REVIEW

Anaerobic fungi in herbivorous animals

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The conviction that all fungi required oxygen for growth was summarized in 1949 by J. N. Foster when he wrote, 'One of the major metabolic differences between moulds and bacteria is that there are no anaerobic moulds, either obligate or facultative'. For obligate anaerobes, this view held until 1975, when C. G. Orpin demonstrated that certain motile cells in the rumen of sheep, previously believed to be protozoan flagellates, were in fact zoospores of an obligately anaerobic fungus. Prior to this discovery it was assumed that only anaerobic bacteria and protozoa were involved in the hydrolysis of plant biomass in the rumen, but now it is acknowledged that anaerobic fungi participate in this process both in ruminants such as sheep and hindgut-fermenting herbivores such as horses. This review describes the life-cycles and physiology of anaerobic fungi, details their interactions with other rumen micro-organisms and assesses their contribution to the digestion of plant material in herbivores.

HERBIVORY AND THE RUMINANT DIGESTIVE TRACT

Few invertebrates and no vertebrates are able to produce cellulases and/or hemicellulases. Instead, many herbivorous animals have evolved symbiotic associations with bacteria, protozoa and fungi which produce these enzymes and are therefore able to degrade plant polymers. This situation led Attenborough (1990) to write: 'Most large animals, in fact are not the single individuals they seem to be. They are walking menageries, whole communities of different species which, in their various ways, are committed by evolution, for better or for worse, in sickness and in health, to live together'. In return for provision of a relatively constant environment and continual supply of plant material, the microbial population in the digestive tract of herbivores supplies the animal with easily utilizable forms of carbon and energy and, in the case of ruminants, with a source of microbial protein.

Two main types of herbivory exist among mammals. The first consists of the ruminants (cloven-hoofed mammals of the order Artiodactyla), which are capable of extensive plant fibre digestion during prolonged retention (60–90 h) of the feed in the rumen (Church, 1969). The second consist of the hindgut-fermenting herbivores such as the Equidae (horses) and Elephantidae (elephants), in which plant feed passes through the digestive tract more rapidly (30–40 h) and is consequently not digested so extensively as in ruminants (Warner, 1981). In these hindgut-fermenting herbivores a greater portion of the nutrient supply to the animal is obtained from the contents rather than the walls of plant cells (Hume, 1989). However, both types of herbivory rely on micro-organisms for the digestion of plant biomass; whereas ruminants depend upon a predominantly pre-gastric fermentation in the rumen (Fig. 1), microbial digestion in the hindgut-fermenting herbivores occurs predominantly in the caecum and large intestine and follows gastric digestion (Fig. 2). Pre-gastric fermentation also occurs in other mammals, e.g. *Macropus* spp. (kangaroos), but the digestive tract modifications in these animals are not as great as in the ruminants.

The foregut of ruminants consists of a system of four chambers (Fig. 1). The first three (the rumen, reticulum and omasum) are pre-gastric chambers formed from modifications of the oesophagus. The fourth, the abomasum, is the site of gastric digestion and is equivalent to the single stomach of a monogastric mammal. The rumen is the largest pre-gastric chamber and, together with the reticulum, forms a large fermentation vessel (with a volume of 100–150 l in cattle and 10 l in sheep; Hobson & Wallace, 1982) containing a dense population of micro-organisms (Figs 1, 3). The reticulum, which is small in comparison to the rumen, forms a forward pouch to the rumen into which it opens (Fig. 1).

During feeding, a few bites of plant material are mixed with saliva by the ruminant and swallowed as a bolus without further chewing. The bolus, which has an average weight of 100 g in sheep (Hungate, 1966), is carried down the oesophagus by peristalsis and forcefully ejected into the rumen, thus by-passing the reticulum. The copious quantities of saliva produced by ruminants (6–16 l d^{-1} in sheep and 98–190 l d^{-1} in cattle; Hobson, 1971) serve to lubricate the passage of boli down the oesophagus. Saliva contains a

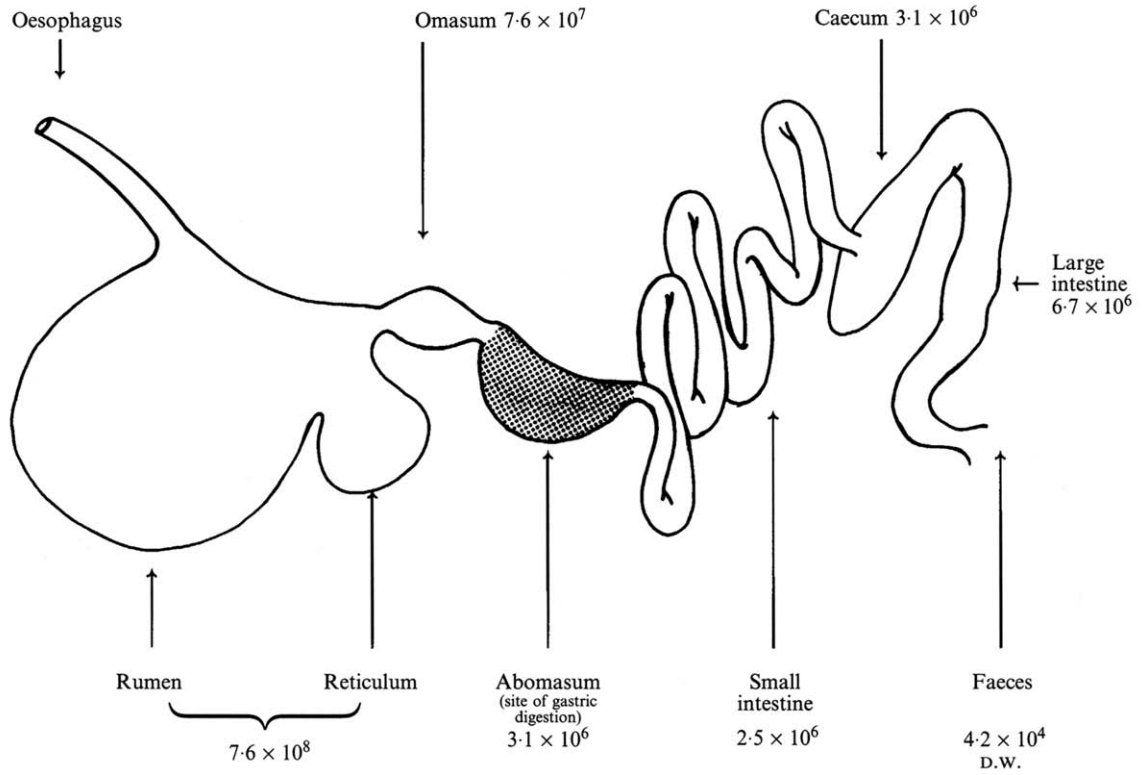

Fig. 1. Diagrammatic representation of the digestive tract of a ruminant such as cattle or sheep in which microbial digestion precedes the gastric and small-intestine digestive system (adapted from Hobson, 1971). Estimates are given of the total number of thallus-forming units of anaerobic fungi in each part of the digestive tract of cattle and of the concentration of anaerobic fungi in faeces of cattle (from Davies *et al.*, 1993).

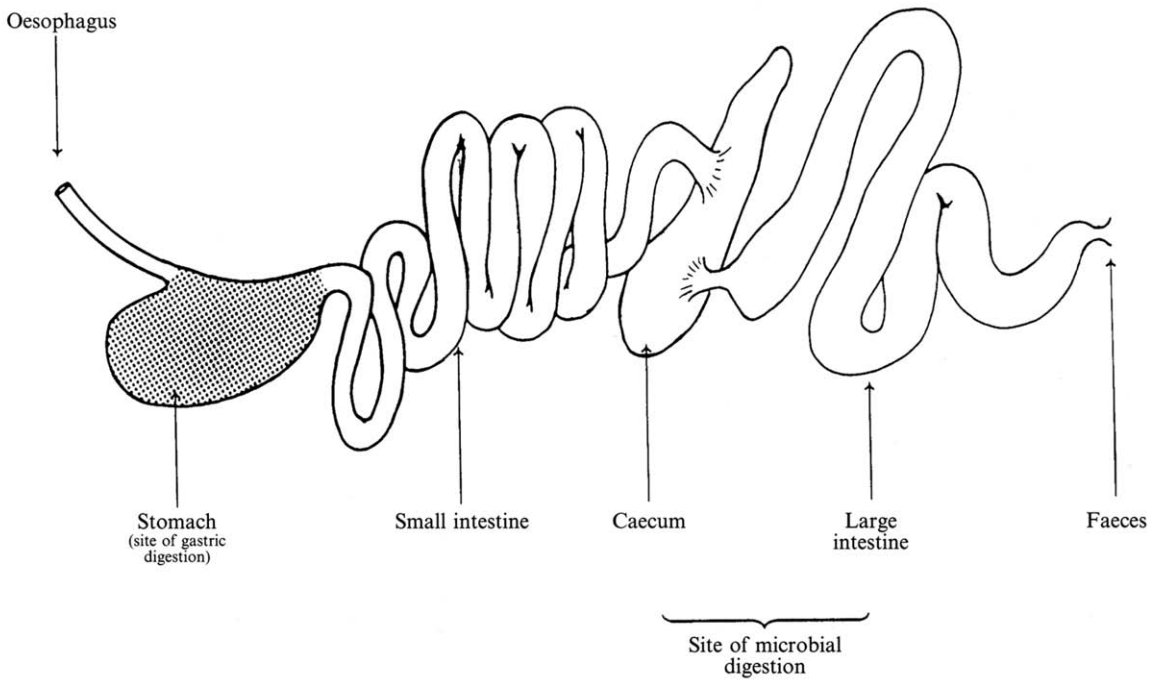

Fig. 2. Diagrammatic representation of a hindgut-fermenting herbivore such as the horse, in which microbial digestion occurs in an enlarged caecum and large intestine after gastric and small-intestine digestion (adapted from Hobson, 1971).

number of salts, but most importantly it contains a bicarbonate/phosphate buffer which helps to maintain the rumen at a pH between 6 and 7. Once in the rumen, the bolus is mixed with digesta by contractions of the reticulo-rumen wall, distributing saliva, enhancing absorption of fermentation acids, and possibly reducing the size of plant particles and thereby facilitating their passage from the rumen and omasum to the abomasum. Gases produced as a result of microbial fermentation in the rumen are removed via the animal's mouth by eructation; contraction of the rumen wall results in the gas

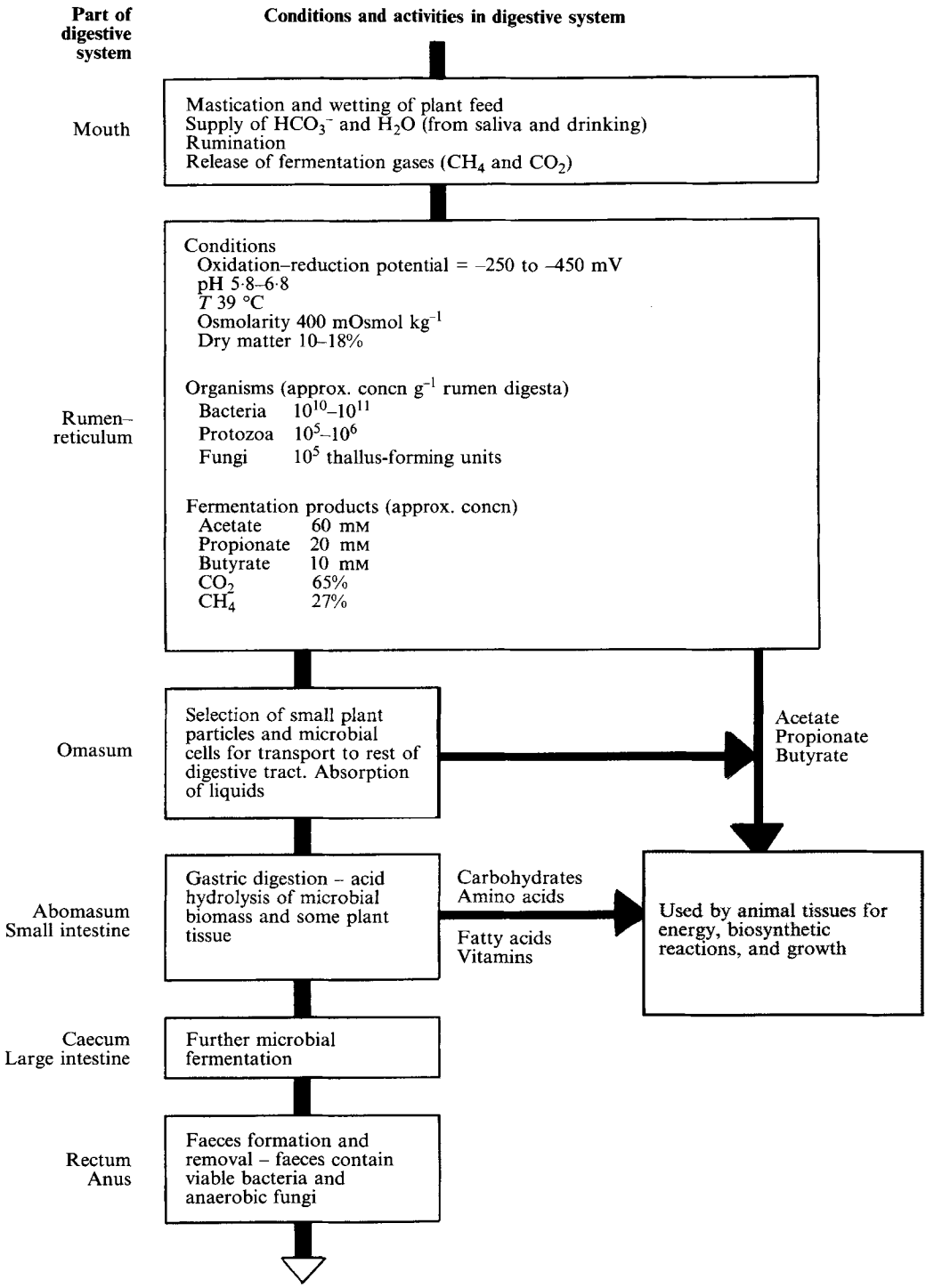

Fig. 3. Diagrammatic representation of the digestive tract of a ruminant as a system for the conversion of plant material to products which can be used by the animal (adapted from Lin *et al.*, 1985).

above the digesta being displaced into the oesophagus and out of the animal.

