

## Studies on the Rumen Flagellate *Neocallimastix frontalis*

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### SUMMARY

The vast increase in the population density of the rumen flagellate *Neocallimastix frontalis* shortly after the host animal has commenced eating is caused by stimulation of a reproductive body on a vegetative phase of the organism to differentiate and liberate the flagellates. The stimulant is a component of the host's diet. The vegetative stage of *N. frontalis* bears a strong morphological resemblance to that of certain species of aquatic phycomycete fungi, and consists of a reproductive body borne on a single, much branched rhizoid. The flagellates liberated *in vivo* within 15 to 45 min of feeding lose their motility within 1 h and develop into the vegetative phase, thus producing a rapid decrease in population density of the flagellates. Conditions for maximum flagellate production are similar to those occurring in the rumen: pH 6.5, 39 °C, absence of O<sub>2</sub>, presence of CO<sub>2</sub>. Differentiation of the reproductive body is inhibited by compounds affecting membrane structure and function, but not by inhibitors of protein synthesis. The organism was cultured *in vitro* in an undefined medium in the absence of bacteria or other flagellates.

### INTRODUCTION

Four species of oval or bean-shaped protists bearing 3 to 15 flagella have been described and grouped in the genus *Callimastix* Weissenberg, in the family Callimastigidae da Fonseca of the protozoan order Polymastigina (Kudo, 1954). These organisms are: *Callimastix cyclopi*s (Weissenberg, 1912), parasitic on fresh-water copepods; *C. frontalis* (Braune, 1913), inhabiting the rumen; *C. equi* (Hsuing, 1930), from the horse caecum, possibly synonymous with *C. frontalis* (Vavra & Joyon, 1966); and *C. jolepsi* (Bovee, 1961), in the intestine of the fresh-water snail *Helisoma duryi*. Weissenberg (1950) suggested that *C. cyclopi*s might be a zoospore of a primitive fungus. This was borne out by Vavra & Joyon (1966), who found that *C. cyclopi*s flagellates were zoospores from a vegetative phase of a simple fungus occurring in the body cavity of infected copepods; they were released through a rupture in the cephalothorax of the host. Ultrastructural studies showed that the zoospores possessed a structure similar to that of the zoospore of the phycomycete family Blastocladales. The genus *Callimastix*, with *C. cyclopi*s as type species, was accordingly placed in this family. The authors were of little doubt that the remaining *Callimastix* species were true flagellate protozoa, and grouped them in the new genus *Neocallimastix* with *N. frontalis* as type species.

*Neocallimastix frontalis* is widely distributed amongst ruminants, occurring in the rumen of sheep, cattle, goats (Braune, 1913; Das Gupta, 1935; Eadie, 1962) and a llama (personal observations). The population density was usually in the range  $4 \times 10^2$  to  $10^5$ /ml, but fluctuated greatly and rapidly over the feeding period of the host animal. Warner

(1966) demonstrated that most of these fluctuations consisted of an up to 25-fold increase one hour after the animal was fed, falling to a minimum 8 to 10 h later. Orpin (1974) showed the fluctuation to be more rapid and larger, with an average population density 67 times higher 20 min after feeding than before feeding. The highest maximum to minimum (pre-feeding) population density ratio determined was 296. Fluctuations as high as this are abnormal for rumen organisms, which normally divide only 2 to 4 times a day (Hungate, 1966).

Warner (1966) suggested that the cause of this dramatic fluctuation was that the neocallimastix were normally sequestered on the rumen wall, entered the rumen fluid in response to a chemical stimulus in the diet and later returned to the wall by another chemotactic response, possibly to a compound diffusing from the blood into the rumen. This theory was discounted by Orpin (1974) who showed that the neocallimastix population density could be increased *in vivo* and *in vitro* by an extract of oats. This extract was incapable of inducing the increase of neocallimastix populations in the absence of particulate material.

This paper describes an investigation into the reason for these unusual population density fluctuations, and consequently into the life-history of *N. frontalis*.

