 cv=70%	
	D	0	5 ± 2 cv=40%	30 ± 8 cv=27%	31 ± 15 cv=48%	25 ± 13 cv=52%	

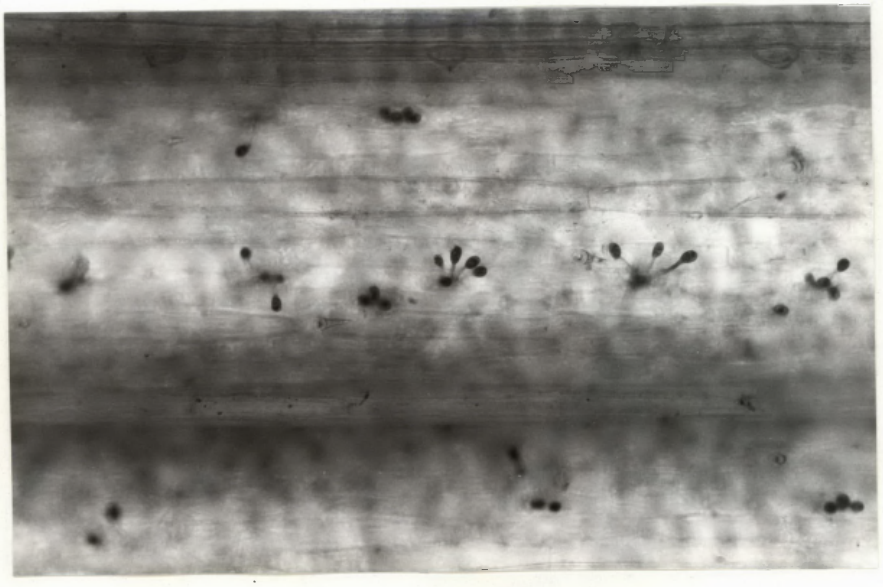


Plate 4.1 Sporangia of rumen fungi colonizing grazed oat leaves (8 h incubation); penetration is through the stomata (Magnification 200 X)

Plate 4.2 Sporangia of rumen fungi are found principally along the mid ribs of kikuyu leaves (24 h incubation, magnification 200 X)

Plate 4.3 Unstained straw showing an early stage of development of rumen fungi (arrow) colonizing the inner surface of the stem (Magnification 100 X)