The flow of digesta from the rumen is selective, with the reticulo-omasal orifice acting as a filter for plant particles above a certain size. This orifice channels liquid, small particles, typically 1·5–2 mm for cattle and 1 mm for sheep (Ulyatt *et al.*, 1986; Ulyatt, Baldwin & Koong, 1976), and free floating microbial cells into the omasum where water and fermentation acids are absorbed by the animal. Larger plant particles remain in the reticulum, where they trigger rumination, regurgitation, mastication and swallowing of the feed bolus. Retention times for liquids and small particles, including microorganisms, in the rumen are in the range 10–24 h, whereas larger plant particles may remain in the rumen for 2–3 d, allowing time for extensive microbial digestion of plant fibres (Hobson & Wallace, 1982).

The digestive physiology of ruminants distal to the omasum is similar to that in monogastric mammals. Digesta passes from the omasum into the abomasum where pepsin and HCl are secreted and protein digestion occurs: enzymic and

acid hydrolysis causes digestion of microbial biomass (and of some undegraded plant material) with the release of amino acids, fatty acids and vitamins, which then become available to the animal (Fig. 3). Alkaline digestion occurs in the small intestine. The caecum and large intestine are sites where further microbial fermentation of undigested food occurs, prior to the formation and elimination of faeces. The activities in various parts of the digestive tract of ruminants are summarized in Fig. 3.

Environmental conditions in the rumen

In ruminant herbivory, the animal relies upon micro-organisms to convert plant biomass to utilizable products for energy and growth. In turn, the micro-organisms rely upon the animal to provide a continuous supply of plant material and a more or less constant environment conducive to microbial growth. Thus the rumen is a continuous culture system (Fig. 3), which due to the heterogeneous nature of the substrate (plant material), particularly with respect to particle size and specific gravity, may become stratified. Micro-organisms in the rumen are therefore subject to a variety of environmental conditions, and consequently the equations used to describe a well-mixed chemostat (Pirt, 1975) cannot be applied to the rumen (Hobson & Wallace, 1982).

The rumen is maintained at a temperature of *ca* 39 °C (Lin, Patterson & Ladisch, 1985), mainly by the heat from the animal's aerobic tissue metabolism, but partly by the heat of the microbial fermentation within the rumen. Rumen fluid has a pH of between 5·8 and 6·8, the exact value being dependent on the animal's diet and frequency of feeding. Fermentation of complex plant polymers to simpler products by rumen micro-organisms results in the production of three main volatile fatty acids, normally present in the following proportions (Lin *et al.*, 1985): acetate (56–70%), propionate (17–29%) and butyrate (9–19%). The gaseous phase in the rumen above the digesta varies in composition but typically contains CO_2 (65%), CH_4 (27%), N_2 (7%), O_2 (0·6%), H_2 (0·2%) and H_2S (0·1%) (Hobson, 1971). Air is taken into the rumen during feeding, but the oxygen is rapidly scavenged by facultative anaerobic bacteria, and this keeps the redox potential of the rumen digesta between −250 and −450 mV (Hobson & Wallace, 1982). The environmental conditions in the rumen are summarized in Fig. 3.

Micro-organisms in the rumen

At birth young ruminants lack the complex microbial biota of adult animals. Milk bypasses the rumen by way of the oesophageal groove, and digestion occurs as in any young mammal, with lactobacilli and streptococci as the major gut microbes. However, as the animal begins to graze the rumen enlarges and the animal acquires the micro-organisms essential to its future existence. When fully developed the rumen contains a large number of bacteria, protozoa and fungi which are present in the liquid phase, associated with plant fragments, and as a lining on the rumen epithelium (Latham, 1980). The concentrations of these populations in rumen fluid are in the range 10^9–10^{10} ml^{-1} for bacteria, 10^5–10^6 ml^{-1} for protozoa (Hungate, 1966), and *ca* 1×10^1 ml^{-1} for fungal zoospores (Theodorou *et al.*, 1990). Anaerobic fungi were first observed in the rumen (Fonty *et al.*, 1987) and faeces (Theodorou *et al.*, 1994) of lambs 8 d and 5 wk after birth respectively, and were first observed in the faeces of steers (Theodorou *et al.*, 1994) 4 wk after birth. Surprisingly, Fonty *et al.* (1987) found that anaerobic fungi disappeared from the rumen in 9 of the 11 lambs studied after solid diet was given (on day 21). According to Eadie (1962) and Lowe *et al.* (1987*b*), protozoa, bacteria and anaerobic fungi brought into the mouth of the parent during rumination are passed to offspring during licking and grooming. Rumen bacteria are also transferred from adult to offspring in aerosols (Hobson, 1971) and feeds (Becker & Hsuing, 1929). By one means or another, at the time of weaning the rumen is completely functional and able to digest the fibrous plant diet consumed by the adult.

More than 200 species of rumen bacteria have been described, but the predominant species involved in cellulose decomposition in the rumen are *Bacteroides succinogenes, Ruminococcus albus, R. flavefaciens* and *Eubacterium cellulosolvens*. These bacteria adhere to the surfaces of plant cell walls (Latham *et al.*, 1978; Stack & Hungate, 1984), forming pits as they degrade the cellulose (Akin, 1980). Hemicellulose is degraded by the cellulolytic bacteria, as well as by such species as *Butyrivibrio fibriosolvens* and *Bacteroides ruminicola* (Hungate, 1966; Dehority & Scott, 1967). Other components of the plant material utilized by bacteria include pectin (by *Lachnospira multiparus*), starch (by *Bacteroides amylophilus*) and lipid (by *Anaerovibrio lipolytica*) (Hobson, 1971; Hobson & Wallace, 1982). In addition, some rumen bacteria utilize the fermentation products produced by other rumen micro-organisms. For example, *Veillonella alcalescens, Megasphaera elsdenii* and *Selenomonas ruminantium* var. *lactilytica* utilize lactate or succinate and produce acetate or propionate as fermentation end-products. Methanogenic bacteria, such as *Methanobacterium ruminantium* and *M. mobilis*, utilize either formate or H_2 and CO_2 as substrates for growth and methane production.

Three groups of protozoa occur in the rumen, the rumen flagellates, the entodiniomorphids and the holotrichs (Williams, 1986). Most do not rely solely on plant biomass as substrates for growth, but instead feed by predation on other rumen micro-organisms. Of the more than 100 species of rumen protozoa which have been described, none has been grown axenically, although *ca* 20 species have been grown *in vitro* in the presence of bacteria. Animals lacking protozoa seem to remain healthy (Hobson & Wallace, 1982), suggesting that bacteria and anaerobic fungi are the principal organisms responsible for digestion of plant material in herbivores.

DISCOVERY OF ANAEROBIC FUNGI

Braune (1913) and Hsuing (1930) described *Callimastix frontalis* and *C. equi* as protozoans. These polyflagellated organisms, which were isolated from the horse, were placed in the same genus as *C. cyclopsis*, a parasite of freshwater copepods (Weissenberg, 1912), and *C. jolepsi*, found in the intestine of a freshwater snail (Bovee, 1961), whilst monoflagellated protozoans found in the rumen were assigned to the genera *Piromonas* and *Sphaeromonas* (Liebetanz, 1910; Braune, 1913).

Table 1. Specific names of anaerobic fungi isolated from herbivores

Genus: characteristics	Species	Source of isolate	Reference
Caecomyces			
Monocentric or polycentric; uniflagellate zoospores; spherical holdfasts	*Caecomyces communis*[1]	Sheep	Gold *et al.* (1988)
	Caecomyces equi	Horse	Gold *et al.* (1988)
Piromyces			
Monocentric; uniflagellate zoospores; filamentous rhizomycelium	*Piromyces communis*[2]	Sheep	Gold *et al.* (1988)
	Piromyces mae	Horse	Li, Heath & Bauchop (1990)
	Piromyces dumbonica	Elephant	Li, Heath & Bauchop (1990)
	Piromyces rhizinflata	Saharan ass	Breton *et al.* (1991)
	Piromyces minutus	Deer	Ho *et al.* (1993*c*)
	Piromyces spiralis	Goat	Ho *et al.* (1993*d*)
Neocallimastix			
Monocentric; polyflagellate zoospores; extensive, filamentous rhizomycelium	*Neocallimastix frontalis*	Sheep	Heath *et al.* (1983)
	Neocallimastix patriciarum[3]	Sheep	Orpin & Munn (1986)
	Neocallimastix hurleyensis	Sheep	Webb & Theodorou (1991)
	Neocallimastix variabilis	Cow	Ho *et al.* (1993*a*)
Anaeromyces			
Polycentric; uniflagellate zoospores; filamentous rhizomycelium	*Anaeromyces elegans*[4]	Cow	Ho *et al.* (1993*b*)
	Anaeromyces mucronatus	Sheep	Breton *et al.* (1990)
Orpinomyces			
Polycentric; polyflagellate zoospores; filamentous rhizomycelium	*Orpinomyces joyonii*[5]	Sheep	Breton *et al.* (1989)

Originally called: [1] *Sphaeromonas communis* (Orpin, 1976); [2] *Piromonas communis* (Orpin, 1977*b*); [3] *Neocallimastix frontalis* (Orpin, 1975); [4] *Ruminomyces elegans* (Ho *et al.*, 1990); [5] *Orpinomyces bovis* (Barr *et al.*, 1989); and *Neocallimastix joyonii* (Breton *et al.*, 1989).

Weissenberg (1950) suggested that *C. cyclopsis* might be a zoospore of a fungus, not a protozoan, and Vavra & Joyon (1966) substantiated this hypothesis when they discovered the vegetative thallus of the fungus. As a result of this discovery, the remaining polyflagellate species of *Callimastix* were grouped into a new 'protozoan' genus, *Neocallimastix*, with *N. frontalis* (formerly *Callimastix frontalis*) as the type species (Vavra & Joyon, 1966).

Warner (1966) observed a 25-fold increase in the number of flagellated cells in the rumen of sheep 1 h after feeding and suggested that this was due to organisms such as *N. frontalis* migrating from the rumen wall to the rumen fluid in response to feeding; this 'sequestration' hypothesis explained the observed increase in size of the 'protozoan' flagellate population in the rumen, which could not be accounted for by binary fission alone. However, Orpin (1974) showed that although the *N. frontalis* flagellate population increased in size *in vitro* when digesta was treated with an extract of oats, in the absence of particulate digesta the oat extract failed to induce the requisite increase in flagellate numbers. Orpin (1975) concluded that the flagellated cells were not sequestered on the rumen wall, but were in fact zoospores of a chytrid-like fungus which, in response to the oat treatment, were released from zoosporangia associated with plant fragments in the digesta. Following this discovery, Orpin identified three anaerobic fungi (he called them *Neocallimastix frontalis*, *Sphaeromonas communis* and *Piromonas communis*, but see below for subsequent change in nomenclature) in the rumen of sheep, each of which had a motile stage (the zoospore) and a non-motile thallus supporting a zoosporangium (Orpin, 1975, 1976, 1977*b*). Orpin (1977*c*) subsequently showed that the cell walls of the three anaerobic fungi contained chitin, a polymer which, amongst non-photosynthetic microbes, is only found in fungi (Rogers & Perkins, 1968). To date fifteen species of anaerobic fungi have been described (Table 1).

The delay in recognizing this group of fungi has been attributed both to mycological dogma (that fungi require oxygen to grow), and to the practice among rumen microbiologists of working with strained rumen fluid and usually discarding the solid digesta: most of the plant fibre-associated fungal biomass was therefore discarded with the solid fraction of the rumen digesta (Bauchop, 1983).

CLASSIFICATION OF ANAEROBIC FUNGI

The ultrastructural uniqueness of the anaerobic fungi, their adaptation to the digestive tract of herbivores and their distribution throughout phylogenetically diverse animals (Table 2) implies that they could have existed as a separate group since the time these mammals began to diverge at least 120 million years ago (Munn, 1994). Although comparatively little is known about the taxonomy of anaerobic fungi, it is generally agreed that they are zoospore-producing fungi and should be assigned to the class Chytridiomycetes. The order Spizellomycetales in the Chytridiomycetes was established by Barr (1980) by subdivision of the Chytridiales to take account of differences in zoospore ultrastructure of the incumbent species. There are, however, many similarities between the