#### METHODS

*Animals.* The sheep used were Clun Forest Wethers, each fitted with a permanent rumen cannula, and fed 800 g of hay chaff and 200 g of crushed oats once daily. Defaunation was by the method of Abou Akkada *et al.* (1968) using dioctyl sodium sulphosuccinate, except that food was withdrawn on the day when dioctyl sodium sulphosuccinate was administered and 800 g of hay fed on the next day. Normal feeding was resumed on the third day.

*Sampling and counting.* Samples were taken *in vivo*, and the neocallimastix flagellates and sporangia counted as described by Orpin (1974). Experiments *in vitro* using fresh rumen fluid were sampled and the neocallimastix flagellates or sporangia counted after the experimental material had been mixed by inverting the tube containing it.

*Preparation of ovine rumen fluid fractions.* Fresh rumen fluid was strained through one layer of muslin. The material retained was washed twice with centrifuged rumen fluid (CRF) (see below), resuspended in CRF and designated coarse food particles (CFP). The filtrate (FRF) was centrifuged at 1000g for 15 min at room temperature and the sediment washed twice with and resuspended in CRF. This fraction was designated the large particulate (LP) fraction. The supernatant of the centrifuged FRF was then centrifuged at 20000g for 1 h at 2 °C and the supernatant designated centrifuged rumen fluid (CRF).

*Extraction of inducer.* The stimulatory component of oats was extracted by the method used by Orpin (1974). The volume of the final aqueous extract was adjusted so that 1 ml represented the extract from 1 g dry wt of oats. The extract was stored at -20 °C, and is the 'inducer' referred to in this paper. The CFP fraction of rumen fluid was also extracted by the same method, after the fraction had been washed twice with water (20 ml/g wet wt of CFP) on the filter.

*Assay in vitro for neocallimastix production.* FRF (1.0 ml) was incubated with 0.1 ml of inducer in a stoppered, CO<sub>2</sub>-gassed test-tube, in a water bath at 39 °C. The population density was determined at the start of the experiment and after 20 min, and compared with a blank tube containing 0.1 ml of water in place of the inducer.

*Experiments with different gas phases.* These were conducted in the same manner using

the standard assay techniques, except that the experimental gas was bubbled through the FRF for 2 min before stoppering and incubation under that gas.

*Experiments with inhibitors.* These were conducted as before with the addition of inhibitor to give a final concentration of 50, 100 or 200 µg/ml.

*Experiments with pH variation.* These were conducted after the addition of the pre-determined quantity of 0.1 M-HCl or 0.1 M-NaOH to bring the pH of the FRF to that required for the experiment.

*Experiments with temperature variation.* Experimental tubes containing the standard assay materials were prepared as described before, and incubated under CO<sub>2</sub> in water-baths of the desired temperature.

*Measurement of turnover rate.* The turnover rate of the contents of the rumen was determined by using polyethylene glycol (PEG) (Huyden, 1955).

*Culture in vitro.* The medium used was based on that of Diamond (1957) and consisted of (g/100 ml final volume): tryptone, 2.0; yeast extract, 1.0; maltose, 0.2; L-cysteine, 0.1; L-ascorbic acid, 0.02; agar, 0.1. These ingredients were dissolved and made up to 79 ml with distilled water; 10 ml of fresh centrifuged rumen fluid was added and the medium autoclaved (10 lb/in<sup>2</sup>, 20 min). The medium was allowed to cool to 50 °C, and 1 ml of sterile solution containing 100 000 units of sodium benzylpenicillin and 0.1 g of streptomycin sulphate was added, followed by 10 ml of inactivated (56 °C for 30 min) sheep serum previously sterilized by filtration. The medium was dispensed aseptically into sterile 12.7 × 1.3 cm (5 × ½ in) rimless test-tubes to within 2.5 cm of the top; the tubes were gassed with sterile O<sub>2</sub>-free CO<sub>2</sub>, stoppered and allowed to gel.

The initial isolation was made by overlaying this medium with 0.2 ml of the LP fraction of rumen fluid, followed by gassing with O<sub>2</sub>-free CO<sub>2</sub> and incubating at 30 °C. Within 2 days, neocallimastix flagellates and many fungus-like growths were observed within the medium. The top 5 cm of medium was then removed aseptically by aspiration, and drops of the underlying sloppy agar containing neocallimastic flagellates overlaid on fresh culture medium. The new culture was stoppered and shaken gently to distribute the inoculum in the top 6 mm of medium, and the tube incubated at 39 °C. The next day growth could be observed in the upper part of the tube. Successive subcultures eliminated bacteria, leaving neocallimastix as the sole flagellate. At this stage it was possible to omit antibiotics from the growth medium which resulted in a greatly increased rate of growth; however, antibiotics were not omitted from stock cultures in order to reduce the risk of contamination by bacteria.