Table 2. Reports of the isolation of anaerobic fungi from herbivores

Herbivore		
Specific name	Common name	Source of isolate and reference
Ruminants – also known as pre-gastric or foregut-fermenting herbivores		
Aepyceros melampus	Impala	Rumen[1]
Bos taurus	Domestic cattle	Rumen, omasum, abomasum, small intestine, caecum, large intestine, faeces[2,14]
Bos sp.	Ethiopian (Zebu) cattle	Faeces[2]
Bos gaurus	Gaur	Faeces[2]
Bos indicus	Kedah kelantan cattle	Rumen[16]
Boselaphus tragocamelus	Nilgai	Faeces[17]
Bubalus bubalis	Water buffalo	Rumen[10,11]
Camelus bactrianus	Bactrian camel	Faeces[2]
Capra hircus	Domestic goat	Rumen, faeces[3,4]
Cephalophus monticola	Blue duiker	Rumen, caecum[13]
Cervus axis	Axis deer	Faeces[17]
Cervus canadiensis	Wapiti	Faeces[17]
Cervus elaphus	Red deer	Rumen[1]
Elaphunis davidianus	Père David's deer	Faeces[17]
Hippotragus equinus	Roan antelope	Faeces[2]
Hydropotes inermis	Chinese water deer	Not specified[16]
Lama glama	Llama (all in the same	Faeces[2]
Lama pacos	enclosure except[15])	Faeces[2]
Lama guanicoe		Faeces[2,15]
Macropus giganticus	Grey kangaroo	Fore stomach[6,7]
Macropus robustus	Wallaroo	Fore stomach[6]
Macropus rufogriseus	Redneck wallaby	Fore stomach[6]
Oryx dammah	Scimitar-horned oryx	Faeces[17]
Oryx leucoryx	Arabian oryx	Faeces[2]
Ovibos muschanus	Musk ox	Not specified[16]
Ovis sp.	Ethiopian (fat-tailed) sheep	Faeces[2]
Ovis aries	Domestic sheep	Oesophagus, rumen, omasum, abomasum, small intestine, caecum, large intestine, faeces[2,5,14]
Rangifer tarandus	Reindeer	Rumen[1]
Rangifer tarandus platyrhynchus	Svalbard reindeer	Rumen[9]
Taurotraous eurycerus	Bongo	Faeces[2]
Tragelaphus strepsiceros	Greater Kudu	Faeces[2]
Vicugna vicugna	Vicuna	Faeces[2]
Wallubia bicolor	Swamp wallaby	Fore stomach[6]
Hindgut fermenting animals – also known as monogastric herbivores		
Diceros bicornis	Black rhinoceros	Not specified, faeces[3,12]
Dolichotis patagonum	Mara	Faeces[12]
Elephas maximus	Asian elephant	Faeces[2]
Equus burchelli	Common zebra	Faeces[2]
Equus caballus przewalskii	Przewalski's horse	Faeces[17]
Equus caballus	Horse	Faeces, caecum[1,8]
Equus hemionus onager	Onager	Faeces[17]
Loxodonta africana	African elephant	Faeces[1]
Rhinoceros unicornis	Indian rhinoceros	Faeces[12]

[1] Bauchop (1979*b*); [2] Milne *et al.* (1989); [3] Orpin & Joblin (1988); [4] D. Davies, M. K. Theodorou & A. P. J. Trinci (unpublished); [5] Lowe *et al.* (1987*b*); [6] Bauchop (1983); [7] M. K. Theodorou & C. G. Orpin (unpublished); [8] Orpin (1989); [9] Orpin *et al.* (1985); [10] Ho *et al.* (1988*a*); [11] Foong *et al.* (1987); [12] Teunissen *et al.* (1991); [13] Dehority & Varga (1991); [14] Davies *et al.* (1993); [15] Marvin-Sikkema *et al.* (1990); [16] Orpin (1981*a*); [17] Lawrence (1993).

families and genera of both orders (the Spizellomycetales and the Chytridiales). At present, anaerobic fungi are classified (Barr, 1988; Barr *et al.*, 1989) as follows.

Division: Eumycotina
Sub-division: Mastigomycotina
Class: Chytridiomycetes
Order: Spizellomycetales*
Family: Neocallimasticaceae
Genera: *Caecomyces, Piromyces, Neocallimastix, Anaeromyces* and *Orpinomyces*

(* Li, Heath & Packer (1993) suggest that anaerobic fungi should be assigned to a new order, the Neocallimasticales.)

Recently, Li & Heath (1992) and Li, Heath & Packer (1993) suggested that the Chytridiales and aerobic Spizellomycetales are more closely related to each other than to anaerobic fungi, and that consequently the latter may not belong to the Spizellomycetales. Genera of anaerobic fungi are defined on the basis of thallus morphology (monocentric or polycentric), rhizoid type (filamentous or bulbous) and number of flagella per zoospore, and species are delimited mainly on details of

zoospore ultrastructure (Munn, Orpin & Greenwood, 1988; Munn, 1994). It is probable that, as more anaerobic fungi are discovered, the number of anaerobic and aerobic species with similar morphologies will increase (Theodorou, Lowe & Trinci, 1992).

If an evolutionary relationship between anaerobic fungi and aerobic chytrids is to be established and if the classification of the Chytridiomycetes is to be based upon phyeogenetic relationships, it is important to obtain information about (*a*) the GC ratios and (*b*) the 5s and 18s ribosomal RNA sequences (Hori & Osawa, 1987) of aerobic and anaerobic chytridiomycetes. Using different extraction methods, Billon-Grand *et al.* (1991) obtained GC contents for anaerobic fungi ranging from 15% (*Anaeromyces mucronatus*) to 22% (*Caecomyces communis*). These values compare well with the non-repetitive fraction of the DNA of *Neocallimastix* (LM2), which has a GC content of only 13%, the lowest value recorded for any organism so far described (Brownlee, 1989). The presence of satellite DNA has been observed in all strains. This type of DNA is characteristic of most fungi and usually represents mitochondrial or ribosomal cistronic DNA. Rumen fungi do not have mitochondria, so the satellite DNA most probably codes for ribosomal RNA genes. The ribosomal genes of *Neocallimastix* are arranged as tandem repeats with a size of 9·4–10·0 kb, the average GC content of this region being *ca* 30% (Brownlee, 1989). Therefore, although these organisms have different phenotypes (monocentric or polycentric thalli, uniflagellate or polyflagellate zoospores, etc.), it seems that they form a homogeneous group with respect to GC content. Few similar studies have been made with aerobic chytrids, but Mandel (1968) found that four species had a percentage GC content ranging from 44% (*Rhizophlyctis rosea*) to 66% (*Blastocladiella emersonii*). It is possible that the nature of the rumen with its highly competitive anaerobic environment played an important part in shaping the base composition changes that have caused the DNA of this group of fungi to diverge so dramatically. Significantly, the coding sequences of two *N. frontalis* genes have ca 45% GC whereas their associated introns have ca 15% GC (Dr. R. Durand, pers commun.)

Analysis of 18s ribosomal RNA sequences has been used to clarify the phylogenetic relationships between the anaerobic fungi, the aerobic chytrids and other eukaryotes. Doré & Stahl (1991), Bowman *et al.* (1992) and Li & Heath (1992) have used partial 18s rRNA sequence analysis to support the assignment of the anaerobic rumen fungi to the Chytridiomycetes, as opposed to the protists, and the inclusion of the Chytridiomycetes with the fungi. It is agreed that anaerobic rumen fungi make up a monophyletic group with 97–99% sequence similarity (Doré & Stahl, 1991), although relationships within the group are not yet clear. Analysis of the internal spacer I (ITSI) region of the rRNA gene sequence suggests that *Neocallimastix* (polyflagellate zoospores), *Piromyces* (monoflagellate zoospores) and *Orpinomyces* (polyflagellate zoospores) are closely related, whereas *Anaeromyces* (monoflagellate zoospores) is more distant from these genera (Li & Heath, 1992). However, Munn (1994) considers that the possession of polyflagellated or monoflagellated zoospores is not a trivial difference, and suggests that a family (separate from the Neocallimasticaceae which would continue to contain polyflagellate anaerobic fungi) should be erected to accommodate the monoflagellated species of anaerobic fungi. Sequence data alone cannot solve all the taxonomic questions raised by the anaerobic fungi; for example, there is a need to compare the results of different tree-generating algorithms because there is a significant difference of opinion on which gives the most accurate results (Hasegawa, Kishino & Saitou, 1991). Cladistic analysis of sequence data, morphological, ultrastructural and other related characteristics will in the future lead to a better understanding of the taxonomic status of these unique micro-organisms.

CHANGES IN NOMENCLATURE

The generic and specific names of anaerobic fungi have undergone changes since their fungal status was first recognized (Table 1). Bauchop & Mountfort (1981) isolated an organism (PN1) similar to the *N. frontalis* described by Orpin (1975), and this isolate was formally classified as *N. frontalis* (Heath, Bauchop & Skipp, 1983). Unfortunately, the '*N. frontalis*' isolated by Orpin was subsequently found to be different from PN1, so the Orpin isolate was renamed *N. particiarum* (Orpin & Munn, 1986). Further controversy arose over the use of the generic names *Sphaeromonas* and *Piromonas* for anaerobic fungi. On the assumption that the anaerobic fungi he isolated from the rumen were the same as Liebetanz's 'protozoans', Orpin (1976, 1977*b*) retained the generic names *Sphaeromonas* and *Piromonas* first used by Liebetanz (1910). However, Gold, Heath & Bauchop (1988) questioned this assumption because Liebetanz's isolates were anteriorly flagellated, obtained nutrition by phagocytosis and divided by binary fission, whereas Orpin's isolates were posteriorly flagellated, rhizoid-producing saprotrophs, and did not undergo binary fission. For these reasons, and to stress their fungal affinity, *Sphaeromonas* was renamed *Caecomyces* and *Piromonas* was renamed *Piromyces* (Gold *et al.*, 1988). The three polycentric anaerobic fungi isolated to date have been placed in two different genera based on the flagellation of their zoospores, and again the generic names of some isolates have been changed since they were first described (Table 1).

LIFE CYCLES

Monocentric and polycentric life cycles

In the Spizellomycetales, families are classified on the basis of morphology and development of the thallus, whereas genera are classified primarily on zoospore ultrastructure (Barr, 1980, 1988). In a monocentric fungus, either the encysted zoospore retains the nucleus and enlarges into a zoosporangium (called endogenous zoosporangial development; Karling, 1978; Barr *et al.*, 1989), or the nucleus migrates out of the zoospore, and the zoosporangium is formed in the germ-tube or rhizomycelium (called exogenous zoosporangial development; Karling, 1978; Barr *et al.*, 1989). In both types of monocentric development, only one zoosporangium is formed per thallus and only the zoosporangium contains nuclei (Fig. 4A, B). In a polycentric fungus the nucleus migrates out of the encysted

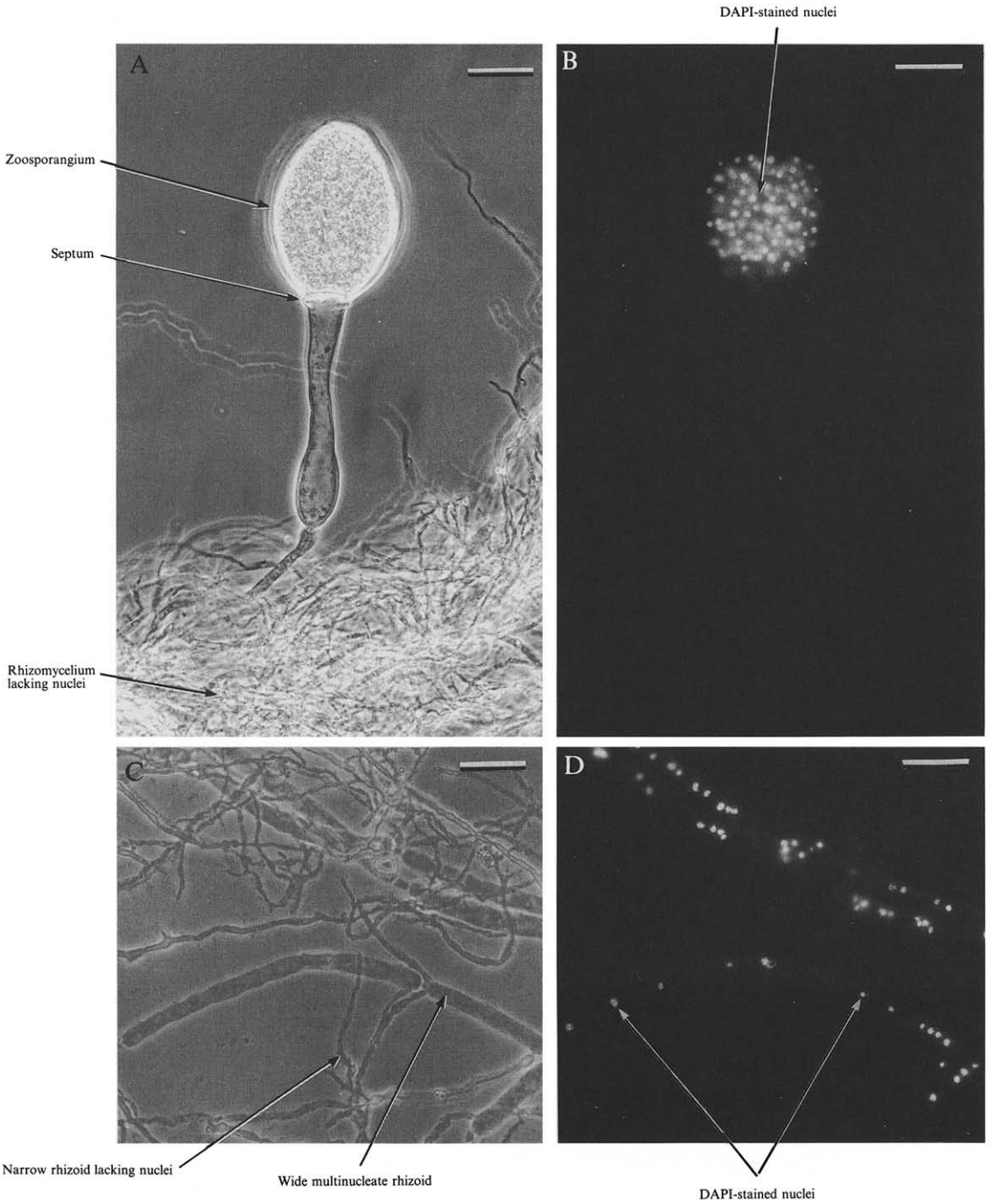

Fig. 4. Phase contrast (A, C) and DAPI-stained (B, D) thalli of a monocentric (A, B) and a polycentric (C, D) anaerobic fungus isolated from the faeces of a water buffalo (in Malaysia) and a castrated male sheep (in England), respectively. DAPI staining shows that nuclei occur in parts of the rhizomycelium of the polycentric isolate (designated SR2), but not in the rhizomycelium of the monocentric isolate (PC2-2). Scale bars = 40 μm.

zoospore (exogenous zoosporangial development) and undergoes mitosis in the rhizomycelium, which subsequently forms several zoosporangia. Thus, in polycentric fungi, both the zoosporangia and the rhizomycelium (Fig. 4C, D) contain nuclei.

Neocallimastix spp.

Zoospores of *Neocallimastix* spp have *ca* 18 or more posteriorly directed flagella (Orpin, 1976; Lowe *et al.*, 1987*a*); however, for cytological reasons, the higher figure is likely to be 16 rather than 18. By observing individual thalli in roll-tube cultures, Lowe *et al.* (1987*a*) made a detailed study of the life cycle of *N. hurleyensis*. Their results, which are summarized in Fig. 5, provide a general description of the life cycle of a monocentric fungus. It shows that *N. hurleyensis* exhibits endogenous zoosporangial development, i.e. the encysted zoospore retains the nucleus and enlarges into a zoosporangium. During the first 6·5 h of growth of the thallus illustrated in Fig. 5, there was rapid development of an

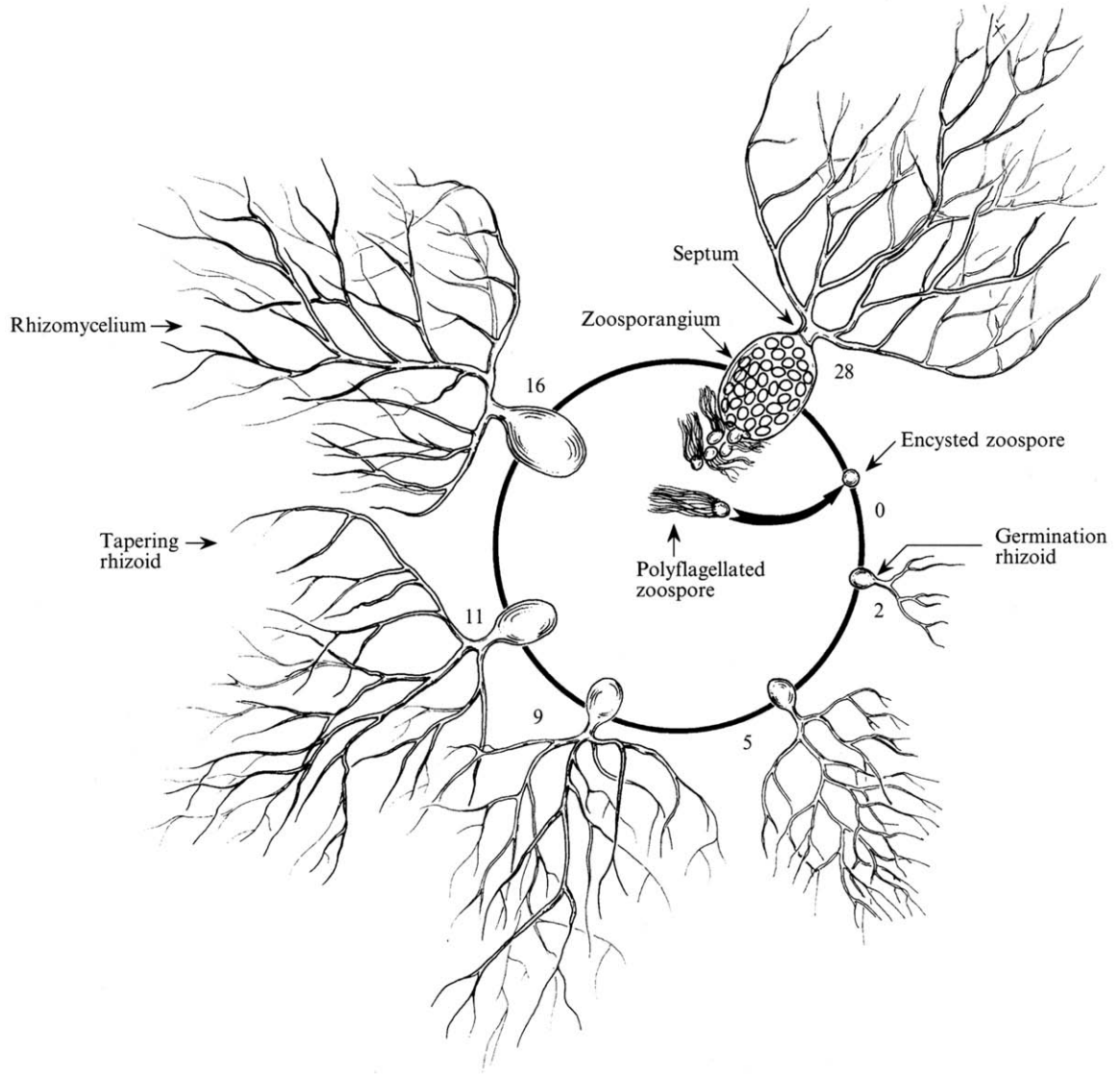

Fig. 5. Diagrammatic representation of the life cycle of the monocentric, anaerobic fungus, *Neocallimastix hurleyensis* originally isolated from the rumen of sheep (after Theodorou *et al.*, 1992*a*). The numbers represent hours after encystment of the zoospore.

extensive, non-septate, non-nucleate (Fig. 4 B), highly branched rhizomycelium; during this period the 'main' rhizoid increased in length exponentially, with a doubling time of 2·49 h. Between 6·5 and 9·5 h after inoculation, the rate of extension of the main rhizoid declined, and no further extension occurred after 9·5 h. The zoosporangium initially increased in volume at an exponential rate with a doubling time of 1·56 h, but between 14 and 20 h growth of the zoosporangium decelerated and little growth occurred after 20 h: the zoosporangium in Fig. 5 had a final volume of $2{\cdot}5 \times 10^5$ μm^3. At *ca* 21 h after encystment, a septum was formed at the base of the zoosporangium, and this event was correlated with a cessation of zoosporangial growth and the onset of zoosporogenesis (at 27 h). The formation of the septum presumably prevented cytoplasm and/or nutrients moving from the rhizomycelium to the zoosporangium. During zoosporogenesis, the protoplasm cleaved to produce uninucleate zoospores, which were eventually liberated through a pore formed in the zoosporangial wall opposite the 'main' rhizoid. By contrast, zoospores of *N. frontalis* are liberated by dissolution of the entire zoosporangial wall (Heath *et al.*, 1983). About 3 h after zoospore release, the rhizomycelium became less refractive, suggesting that autolysis had occurred. Thus *N. hurleyensis* has a determinate life cycle.

When swimming, the flagella of *Neocallimastix* spp. beat together in a clockwise direction as if they were a single flagellum, and thus propel zoospores forward in a spiral manner (Wubah, Fuller & Akin, 1991). Alternatively, the zoospores migrate by amoeboid movement (Lowe *et al.*, 1987*a*), a type of movement also observed in zoospores of aerobic chytrids (Karling, 1978). Depending on conditions, zoospores remain motile from between a few minutes to up to 2 h after liberation from the zoosporangium (Lowe *et al.*, 1987*a*), but eventually they are attracted to a suitable substrate (typically a plant fragment) by chemotaxis (Orpin & Bountiff, 1978), attach to it, shed or absorb their flagella (Munn, Orpin & Hall, 1981; Heath *et al.*, 1983; Lowe *et al.*, 1987*a*), and encyst. The most remarkable fact about the loss of flagella from encysting zoospores is that there is also loss of kinetosomes and the perikinetosomal apparatus (Heath,

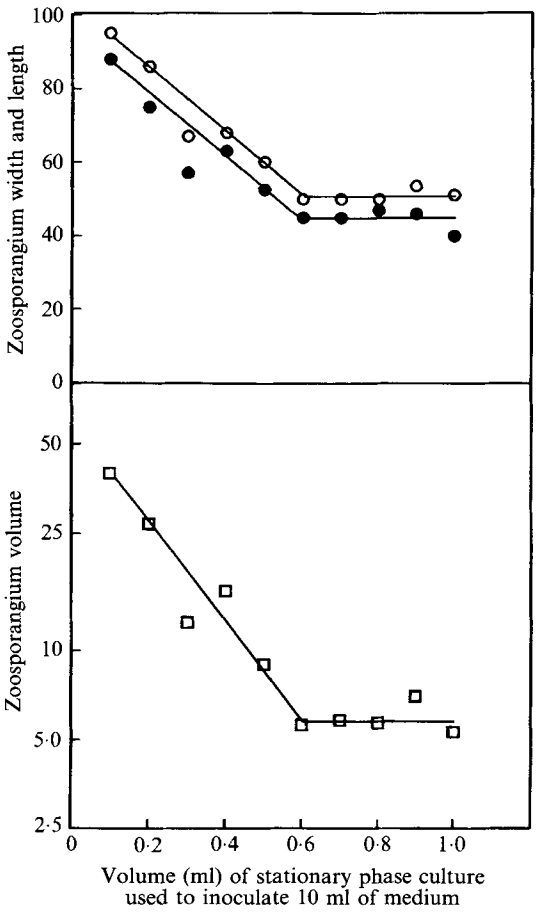

Fig. 6. Effect of zoospore concentration on the final volume (i.e. volume at zoosporangenesis) of zoosporangia of *N. hurleyensis*. 'Coverslip' cultures (Lowe *et al.*, 1987*a*) were grown at 39° in 10 ml volumes of defined medium B containing 25 mM glucose inoculated with various volumes of the culture supernatant of a stationary-phase (3-d-old) culture grown under the same conditions as the experimental culture; each point is the mean of five replicates (N. Fernando, M. K. Theodorou & A. P. J. Trinci, previously unpublished result). □, Volume ($\mu m^3 \times 10^{-4}$, log scale); ●, width; ○, length (μm).

Kaminsky & Bauchop, 1986). The implications of this are twofold; first, it implies that kinetosomes are not autonomous but arise *de novo* during zoosporogenesis, and secondly, it implies that a mechanism must exist for a rapid fusion of plasma membrane around the site of each deleted kinetosome (Munn, 1994).

The cyst germinates to form rhizoids which invade the plant material (Bauchop 1979*a*, *b*, 1980). Longitudinally arranged microtubules are abundant in the core region of filamentous rhizoids of *Neocallimastix* sp. (Munn *et al.*, 1988; Munn, 1994) and hydrogenosomes are frequently associated with microtubules, and all rhizoids contain characteristic entities called 'crystals with spots' (Munn *et al.*, 1988).

At 39°, and under conditions for growth which are unrestricted (nutrients present in excess, and absence of growth inhibitors), the life cycle of *N. hurleyensis* lasts 29–31 h and culminates in the release of an average of 88 zoospores per zoosporangium (Lowe *et al.*, 1987*a*). Thus, assuming that nuclear division in the zoosporangium is synchronous (Gaillard, Breton & Bernalier, 1989), most thalli form 64 or 128 zoospores per zoosporangium. Fig. 6 shows the inoculum concentration has an appreciable effect on the final size of zoosporangia of *N. hurleyensis*, and hence on the number of zoospores produced per zoosporangium. Others (Heath *et al.*, 1983; Orpin, 1976) have shown that zoosporangia can produce between 1 and 114 zoospores.

Piromyces spp.

Zoospores of *Piromyces* spp. have a single posteriorly directed flagellum. Zoosporangial development is either endogenous or exogenous. In the latter case there is two-sided germination of the zoospore cyst; initially, a germ-tube develops into an extensive, filamentous rhizomycelium as in *Neocallimastix*, but then a tubular outgrowth develops on the side opposite the main rhizoid into which the nucleus migrates to form a zoosporangium at its tip (Barr *et al.*, 1989).

Caecomyces spp.

Caecomyces spp. produce zoospores which each have a single posterior flagellum. Gold *et al.* (1988) and Wubah & Fuller (1991) described the life cycles of *C. equi* and *C. communis* respectively. Upon germination, a cyst of *C. equi* forms a broad germ-tube (Fig. 7) which enlarges into a branched or unbranched spherical holdfast (Karling, 1978), which is highly vacuolate and adheres to the substrate (Gaillard & Citron, 1989); this structure has also been called a spherical body (Lowe *et al.*, 1987*b*) vesicle (Gaillard & Citron, 1989) and vegetative cell (Wubah & Fuller, 1991). Microtubules are abundant around the rim of the holdfast (Munn *et al.* 1988; Munn, 1994). Development of *C. equi*, like *N. hurleyensis*, is endogenous, i.e. the encysted zoospore retains the nucleus and enlarges into a zoosporangium. Consequently, at maturity, the thallus consists of a multinucleate zoosporangium and a non-nucleate spherical holdfast (Fig. 7). For *C. communis*, Orpin (1976) observed 'only vegetative structure bearing a *single* sporangium in samples taken from the rumen', but thalli bearing two to three zoosporangia were observed in axenic culture. Similarly, although Wubah & Fuller (1991) found that *in vitro* some zoospores of *C. communis* developed endogenously like *C. equi* (Fig. 7), others developed exogenously, i.e. the nucleus migrated out of the zoospore into the holdfast. When the latter event occurred, the holdfast contained nuclei and developed two to four sporangial stalks which, at maturity, were terminated in zoosporangia. It may be significant that this polycentric type of development has only been observed *in vitro* (Orpin, 1976).

Fig. 8 shows the growth of a thallus of *C. communis* which eventually formed four zoosporangia. For the first 16 h after germination, the thallus (holdfast plus zoosporangia) increased in volume exponentially with a doubling time of 3·16 h. At this time, growth of the holdfast ceased although growth of the zoosporangia continued until 22 h after germination. Zoospores of *Caecomyces* spp. are probably released by dissolution of the entire zoosporangial wall (Orpin, 1976; Gold *et al.*, 1988).