*Photomicrography.* Photographs were taken by phase-contrast lighting with the exception of Fig. 7 which was taken by reflected light.

## RESULTS

### *Observations in vivo*

Neocallimastix flagellates (Fig. 1) were found in the rumens of normally faunated sheep and cattle and in sheep defaunated of ciliate protozoa. It had previously been found that the organisms were associated with the LP fraction of rumen fluid before the population increase (Orpin, 1974). This fraction contained large numbers of ciliate protozoa when prepared from rumen fluid taken from a normally faunated sheep; to simplify the counting of neocallimastix flagellates in population studies, defaunated sheep were used for these experiments.

All the sheep examined (18) contained neocallimastix and each population of neocalli-

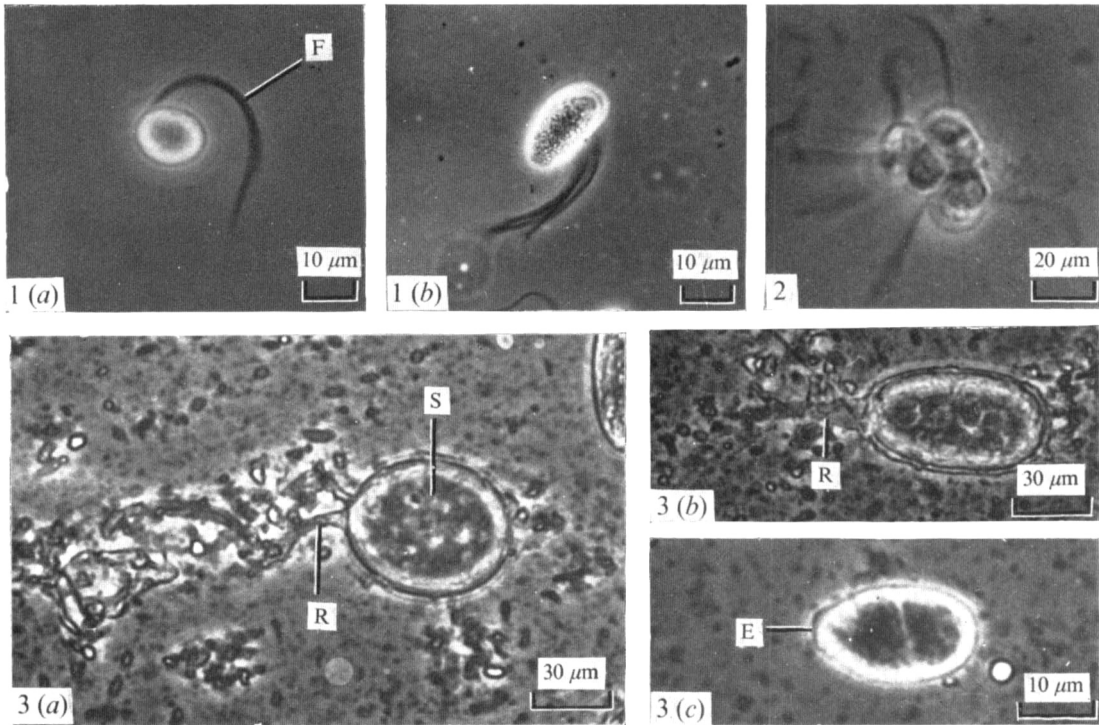


Fig. 1. *Neocallimastix frontalis* flagellates. F, locomotory organelle. (a) Living preparation; (b) fixed with 2% HCHO.

Fig. 2. Association of several neocallimastix flagellates; living preparation.