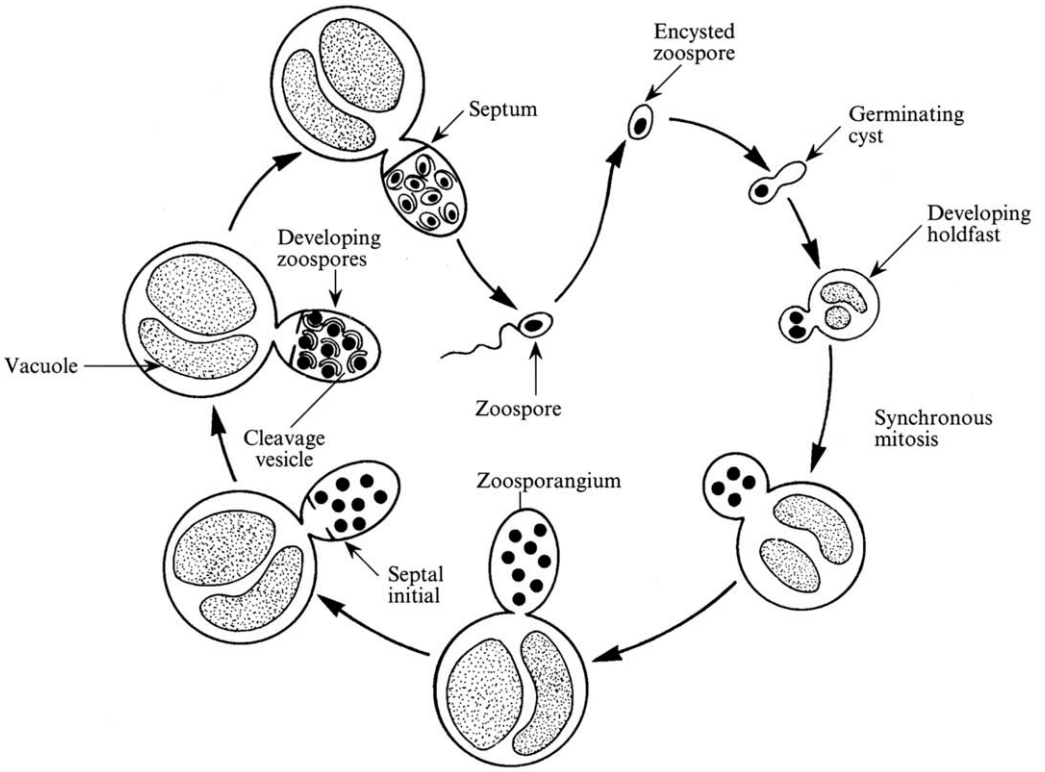

Fig. 7. Diagrammatic representation of the life cycle of *Caecomyces equi* isolated from the caecum of the horse (after Gold *et al.*, 1988).

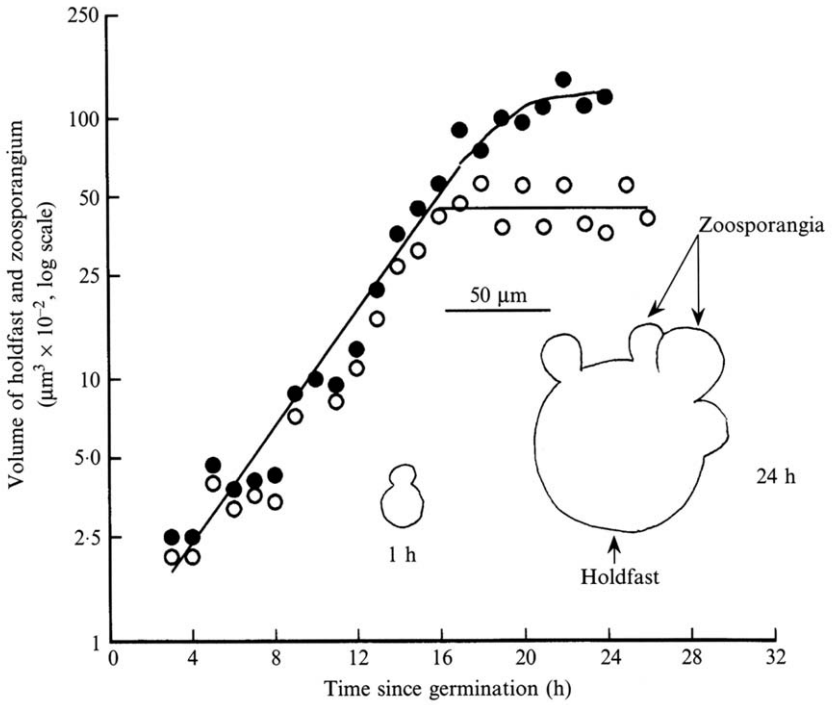

Fig. 8. Growth of a thallus of *C. communis* at 39° in a roll tube on defined medium B containing 25 mM glucose. The isolate was kindly provided by Professor M. Fèvre and was grown in a roll tube as described by Lowe *et al.* (1987*a*); the volume of the thallus was calculated assuming that the holdfast and the zoosporangia were perfect spheres (D. R. Davies, M. K. Theodorou & A. P. J. Trinci, previously unpublished result). The inset diagrams show the morphology of the thallus at various times after germination. ○, Holdfast; ●, holdfast and zoosporangium.

Life-cycle of polycentric anaerobic fungi

Upon encystment, the zoospore of a polycentric anaerobic fungus forms a germination rhizoid into which the nucleus migrates (Barr *et al.*, 1989; Gaillard *et al.*, 1989); the zoospore then becomes redundant (Breton *et al.*, 1989). As with some monocentric fungi, a highly branched rhizomycelium develops, but in this case some of the rhizoids contain nuclei (Fig. 4C, D). Zoosporangia are formed on sporangiophores produced by the rhizomycelium either singly or in groups of up

to six (Barr *et al.*, 1989; Ho *et al.*, 1990); the sporangiophores develop either intercalary or terminally on the rhizoids (Barr *et al.*, 1989; Breton *et al.*, 1989; Ho *et al.*, 1990). When mature, the zoosporangium releases zoospores which have 1–16 flagella (Breton *et al.*, 1989, 1990; Ho *et al.*, 1990), *Anaeromyces* spp. producing monoflagellated zoospores, and *Orpinomyces joyonii* producing polyflagellated zoospores (Table 1). Barr (1983) considered the development of polycentric thalli to be a major step in chytridiomycete evolution, as such thalli produce many zoosporangia and have the capability of vegetative reproduction by fragmentation of the rhizomycelium: thus, unlike monocentric fungi, polycentric fungi have indeterminate life-cycles and are not dependent upon the formation of zoospores for their continued survival. For example, the SR2 polycentric isolate illustrated in Fig. 4C, D has been maintained in culture despite not producing zoosporangia or zoospores.

An important difference between anaerobic fungi with endogenous and exogenous zoosporangial development is that, although mitosis proceeds in both groups, nuclear migration only occurs in the latter. Osmani, Osmani & Morris (1990) identified a gene product (a 22 kDa protein coded by *nudC*) in *Aspergillus nidulans*, which is specifically required for nuclear migration. It would be interesting to see if a gene similar to *nudC* is present in displaying exogenous anaerobic fungi zoosporangial development.

Sexual reproduction

In the aerobic chytridiomycetes, sexual reproduction occurs by the fusion of isoplanogametes or aplanogametes, or by the fusion of gametangia (Karling, 1978). To date, no sexual stage of an anaerobic fungus has been described, and only mitotic nuclear divisions have been observed (Heath & Bauchop, 1985). However, the fact that *Callimastix cyclopsis* has an alternation of generations, with an asexual stage in copepods and a sexual stage in mosquito larvae (Whisler, Zebold & Shemanchuk, 1975), suggests that the anaerobic fungi may also have a sexual stage, perhaps in an alternative host.

Resistant stages in the life-cycle

In the aerobic chytrids, resistant structures are formed following sexual reproduction, or by the formation of resistant zoosporangia or cysts (Karling, 1978). However, the occurrence of such structures in anaerobic fungi has not been confirmed, although the ability to isolate anaerobic fungi from air-dried faeces of both ruminant and monogastric herbivores suggests they probably exist (Milne *et al.*, 1989; Davies, Theodorou & Trinci, 1990; Theodorou *et al.*, 1990, 1994; Davies *et al.*, 1993). After drying, populations of anaerobic fungi in faeces decline very slowly, and isolations can still be made for up to 10 months after the onset of drying (Milne *et al.*, 1989; Theodorou *et al.*, 1990). Anaerobic fungi have even been isolated in Ethiopia from sun-baked dung of cattle and sheep (Milne *et al.*, 1989).

Thus the anaerobic fungi appear to have the ability to pass through the entire digestive tract of ruminants to be voided ultimately in faeces, and indeed Davies *et al.* (1993) have isolated anaerobic fungi from each part of the digestive tract of cattle (Fig. 1). Significant populations of anaerobic fungi were isolated by Davies *et al.* (1993) from dried digesta from most organs of the digestive tract, including the omasum and abomasum, but not from the rumen. To account for these observations, Davies *et al.* (1993) suggested that the generally accepted life-cycles (Figs 5 and 7) of anaerobic fungi should be altered to include a resistant stage (cyst or zoosporangium). Although resistant structures have yet to be positively identified in anaerobic fungi, Wubah *et al.* (1991) demonstrated the formation of a putative resistant zoosporangium in a *Neocallimastix* sp. which had a melanized wall and contained nuclei with four times the DNA content of nuclei in zoospores. However, so far germination of these structures has not been observed (Wubah *et al.*, 1991).

DISTRIBUTION OF ANAEROBIC FUNGI

Geographical distribution

Since their first isolation in the U.K. from the rumen of sheep (Orpin, 1975), anaerobic fungi have been isolated from animals in Australia (Phillips, 1989; Lawrence, 1993), Canada (Kudo *et al.*, 1990), Chile (Dr M. K. Theodorou; personal communication), Czechoslovakia (Novozamska, 1987), Ethiopia (Milne *et al.*, 1989), France (Fonty *et al.*, 1987), Holland (Teunissen *et al.*, 1991), Indonesia (Dr M. K. Theodorou; personal communication), Japan (Ushida, Tanuka & Kojima, 1989), Malaysia (Ho, Abdullah & Jalaludin, 1988*a*, *b*; Lawrence, 1993), New Zealand (Bauchop, 1979*a*, *b*), Norway (Orpin *et al.*, 1985), Russia (Kostyukovsky, Okunev & Tarakanov, 1991), Tanzania (Breton *et al.*, 1991), and the U.S.A. (Akin, Borneman & Windham, 1988). It would appear, therefore, that anaerobic fungi have a worldwide distribution.

Distribution amongst herbivores

Table 2 shows that anaerobic fungi have been isolated from the smallest herbivore, the blue duiker (Dehority & Varga, 1991), to the largest herbivore, the elephant (Milne *et al.*, 1989); they have even been isolated from a large rodent, the mara (Teunissen *et al.*, 1991). These reports suggest that anaerobic fungi are ubiquitous amongst ruminant and hindgut-fermenting herbivores (Table 2). However, anaerobic fungi have not been isolated from habitats other than the digestive tract of herbivorous mammals and their faeces; attempts to isolate them from mud and from landfill sites have proved unsuccessful (Orpin & Joblin, 1988; Theodorou & King-Spooner, 1989).

Oxygen tolerance and transfer of anaerobic fungi between animals

In order to survive, rumen micro-organisms must be transferred from adult to offspring and in so doing may be exposed to oxygen. The toxicity of oxygen is related to the partly reduced, reactive intermediates generated during the univalent reduction of oxygen to water, namely the superoxide radical

(O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^\bullet$). Small amounts of both O_2^- and H_2O_2 are normal products of oxygen reduction. In oxygen-tolerant organisms O_2^- is eliminated by superoxide dismutases while H_2O_2 is removed by hydroperoxidases (Hassan, 1989). Oxygen is toxic to obligately anaerobic bacteria because they are unable to synthesize superoxide dismutase, and presumably anaerobic fungi also lack this enzyme. However, Lowe *et al.* (1987*b*) isolated anaerobic fungi from saliva taken from sheep, and Milne *et al.* (1989) showed that anaerobic fungi survived for up to 8 h in sheep saliva stored in air at 39°. Thus, anaerobic fungi can tolerate exposure to oxygen for short periods. The results of Lowe *et al.* (1987*b*) and Milne *et al.* (1989) suggest that, like bacteria (Hungate, 1966), anaerobic fungi may be present in aerosols formed from saliva and by this means become dispersed between ruminants. However, direct contact between mother and offspring during grooming or through shared food, is a more likely route for transfer of anaerobic fungi in nature.

Anaerobic fungi have been isolated from fresh faeces (Lowe *et al.*, 1987*b*) and dried faeces stored in air for a number of months (Milne *et al.*, 1989; Theodorou *et al.*, 1990). These results, together with those of Davies *et al.* (1993), suggest that faeces may serve as a route for transfer of anaerobic fungi between herbivores: ruminants are not coprophagic, but accidental contact with fresh or dried faeces may occur, facilitating transfer of anaerobic fungi between ruminants and non-ruminants alike.

By successfully inoculating two 12-week-old Australian lambs with a *Neocallimastix* sp. isolated from a Norwegian deer and a *Piromyces* sp. isolated from a French horse, Orpin (1989) showed that these anaerobic fungi were not host-specific. However, further studies are required before deciding whether the same fungal species are present in all herbivores, or whether there is some degree of host specificity.

ENERGY METABOLISM AND FERMENTATION CHARACTERISTICS

Like many eukaryotes adapted to anaerobic environments, anaerobic fungi lack mitochondria (Yarlett *et al.*, 1986; Munn *et al.*, 1988; Gaillard & Citron, 1989). Instead they obtain energy by the anaerobic fermentation of carbohydrates, which act as both electron acceptors and electron donors (Stanier *et al.*, 1987). Anaerobic fermentation is a less efficient energy-yielding process than aerobic respiration because part of the energy present in the substrate remains in fermentation end-products such as ethanol, lactic acids, formate, etc. (Stanier *et al.*, 1987). By contrast, substrates utilized for aerobic respiration are completely oxidized to H_2O and CO_2. Degradation of one mole of glucose gives 36 molecules of ATP by aerobic respiration, but only two molecules of ATP by anaerobic fermentation (Stanier *et al.*, 1987). Consequently, the yield coefficient (mg biomass produced mg^{-1} glucose utilized) for a fungus like *N. hurleyensis* (0·14) grown anaerobically (Lowe *et al.*, 1987*c*) is much lower than for a fungus like *Geotrichum candidum* (0·45) grown aerobically (Caldwell & Trinci, 1973).

Anaerobic fungi have mixed-acid fermentation profiles similar to those of enterobacteria such as *Escherichia coli* (Table 3). Thus they convert hexose to formate, acetate, lactate succinate, ethanol, CO_2 and H_2 (Bauchop & Mountfort, 1981; Lowe, Theodorou & Trinci, 1987*c*; Phillips & Gordon, 1988; Borneman, Akin & Ljungdahl, 1989). However, at least one species, *Neocallimastix patriciarum,* produces only trace amounts of formate and ethanol from glucose (Orpin & Munn, 1986). Little is known about the fermentation profiles of anaerobic fungi on substrates other than glucose, but Lowe *et al.* (1987*b*) showed that the utilization of xylose by *N. hurleyensis* yielded the same end-products as those for fermentation on glucose (Table 3), except that acetate rather than formate was the main end-product of the fermentation.

Like rumen protozoa (Müller, 1980), anaerobic fungi contain hydrogenosomes (Fig. 9). Hydrogenosomes are characterized by the possession of a pyruvate:ferredoxin oxidoreductase and hydrogenase (Yarlett *et al.*, 1986), and were first described in *N. patriciarum* as amorphous globules, each consisting of a homogeneous, electron-dense matrix enclosed by a single membrane. In all free zoospores studied, hydrogenosomes

Table 3. Fermentation profiles of *Escherichia coli* and monocentric anaerobic fungi (mole product formed per 100 mole glucose fermented)

Fermentation end-product	*Escherichia coli*[a]	*Neocallimastix hurleyensis*[b]	*Piromyces* MC1[c]	*Caecomyces* NM1[d]
Formate	3	77	92	40
Acetate	36	70	83	33
Ethanol	50	26	62	15
D(−)-Lactate	79	48	44	67
L(+)-Lactate	ND	ND	15	0·6
Succinate	11	ND	0	TR
CO_2	88	19	57	7
H_2	75	62	62	ND
Carbon recovery (%)	ND	87	102	58

[a] Stanier *et al.* (1987); [b] Lowe *et al.* (1987*c*); [c] Borneman *et al.* (1989); [d] Phillips & Gordon (1988).

TR, trace amount; ND, not determined.

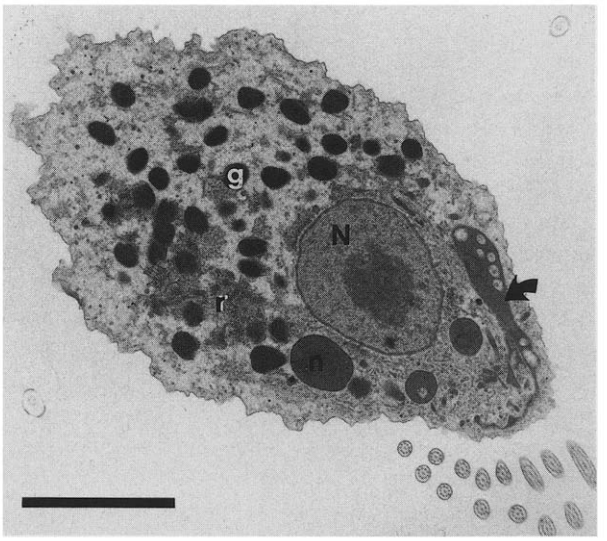

Fig. 9. Section through a zoospore of *Neocallimastix hurleyensis* showing granular microbodies (g), non-granular microbodies (n), ribosomes (r), nucleus (N) and hydrogenosome (arrow). Scale bar, 2·5 μm.

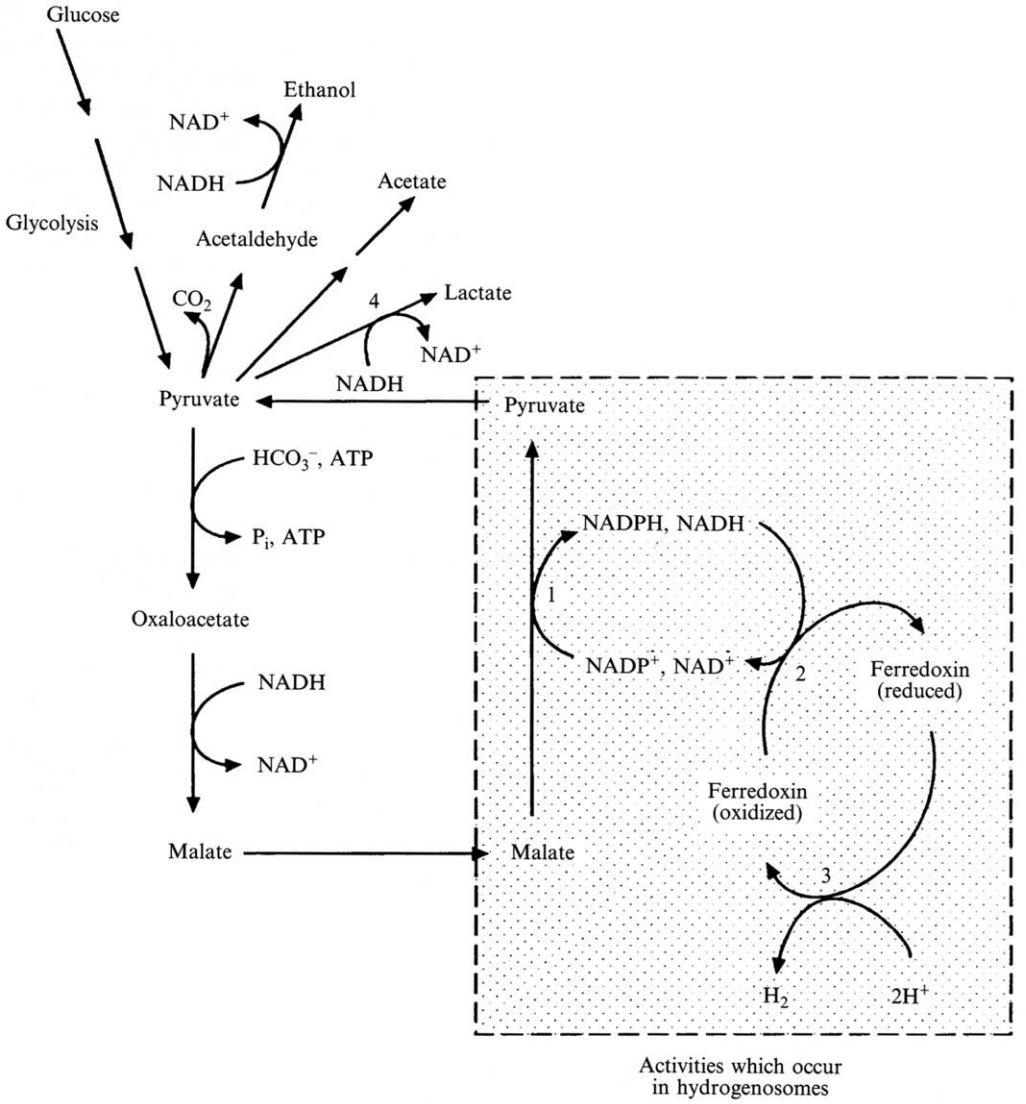

Fig. 10. Conversion of glucose into acetate, ethanol and lactate by *Neocallimastix frontalis*. Numbers designate the following enzymes: 1, 'malic' enzyme; 2, NADH(NADPH):ferredoxin oxidoreductases; 3, hydrogenase; 4, lactate dehydrogenase (adapted from O'Fallon *et al.*, 1991).

occupy the same region of the cell and, due to their close proximity to the flagella (and by analogy with mitochondria in aerobic zoospores) it is assumed that their primary function is to provide energy for motility in the form of ATP for driving the flagella.

The carbon and electron flow pathways for glucose metabolism have been demonstrated for *N. patriciarum* and *N. frontalis* (Yarlett *et al.*, 1986; O'Fallon, Wright & Calza, 1991). In *N. frontalis* (Fig. 10), O'Fallon *et al.* (1991) showed that the pyruvate produced by glycolysis is converted via pyruvate carboxylase into oxaloacetate, which is reduced by malate dehydrogenase to malate. The malate is transported into the hydrogenosomes (Yarlett *et al.*, 1986), where enzymes generate energy by coupling its oxidation to electron transfer via NADH:ferredoxin oxidoreductase; proton reduction is then achieved via ferredoxin and hydrogenase to proton reduction, with the concomitant generation of H_2. The pyruvate generated by the oxidation of malate is transported into the cytoplasm where it can be converted back into oxaloacetate, reduced by lactate dehydrogenase to lactate, metabolized to acetate, or decarboxylated to acetaldehyde by pyruvate decarboxylase with the subsequent formation of ethanol (Yarlett *et al.*, 1986; O'Fallon *et al.*, 1990). In *N. patriciarum* the synthesis of pyruvate: ferredoxin oxido reductase is suppressed when the organism in cultured under an atmosphere of CO_2 (Yarlett *et al.*, 1986), the usual condition in laboratory-grown cultures. D(−)lactate dehydrogenase has been identified in *Neocallimastix* sp., *Piromyces* sp. and *Caecomyces* sp. (Gleason & Gordon, 1990) and also in a small number of aerobic Chytridiomycetes, namely *Allomyces*, *Blastocladiella* and *Blastocladia*.

The metabolic pathway for glucose metabolism in *N. patriciarum* (Yarlett *et al.*, 1986) differs slightly from that of *N. frontalis* (O'Fallon *et al.*, 1991). Firstly, *N. particiarum* converts glucose to phosphoenol-pyruvate, which is subsequently carboxylated to oxaloacetate before reduction to malate. Secondly, the pyruvate produced in the hydrogenosomes can, in addition to conversion into fermentation products, be converted by a pyruvate kinase to phosphoenolpyruvate.

Gas production has been used as an indicator of biomass to

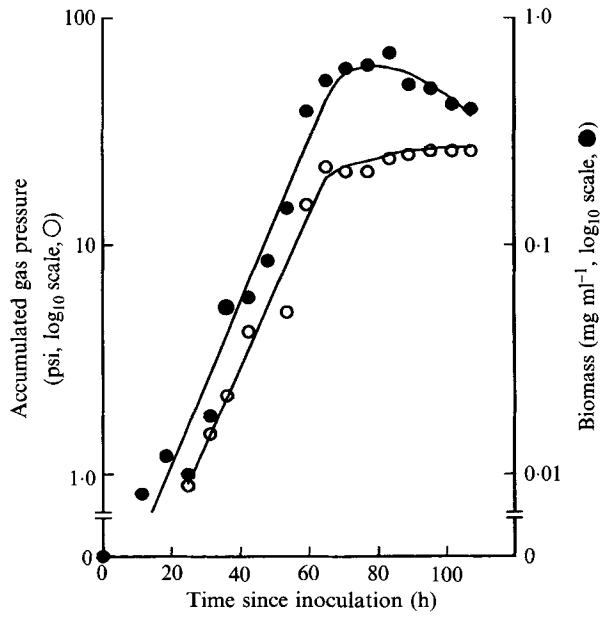

Fig. 11. Growth of *N. hurleyensis* at 39° in 100 ml of defined medium B containing 25 mM glucose. The inoculum was taken from 3-d-old cultures grown on the same medium. The gas pressure measurements were made (prior to harvesting the biomass) using a detachable pressure transducer attached to an LED digital-readout voltmeter. The voltameter was calibrated to read units of pressure in lb in^{-2} (from Lawrence, 1993).

follow the growth of anaerobic fungi in batch culture (Lawrence *et al.*, 1990; Lawrence, 1993). Fig. 11 shows that *N. hurleyensis* grows exponentially in batch culture, with the biomass data giving a specific growth rate of 0·080 h^{-1} (doubling time of 8·7 h) and the biogas data giving a specific growth rate of 0·074 h^{-1} (doubling time of 9·4 h). The figure also shows that during the exponential phase of growth there is a high correlation between biogas pressure and culture dry weight, and both biomass indicators showed that the culture entered stationary phase *ca* 82 h after inoculation. Thus biogas pressure can be used to measure the specific growth rate and yield of anaerobic fungi in batch culture, but cannot be used to follow autolysis (Fig. 11). Biogas pressure is a convenient method of following the growth of anaerobic fungi on insoluble substrates such as plant material (Lawrence *et al.*, 1990; Theodorou *et al.*, 1992b; Lawrence, 1993).

SUBSTRATE UTILIZATION

Anaerobic fungi in the rumen colonize a variety of cultivated plants, including wheat straw, rice straw, maize, soya-bean hulls and both temperate and tropical grasses (Akin, Gordon & Hogan, 1983; Lowe, Theodorou & Trinci, 1987*d*; Grenet & Barry, 1988; Akin, Borneman & Lyon, 1990; Ho, Abdullah & Jalaludin, 1991; Roger *et al.*, 1992). They can even colonize such highly recalcitrant plant materials as palm press fibre and wood (Joblin & Naylor, 1989; Ho *et al.*, 1991).

Cellulose is used by most genera of anaerobic fungi (Table 4), although *Caecomyces* spp. apparently do not degrade this polymer (Fonty, Gouet & Sante, 1988; Hébraud & Fèvre, 1988; Phillips & Gordon, 1988). The major hemicellulosic component of graminaceous plant cell walls, xylan, is also readily utilized by anaerobic fungi (Table 4). Although Orpin (1983/84) showed that 20–40% of the pectin in wheat straw leaves was degraded (solubilized) during fungal growth, pectin and pectin breakdown products are generally not fermented by anaerobic fungi (Phillips & Gordon, 1988). However, several isolates of anaerobic fungi obtained from herbivores in Australia and Malaysia grew on pre-washed apple pectin as the sole carbon source, and some of these also grew on polygalacturonate (Lawrence, 1993). No monocentric anaerobic fungus utilizes arabinose, and only one isolate utilizes galactose. These findings are surprising, because arabinose and galactose are common components of plant cell walls and are liberated during their hydrolysis (Theodorou *et al.*, 1989). Furthermore, pathways for their degradation are known in other micro-organisms (Gottschalk, 1985).