Fig. 3. Sporangia present in rumen fluid. R, rhizoid; S, sporangium; E, squared-off end of sporangium, possibly the point of attachment of a rhizoid broken off during preparation of the sample. (a) Sporangium bearing a rhizoid; (b) sporangium bearing a short length of rhizoid; (c) sporangium without a rhizoid.

mastix showed large increases over the feeding period. Amongst the increased neocallimastix populations produced during several experiments, cells were observed linked by their flagella or adhering to each other at other points; in this way groups of up to fourteen individual cells were found closely associated (Fig. 2), suggesting either a multiple reproductive phase or the aggregation of free organisms.

#### *Microscopic examination of the LP fraction*

Microscopic examination of the LP fraction obtained before feeding did not reveal the presence of any neocallimastix either attached to food particles, aggregated together, or, as far as could be ascertained, within the food particles. A search was therefore conducted for the logical alternative to the secretion of the neocallimastix amongst the food – a reproductive body. Bodies resembling fungal sporangia (referred to as sporangia in this paper), some of which possessed simple hyphae or rhizoids (Fig. 3a), were seen free in the supernatant fluid. Most were oval, though others were more cylindrical (Fig. 3b) or even slightly conical. If no long rhizoid was obvious on first examination, it was normally possible to identify (on sporangia, 35 μm long) a short length of broken rhizoid in a terminal position on the sporangium (Fig. 3b); sometimes it appeared that the entire

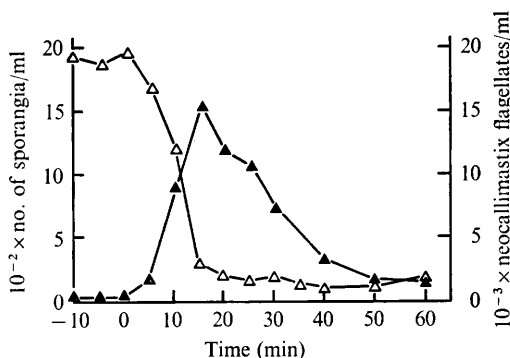


Fig. 4. Population density curves for *N. frontalis* flagellates (▲) and sporangia (△) *in vivo*. Inducer extracted from 200 g crushed oats was added at zero time. Average of four experiments.

rhizoid had been broken off and the flattened surface of the sporangium was visible where the rhizoid would have been expected to be attached (Fig. 3c).

#### *Evidence for the association of the sporangia with Neocallimastix frontalis*

Experiments *in vivo* revealed that in addition to the population of the neocallimastix increasing in the presence of inducer, the population density of the sporangia decreased (Fig. 4). Since such a decrease is common in rumen organisms over the feeding period of the host, due to dilution of the rumen contents (Warner, 1966), the dilution was measured using PEG. This showed that a 25% dilution occurred within 1 h of the animal commencing to eat, and therefore a 25% reduction in sporangia concentration would have occurred. However, a concomitant decrease of 91% in the population density of the sporangia was recorded. Dilution of the rumen contents was therefore not responsible for the loss of most of the sporangia.

To ascertain whether the sporangia in the LP fraction were the reproductive stage in the life-history of neocallimastix, LP fraction (1.0 ml) was mixed with inducer (extracted from 0.1 g dry wt of oats) and 0.01 ml of this suspension placed on a pre-heated (39 °C) microscope slide, sealed with Vaseline to restrict oxygen access and placed on a heated (39 °C) microscope stage. The sporangia were observed over the period when the population increase was expected. Within 10 to 20 min, rounded bodies of increased density became differentiated inside the sporangium. These bodies gradually became more elongated and after 15 to 45 min the sporangium ruptured to release neocallimastix flagellates. Typical development of the sporangium was as follows: At the onset of incubation no internal structure was evident (Fig. 5a). Within 10 min of the addition of inducer, internal differentiation was visible (Fig. 5b). At 15 min, apparently mature neocallimastix were present within the sporangium (Fig. 5c), which ruptured at 18 min (Fig. 5d) to liberate the motile neocallimastix flagellates. The empty sporangium is depicted in Fig. 5e.

Occasionally, differentiation of the flagellates within the sporangium was not complete before their release. The most common malformation produced in this way *in vitro* is depicted in Fig. 6, where the sporangium contents differentiated into only three separate cells, each bearing several flagella. These cells were incapable of directional movement. Cells of this type were also observed in samples of rumen fluid taken shortly after the host animal was fed.