Gordon & Phillips (1988) tested 17 monocentric fungi for carbohydrate utilization, including *N. frontalis* and *P. communis*, whose substrate utilization profiles had previously been determined. The data in Table 4 include results from Gordon & Phillips (1988) and information about carbohydrate utilization by *N. hurleyensis* (Lowe *et al.*, 1987*b*) and *N. patriciarum* (Orpin & Bountiff, 1978; Orpin & Letcher, 1979; Orpin & Munn, 1986; Williams & Orpin, 1987*a*). Monosaccharide utilization by anaerobic fungi appears to be limited to glucose, fructose and xylose, with the possible exception of *N. patriciarum*, which can also use galactose for growth (Orpin & Letcher, 1979; Phillips & Gordon, 1988). The disaccharides cellobiose, gentiobiose, lactose and maltose are used by the majority of anaerobic fungi, although some can use sucrose and the trisaccharide raffinose also (Table 4). Table 4 shows that, with few exceptions, *Neocallimastix* and *Caecomyces* isolates have similar substrate utilization profiles, although the latter isolates utilize fewer substrates than the other monocentric genera (Phillips & Gordon, 1988; Bernalier, Fonty & Gouet, 1990). Substrate utilization profiles of some *Piromyces* isolates resemble those of *Neocallimastix* isolates, whereas others have utilization profiles which resemble *Caecomyces* (Phillips & Gordon, 1988).

Substrate utilization profiles have been determined for two polycentric isolates. *O. joynii* and *A. mucronatus* (Breton *et al.*, 1989, 1990). In addition to the sugars utilized by *Neocallimastix* isolates (Table 4), these fungi utilize D/L-arabinose, D-mannose and pectin (Breton *et al.*, 1989, 1990). However, apart from *A. mucronatus*, which was able grow on cellobiose (Breton *et al.*, 1990), these polycentric fungi were not tested with the disaccharides listed in Table 4.

PLANT CELL-WALL DEGRADATION BY ANAEROBIC FUNGI

Anaerobic fungi produce a wide range of hydrolytic enzymes including cellulases (Lowe *et al.*, 1987*d*; Barichievich & Calza, 1990), hemicellulases (Lowe *et al.*, 1987*d*; Mountfort & Asher, 1989), proteases (Wallace & Joblin, 1985), amylases, amyloglycosidases (Pearce & Bauchop, 1985; Mountfort & Asher, 1988), feruloyl and *p*-coumaroyl esterase (Borneman *et al.*, 1990, 1991), various disaccharidases (Hébraud & Fèvre, 1988) and pectinases (Gordon & Phillips, 1992).

Table 4. Utilization of carbohydrates by *Neocallimastix* spp., *Piromyces* spp. and *Caecomyces* spp.

	N. frontalis (MCH3) [1]	*N. frontalis* (PN1) [2], [3]	*N. patriciarum* [3], [4], [5]	*N. hurleyensis* (R1) [6]	F1 [9]	LM1 [3], [8]	LM2 [3], [8]	LM4 [3], [8]	PN2 [3], [8]	S5 [3], [8]
Monosaccharides										
Arabinose	−	−	ND	−	−	−	−	−	−	−
Fructose	+	+	−	+	+	+	+	+	+	+
Fucose	−	ND	−	ND	ND	−	−	−	−	−
Galactose	−	−	+	−	−	−	−	−	−	−
Glucose	+	+	+	+	+	+	+	+	+	+
Mannitol	ND	ND	+	ND	ND	−	−	−	−	−
Mannose	±	−	−	−	−	−	−	−	−	−
Rhamnose	ND	ND	ND	ND	−	−	−	−	−	−
Ribose	ND	ND	ND	−	−	−	−	−	−	−
Sorbitol	ND	ND	−	ND	ND	−	−	−	−	−
Sorbose	ND	ND	−	−	ND	−	−	−	−	−
Xylitol	ND	−	ND	ND	ND	−	−	−	−	−
Xylose	+	+	+	+	+	+	+	+	+	+
Di/Trisaccharides										
Cellobiose	+	+	+	+	+	+	+	+	+	+
Gentiobiose	±	ND	ND	ND	ND	+	+	+	+	+
Lactose	+	ND	ND	(+)	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+
Raffinose	±	(+)	±	+	ND	+	+	+	+	+
Sucrose	−	+	+	+	+	+	+	+	+	+
Polysaccharides										
Cellulose (filter paper)	ND	+	+	+	+	+	+	+	+	+
Carboxymethyl-cellulose	ND	ND	−	−	−	−	−	−	−	−
Xylan	ND	+	+	+	+	+	+	+	+	+
Starch	ND	+	+	+	+	+	+	+	+	+
Glycogen	ND	+	+	+	ND	+	+	+	+	+
Pullulan (1,6-α-glucan)	ND	+	+	ND	ND	+	+	+	−	+
Pustulan (1,6-β-glucan)	ND	+	+	ND	ND	+	+	−	+	+
Inulin	ND	+	+	+	−	+	+	+	+	+
Pectin	ND	−	−	−	−	−	−	−	−	−
Polygalacturonate	ND	ND	−	ND	ND	−	−	−	−	−

	P. communis								*C. communis*			
	[1], [3] [7], [8]	LM7 [3], [8]	NF1 [3], [8]	SM5 [3], [8]	SM1 [3], [8]	SM2 [3], [8]	SM3 [3], [8]	LM8 [3], [8]	(FG10) [1]	NM1 [3], [8]	NM2 [3], [8]	CN1 [3], [8]
Monosaccharides												
Arabinose	−	−	−	−	−	−	−	−	−	−	−	−
Fructose	+	+	+	+	+	+	+	+	+	+	+	+
Fucose	−	−	−	−	−	−	−	−	−	−	−	−
Galactose	−	−	−	−	−	−	−	−	−	−	−	−
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Mannose	−	−	−	−	−	−	−	−	−	−	−	−
Rhamnose	−	−	−	−	−	−	−	−	−	−	−	−
Ribose	−	−	−	−	−	−	−	−	−	−	−	−
Sorbitol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sorbose	−	−	−	−	−	−	−	−	−	−	−	−
Xylitol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Xylose	+	+	+	+	+	+	−	−	±	+	+	+
Di/Trisaccharides												
Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+
Gentiobiose	+	+	+	+	+	+	+	+	±	+	+	+
Lactose	±	+	+	+	+	+	+	+	±	+	+	+
Maltose	+	+	+	+	−	−	+	+	+	−	−	−
Raffinose	±	−	+	+	−	−	−	−	−	−	−	−
Sucrose	±	−	+	+	−	−	+	+	−	−	−	−

Table 4. *(cont.)*

	P. communis								*C. communis*			
	[1], [3] [7], [8]	LM7 [3], [8]	NF1 [3], [8]	SM5 [3], [8]	SM1 [3], [8]	SM2 [3], [8]	SM3 [3], [8]	LM8 [3], [8]	(FG10) [1]	NM1 [3], [8]	NM2 [3], [8]	CN1 [3], [8]
Polysaccharides												
Cellulose (filter paper)	+	−	+	+	+	+	−	+	ND	−	−	−
Carboxymethyl-cellulose	−	−	−	−	−	−	−	−	ND	−	−	−
Xylan	+	+	+	+	+	+	+	+	ND	+	+	+
Starch	+	+	+	+	−	−	+	+	ND	−	−	−
Glycogen	+	+	+	+	−	−	+	+	ND	ND	ND	ND
Pullulan (1,6-α-glucan)	+	−	+	+	−	−	+	+	ND	−	−	−
Pustulan (1,6-β-glucan)	+	−	+	+	−	−	+	−	ND	−	−	−
Inulin	−	−	−	−	−	−	−	−	ND	−	−	−
Pectin	−	−	−	−	−	−	−	−	ND	−	−	−
Polygalacturonate	−	−	−	−	−	−	−	−	ND	−	−	−

Not utilized; +, utilized; (+), poor utilization; ±, variable characteristic; ND, not determined.

[1] Bernalier *et al.* (1990); [2] Mountfort & Asher (1983); [3] Phillips & Gordon (1988); [4] Orpin & Bountiff (1978); [5] Orpin & Letcher (1979); [6] Lowe *et al.* (1987*c*); [7] Williams & Orpin (1987*a*, *b*); [8] Gordon & Phillips (1989); [9] Lowe *et al.* (1987*b*).

After a prolonged fermentation period (> 72 h) monocentric and polycentric anaerobic fungi produce feruloyl and *p*-coumaroyl esterases and these enzymes may have a role in cleaving ferulic and coumaric acids from lignin/hemicellulose complexes and may be of considerable significance in the dissolution of plant cell walls (Borneman *et al.*, 1990, 1991). However, because of the anaerobic nature of the rumen, it is unlikely that enzymes capable of degrading lignin are produced by anaerobic fungi (Gordon & Ashes, 1984; Windham & Akin, 1984; Theodorou *et al.*, 1989), although some results suggest that lignin may be partly solubilized by these organisms (Orpin, 1981*b*; Akin *et al.*, 1983).

Cellulose is the most abundant biopolymer on earth, but relatively few fungi produce cellulases able to degrade crystalline cellulose. Among them are *Trichoderma* spp. and the anaerobic fungi (Wood, 1991; Pearce & Bauchop, 1985; Hébraud & Fèvre, 1988). Some cellulases from anaerobic fungi have quite remarkable properties, for example, the cellulase from a co-culture of *N. frontalis* and a rumen methanogen was more active (enzymes in the co-culture were able to solubilize 98% of the highly ordered cotton cellulose) than that from *Trichoderma reesei* (strain C-30), previously the source of the most active cellulase known (Wood *et al.*, 1986). Extracellular cellulases are often described as a complex consisting of enzymes which by acting together are able to solubilize cellulose (Person, Tjerneld & Hahn-Hägerdal, 1991; Wood, 1991). There is general agreement that this complex contains exo-1,4-β-D-glucanases (normally cellobiohydrolases), endo-1,4-β-D-glucanases and β-D-glucosidase (Wood, 1991). The combined action of the cellobiohydrolase and endoglucanase results in the conversion of cellulose to oligosaccharides and cellobiose, which is further hydrolysed to glucose by β-glucosidase (Goyal, Ghosh & Eveleigh, 1991). However, the molecular interactions involved in this synergy are poorly understood (Wood, 1989). Recently, Wilson & Wood (1992) showed that a minor component (4% of the total extracellular protein) of the cellulolytic enzymes of *N. frontalis* is present in a cellulosome-like structure which is secreted and absorbed to cellulose. Thus *N. frontalis* is similar to the bacterium *Clostridium thermocellum* in that the component which degrades crystalline cellulose contains several enzymes arranged in a complex (the cellulosome), although the cellulosome of *N. frontalis* (700 kDa) is smaller than that of *C. thermocellum* (2×10^6 kDa), and also differs in other ways. Recently, Xue *et al.* (1992*a*, *b*) have shown the existence of two types of genes that encode for cellulases and other polysaccharide hydrolases in the *N. patriciarum* genome. One type was found in multiple copies, and encodes for cellulases with a specific substrate activity (Xue *et al.*, 1992*a*). Xue *et al.* (1992*a*) found genes for one cellobiohydrolase and two endoglucanases, the expression of which was induced by cellulose. The other type of genes was found as single copies that encoded for a multi-functional polysaccharide hydrolase with three catalytic domains, each domain possessing endoglucanase, cellobiohydrolase and xylanase activities (Xue *et al.*, 1992*b*). This enzyme was constitutively expressed and was not affected by the presence of cellulose in the culture medium. This confirms the results of Lowe *et al.* (1987*d*), who observed some constitutive xylanase production, and Barichievich & Calza (1990), who detected a low level of cellulase activity in glucose-grown cultures.

Xylanases (hemicellulases) of anaerobic fungi have been described by a number of workers (Orpin & Letcher, 1979; Pearce & Bauchop, 1985; Williams & Orpin, 1998*a*; Lowe *et al.*, 1987*d*; Mountfort & Asher, 1989). Although xylan is the most effective inducer for xylanase (hemicellulase) production, xylanases are also produced when anaerobic fungi are grown on wheat straw, cellulose, cellobiose, glucose or xylose (Lowe *et al.*, 1987*d*). This suggests that a basal level of xylanase is constantly produced by anaerobic fungi but that this production is enhanced by xylan or hemicellulose. Mountfort & Asher (1989) found that the xylanase activity in *N. frontalis* was predominantly extracellular, whereas xylobiase activity was mainly cell-associated. Both these enzymes are implicated in the dissolution of xylan since xylose, xylobiase and xylo-oligosaccharides accumulate in culture filtrates during growth on xylan (Lowe *et al.*, 1987*d*; Mountfort & Asher, 1989). The

apparent lack of pectinases in some anaerobic fungi is intriguing. However, Gordon & Phillips (1992) recently demonstrated pectinolytic activity in the culture filtrate of an Australian strain of *N. frontalis*. The enzyme was an endo-acting pectin lyase which was induced in the presence of pectin.

There are few accounts of the production, properties and regulation of amylases produced by anaerobic fungi (Pearce & Bauchop, 1985; Mountfort & Asher, 1988; Phillips & Gordon, 1988). Mountfort & Asher (1988) concluded that α-amylase was mainly responsible for starch degradation by *N. frontalis*, since the products of amylolytic activity were maltose, maltobiose, maltotetraose, longer-chain oligosaccharides, but not glucose. However, in an earlier study by Pearce & Bauchop (1985), partial digestion of starch by crude enzyme extracts from *N. frontalis* led to the accumulation of glucose in the absence of oligosaccharides (= limit-dextrans). Thus, it was concluded that amyloglucosidase and not α-amylase was responsible for starch digestion by this fungus. Enzyme activities in both studies were predominantly extracellular. Like cellulases and hemicellulases, amylolytic enzymes are subject to regulation by simple sugars (Mountfort & Asher, 1988).

Little attention has been given to the proteolytic activity of rumen fungi, although Wallace & Joblin (1985) showed that the proteolytic activity of *N. frontalis* has a trypsin-like specificity with both cell-bound and cell-free activities. The activity of the *N. frontalis* protease(s) was comparable to that of the most active proteolytic rumen bacteria, but was not high when compared to some aerobic fungi. Wallace & Joblin (1985) concluded that proteases in rumen fungi may provide amino acids for growth, modify the activities of other extracellular enzymes, or assist in penetration of plant material. They also observed that the most active cellulolytic rumen bacteria are not usually proteolytic, and the possession of both proteolytic and cellulolytic enzymes may be a special feature of anaerobic fungi.