No differentiation was visible in the sporangia present in fresh rumen fluid, except when

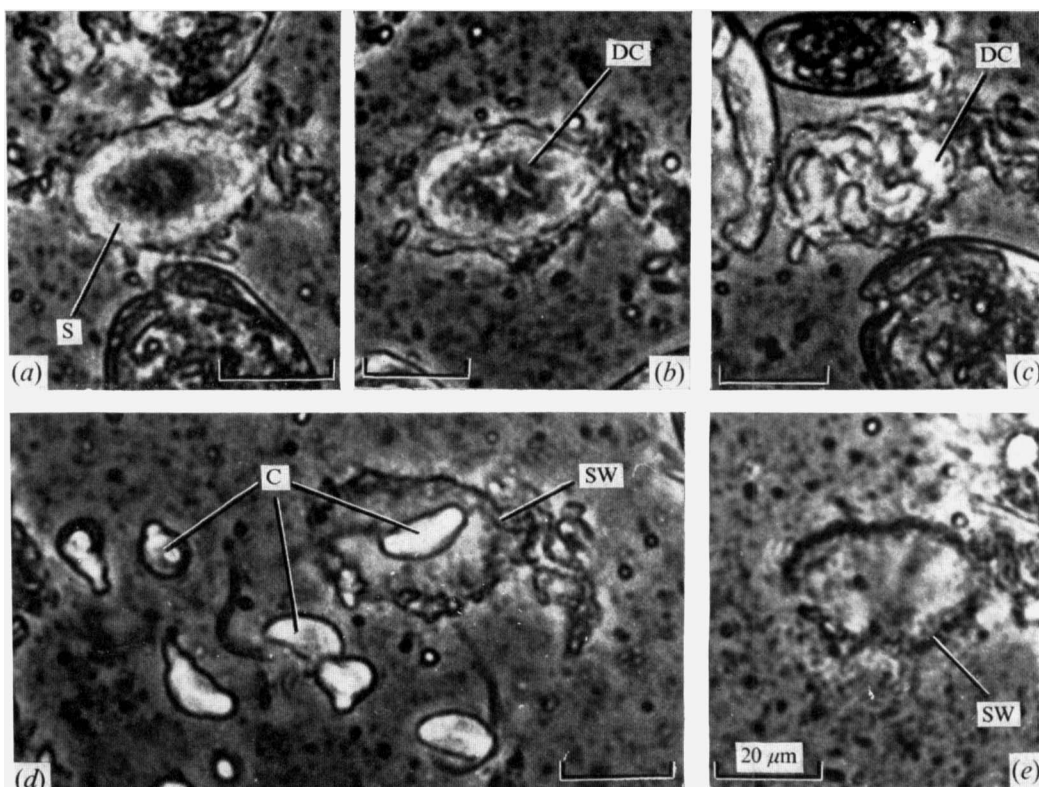


Fig. 5(a)–(e)

Fig. 5. Development of sporangia and liberation of *N. frontalis* flagellates *in vitro* in the presence of the inducer. S, sporangium; SW, sporangium wall; DC, developing neocallimastix flagellates; C, flagellated neocallimastix cells. (a) Two min after the addition of the inducer; no differentiation visible. (b) After 10 min; differentiation visible within the sporangium. (c) After 15 min; the differentiation apparently complete, with evident movements of the separate flagellates within the sporangium. (d) After 18 minutes; the sporangium ruptured, releasing the neocallimastix flagellates. (e) Wall of empty sporangium after egress of flagellates.

taken within 30 min of the animal having fed. Mechanical rupture of the sporangia taken from the rumen before feeding resulted in the release of granular cell contents, amongst which only flagella could be seen. No structure resembling the cell wall of neocallimastix was visible. Production of neocallimastix did not occur when the extract was replaced by distilled water, confirming that a component in the extract stimulated the production of the organisms.