Many of the cellulase, hemicellulase, amylase and glycosidase activities of anaerobic fungi are subject to catabolite repression. Mountfort & Asher (1983), for example, showed preferential utilization of glucose over xylose, and Mountfort & Asher (1985, 1988) and Morrison, Mackie & Kistner (1990) showed that the presence of mono- or disaccharides in cultures containing cellulose, xylan or starch resulted in reduced or zero production of the polymer-degrading enzymes. Induction of extracellular cellulases in *N. frontalis* by switching from glucose to a cellulosic substrate has been demonstrated by Barichievich & Calza (1990). Using various cellulolytic substrates, including Avicel (microcrystalline cellulose), these authors increased cellulolytic activity to 3- to 5-fold as compared to cultures grown on glucose alone.

In addition to the regulation of extracellular cellulases by catabolite repression, secretion is also dependent on *de novo* protein synthesis and protein glycosylation (Calza, 1991*a*, *b*; Li & Calza, 1991). Protein glycosylation appears to be important for the stability and catalytic activity of cellulase isolated from *Neocallimastix frontalis* (EB188) (Calza, 1991*a*, *b*). However, it may be more important for protein secretion than enzyme activity, since active enzymes were synthesized but remained apparently intracellular in cultures that were treated with glycosylation inhibitors (Li & Calza, 1991). The surface of the substrate can also affect the activity of cellulolytic enzymes. Cheng *et al.* (1991) found that attachment of anaerobic fungi to cellulose and cellulose digestion are completely inhibited by the addition of methylcellulose to the culture. In contrast, the addition of methylcellulose does not affect the growth of anaerobic fungi on soluble substrates. This led Cheng *et al.* (1991) to conclude that anaerobic fungi, like cellulose-digesting bacteria, require the special juxtaposition of the cellulolytic organism and its insoluble substrate.

THE IMPORTANCE OF ANAEROBIC FUNGI IN THE RUMEN

Due to the complex nature of the rumen ecosystem and the involvement of so many micro-organisms, the precise role and overall contribution of anaerobic fungi to the degradation and fermentation of plant biomass have yet to be determined. Nevertheless, it is clear that at least for some diets, plant fragments entering the rumen are rapidly and extensively colonized by large populations of anaerobic fungi (Bauchop, 1979*a*, *b*), for example, zoospores attached and encysted on the leaves and stems of guinea-grass fragments within 15 min after inoculation (Ho *et al.*, 1988*a*). In addition, Theodorou, Lowe & Trinci (1988) suggested that anaerobic fungi, by their primary activity on plant particles, may enhance the activity of colonizing bacteria and thus facilitate the degradation of plant biomass.

Orpin (1977*a*, *b*) was the first to show a close association in the rumen between anaerobic fungi and plant biomass, and he demonstrated the uptake by anaerobic fungi of labelled carbon from plant tissues. Engles & Brice (1985) suggested that a tertiary wall of lignified cells in barley straw might act as a barrier to rumen micro-organisms but, according to Akin *et al.* (1989), anaerobic fungi with their rhizoidal mode (Figs 4 and 5) of growth can penetrate and overcome this barrier. Some anaerobic fungi are morphologically adapted to penetrate plant tissues. Ho, Abdullah & Jalaludin (1988*b*), for example, showed that some anaerobic fungi in the rumen of cattle produced appressorial-like structures which invaded undamaged cell walls of guinea grass and rice straw, and Joblin (1989) suggested that holdfasts of *Caecomyces* spp. physically disrupt plant fibres.

Anaerobic fungi are more prevalent in ruminants fed on stalky fibrous diets than in those fed on soft leafy diets such as clover (Bauchop, 1980, 1981). One explanation for this difference is that, compared to a leafy diet, fibrous plant tissues are retained in the rumen for a long period, thus enabling their more extensive colonization by anaerobic fungi. By contrast, diets rich in beet pulp, barley grain or young pastures result in a reduction of the anaerobic fungal population in the rumen (Bauchop, 1981, 1989; Grenet *et al.*, 1989). Such diets are rich in soluble carbohydrates, undergo rapid fermentation and consequently have rapid passage rates through the rumen. Grenet *et al.* (1989) fed dairy cows a beet diet either one or six feeds daily. With the first feeding regime, the pH of the rumen fell to below 5·5, whereas the six times daily regime gave a rumen pH of over 6·2. Interestingly, the fungal population in animals fed six times a day was 10-fold higher than that of the animals fed once a day, suggesting that the

rumen pH may have significant effect on the size of the fungal population.

Akin *et al.* (1983) showed that sheep fed sulphur-fertilized forage had larger populations of anaerobic fungi in the rumen than sheep fed unfertilized forage, whereas bacterial populations in the rumen remained constant regardless of the degree of sulphur fertilization. Similar results were obtained using wheat straw diets with or without the addition of methionine (Gordon, Gulati & Ashes, 1983). Morrison *et al.* (1986) suggested that anaerobic fungi may require sulphur in the form of sulphide, thus accounting for their increased numbers in the presence of added sulphur. In contrast to these results, Akin & Windham (1989) reported no difference in the size of the fungal population in animals fed sulphur-fertilized and unfertilized grasses. Furthermore, Millard *et al.* (1987) found that sulphur-fertilization of Italian ryegrass actually decreased the number of anaerobic fungi in the rumen of sheep.

Phenolic compounds occur naturally in plants, particularly as secondary metabolites, and are generally involved in plant defence mechanisms. Akin & Rigsby (1985) studied the effects of three phenolic acids (*p*-coumaric, ferulic and sinapic acids) on anaerobic fungi. At relatively low concentrations (0·1% w/v), these compounds decreased anaerobic fungal populations *in vitro*, and the dry-weight loss of plant cell walls (the substrate) was reduced by 25–50%. These results suggest that plant phenolics have a detrimental effect on the growth of anaerobic fungi.

Anaerobic fungi may contribute to the physical disruption of plant particles in the rumen; for example, Orpin (1983/84) reported a significant reduction in the size of wheat straw particles in some cultures of anaerobic fungi. In addition, Joblin (1989) suggested that the holdfasts of *Caecomyces* spp. (Fig. 7) may cause mechanical disruption of plant cells, whilst Akin *et al.* (1983) showed that plant material recovered from rumen digesta containing high fungal populations required much less force to break than plant material recovered from digesta with lower fungal populations. Finally, polycentric isolates have been shown to reduce the tensile strength of plant particles to a greater extent than monocentric isolates (Akin *et al.*, 1990).

INTERACTIONS BETWEEN ANAEROBIC FUNGI AND OTHER MICRO-ORGANISMS IN THE RUMEN ECOSYSTEM

The rumen is a highly complex ecosystem, which contains many different microbial species and has a great potential for inter-microbial associations. Interactions are known to occur between anaerobic fungi and methanogenic bacteria, between anaerobic fungi and non-methanogenic bacteria, and between anaerobic fungi and protozoa (Mountfort, Asher & Bauchop, 1982; Romulo, Bird & Leng, 1989; Marvin-Sikkema *et al.*, 1990).

Interactions between anaerobic fungi and methanogenic bacteria

Symbioses between anaerobic fungi and methanogens are probably common in the rumen, but their advantage to the animal host remains unclear: although cellulolysis is increased in co-cultures, the consequential enhanced CO_2 and CH_4 production results in a loss of carbon to the herbivore. Bauchop & Mountfort (1981) showed that cultures of *N. frontalis* degraded filter paper more extensively in the presence of methanogens than in their absence. In the presence of methanogens, the fermentation was shifted from the formation of electron sink products such as lactate and H_2 towards more highly reduced end-products such as acetate, CO_2 and CH_4. Further studies (Mountfort *et al.*, 1982) showed that *N. frontalis* alone gave 53% dry-matter loss, whereas co-cultures of the fungus with *Methanosarcina barkeri* and *Methanobrevibacter* sp. gave losses of 69% and 87%, respectively, whilst a tri-culture of all three organisms resulted in a 98% loss of filter paper D.W., with the end-products being almost entirely CO_2 and CH_4. Hydrogenosomes in anaerobic fungi (Yarlett *et al.*, 1986; Munn *et al.*, 1988; Webb & Theodorou, 1988) are situated near the surface of the plasma membrane and therefore close to the associated methanogens, which are able to utilize H_2 and CO_2 produced by hydrogenosomes as substrates for growth (Yarlett *et al.*, 1986). Methanogen/anaerobic fungus co-cultures with *Piromyces* sp. and *Caecomyces* sp. also show enhanced acetate production (Fonty *et al.*, 1988; Marvin-Sikkema *et al.*, 1990); production of acetate in preference to lactate and ethanol results in an increased ATP yield (from 3 to 4).

Natural plant substrates have been used in some co-culture studies. On barley straw, Joblin *et al.* (1989) showed acetogenic shifts when either *Neocallimastix* sp. or *Piromyces* sp. was grown with *Methanobrevibacter smithii*, a methanogen obtained from sewage sludge. However, although increased cellulolysis was apparent in these co-cultures, it was not as extensive as in the co-cultures of Mountfort *et al.* (1982); Joblin & Naylor (1989) suggested that the presence of plant polymers such as lignin reduced cellulolytic activity in these co-cultures.

Interactions between anaerobic fungi and non-methanogenic bacteria

There are many non-methanogenic bacteria in the rumen, and interactions between anaerobic fungi and these bacteria may involve competition, synergism or symbiosis. *Selenomonas ruminantium*, which consumes H_2 and compounds such as succinate and lactate, enhanced cellulose digestion by *Neocallimastix* sp. (Marvin-Sikkema *et al.*, 1990). *S. ruminantium* also increased the cellulolytic activity of *C. communis*, but decreased cellulolysis and increased lactate production were observed when *P. communis* was co-cultured with this bacterium (Bernalier, Fonty & Gouet, 1991). The reasons for enhanced cellulolysis by *Neocallimastix* sp. and *C. communis* but not *P. communis* in co-cultures containing *S. ruminantium* are not clear, but may be related to lactate production, which in high concentrations is known to exert a negative effect on cellulolysis (Bernalier *et al.*, 1991). Williams, Withers & Joblin (1991) found that xylan degradation by *N. frontalis* was not increased when the fungus was incubated with one of two strains of *S. ruminantium*. However, all three organisms in tri-culture enhanced xylan utilization. A decrease in monosaccharide accumulation in culture filtrates of *N. frontalis* grown on xylan was found when this fungus was co-cultured

with *Veillonella parvula* a lactic acid utilizer, but no increase in xylanolysis occurred (Williams *et al.*, 1991).

The presence of *Bacteroides succinogenes* had no effect on the degradation of straw by mixed cultures of fungi (Irvine & Stewart, 1991), but *B. ruminicola* increased xylan and barley straw degradation when grown with *N. frontalis* (Richardson *et al.*, 1986; Williams *et al.*, 1991). The *N. frontalis* and *B. ruminicola* co-culture appeared to be synergistic, as degradation of xylan in the co-culture was more than three-fold greater than in monocultures of either the bacterium or fungus (Williams *et al.*, 1991). *Succinivibrio dextrinosolvens* co-cultured with *N. frontalis* also degraded xylan synergistically (Williams *et al.*, 1991), whereas *N. frontalis*/*Butyrivibrio fibrosolvens* co-cultures showed only a small increase in xylan degradation (Williams *et al.*, 1991). *Lachnospira multiparus* and *Streptococcus bovis* had little effect on xylan degradation by *N. frontalis*, but did depress its utilization (Williams *et al.*, 1991).

The interactions between anaerobic fungi and non-methanogenic bacteria are complex and will be difficult to elucidate, particularly as mycoplasmas have recently been found associated with a number of anaerobic fungi isolated from a steer (Kudo *et al.*, 1990). The ecological role of the association of mycoplasmas with rumen fungi is still unknown. However, Kudo *et al.* (1990) suggest that the axenic status of all cultures of anaerobic fungi isolated to date needs to be re-examined.

Interactions between anaerobic fungi and rumen protozoa

Anaerobic fungal numbers have been shown to increase in defaunated animals (animals lacking rumen protozoa). Romulo, Bird & Leng (1986, 1989) showed 2- to 4-fold increases in zoospores and zoosporangia of anaerobic fungi in defaunated sheep. Soetanto *et al.* (1985) found increased fungal populations in defaunated animals, as well as observing increased digestion of the high-fibre diet fed to these animals. By contrast, Newbold & Hillman (1990) observed only small increases in fungal zoospores in defaunated ruminants. The increase in fungal populations upon defaunation may be influenced by the animal's diet, as fungal populations are greater in animals fed high-fibre diets than in those fed low-fibre or high-concentrate diets (Bauchop, 1981), whereas the opposite is true for ciliated protozoa. In nature, anaerobic fungi and rumen protozoa may be complementary rather than competitive (Orpin, 1983/84).

CONCLUDING REMARKS

Emerson & Natvig (1981) suggested that anaerobism might be more widely distributed amongst fungi than previously thought, and advanced the hypothesis that many facultative and obligately anaerobic fungi may not yet have been isolated because of the selection procedures commonly used by mycologists. For example, mycologists traditionally bait for aquatic fungi in the oxygenated upper water layers and, as a result, facultative anaerobic fungi present in waters low in oxygen are overlooked; however, by baiting for fungi in a lake at depths low in oxygen, Natvig (1977) was able to isolate the highly fermentative fungus, *Rhipidium*.

The discovery by Orpin (1975) of obligately anaerobic fungi in the rumen of sheep has prompted studies of these organisms in several laboratories worldwide. Prior to Orpin's work, even the idea of the existence of fungi which lacked mitochondria, possessed hydrogenomes and produced formate, acetate, lactate, succinate ethanol and hydrogen, would have been instantly dismissed. What other bizarre (and useful) fungi have yet to be discovered?

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