#### *Observations in vitro*

The neocallimastix flagellates grown in culture *in vitro* were indistinguishable morphologically from those observed *in vivo*. At no time was it possible to culture the flagellates in the absence of the fungus-like growth. The sporangia cultured *in vitro* (Fig. 7) bore a more extensive rhizoid system than those observed *in vivo*, sometimes 1380  $\mu\text{m}$  long and clearly visible to the naked eye. The production of neocallimastix flagellates from the sporangia was observed many times (Fig. 8) but was slower than *in vivo*, probably due to the agar in the culture medium. So far it has not been possible to culture neocallimastix in the absence of agar; however, agar proved beneficial in the preparation of cultures

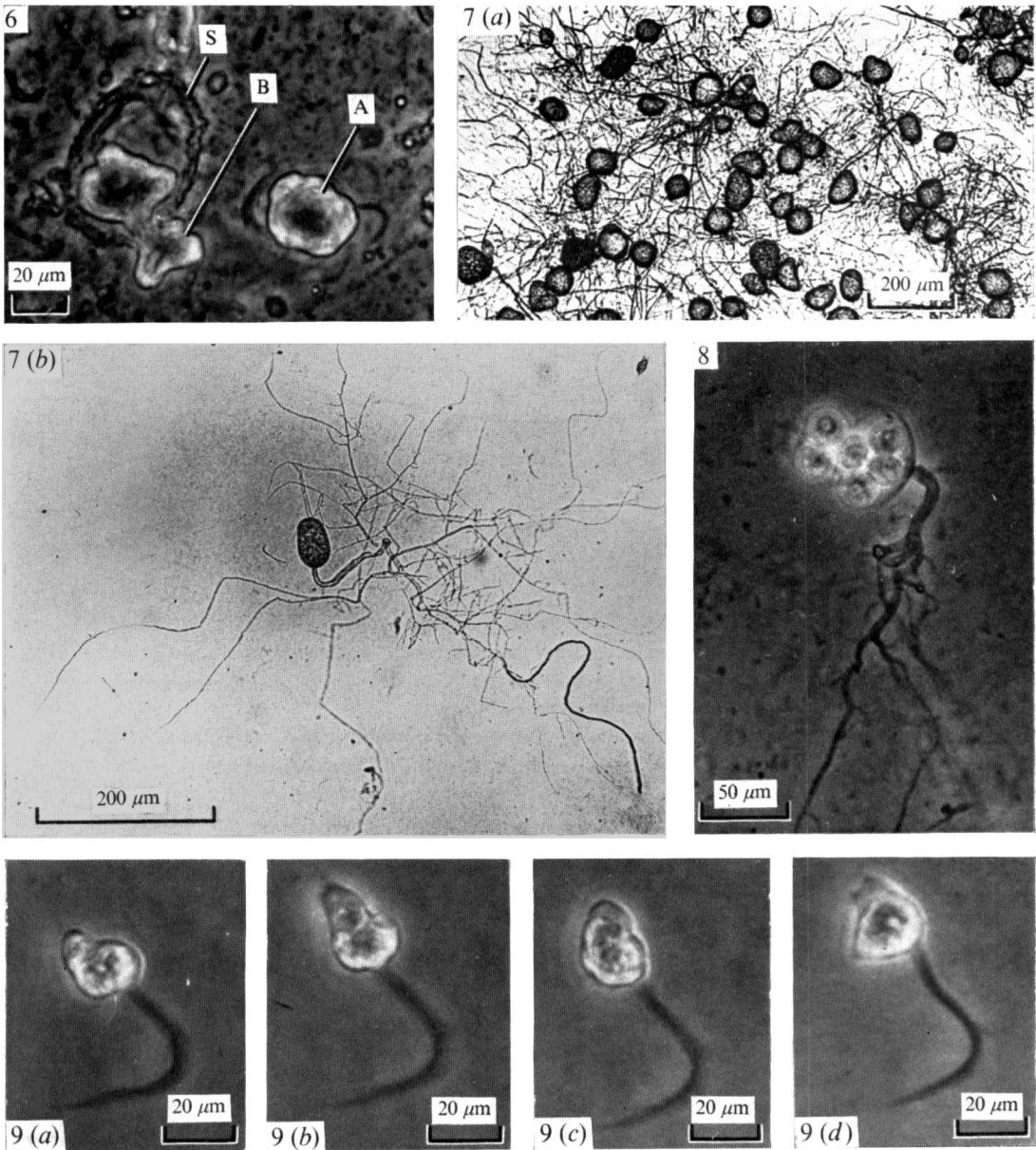


Fig. 6. Liberation of malformed flagellates from a sporangium. S, sporangium wall; A, malformed flagellate with many locomotory organelles; B, similar malformed cell emerging from the sporangium through a rupture in the sporangium wall.

Fig. 7. Vegetative stage cultured *in vitro*. (a) Appearance of typical culture before the release of the flagellates; (b) an individual vegetative stage.

Fig. 8. Liberation of flagellates from a sporangium cultured *in vitro*.

Fig. 9. Amoeboid movement shown by a flagellated cell. Photographs taken under phase contrast at 1 min intervals.

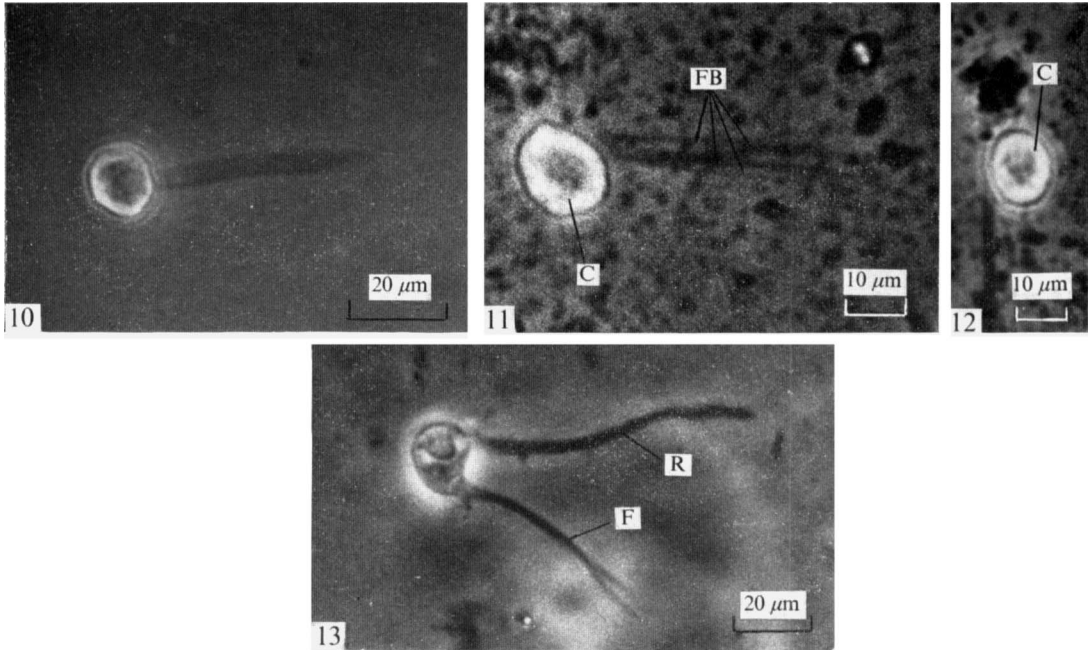


Fig. 10. Flagellated stage, showing locomotory organelle spread in two dimensions.

Fig. 11. Separation of the locomotory organelle of the *N. frontalis* flagellate into smaller flagellar bundles. C, cell of flagellate; FB, flagellar bundles.

Fig. 12. *Neocallimastix frontalis* immediately after the loss of the flagella. C, neocallimastix cell.

Fig. 13. Germination of flagellated stage *in vitro*. R, rhizoid; F, locomotory organelle.

free of bacteria since the flagellates were capable of migrating downwards in the medium faster than the bacteria. The cultures needed to be subcultured every 2 days into fresh medium. The medium could be kept at 4 °C for up to three days; if inoculated with neocallimastix after this period of storage, growth of the vegetative stage occurred but no flagellates were released. Cultures could not be stored at either 4 or -20 °C without loss of viability.

#### *Morphology and life history*

*The Neocallimastix frontalis flagellate.* The neocallimastix flagellates averaged  $20.6 \times 8.7 \mu\text{m}$  ( $n = 100$ ); their shape was variable, but basically ovoid (Fig. 1). Cells with knob-like projections not infrequently occurred. Little internal structure was visible under the light microscope, the cell appearing refractile and the contents granular (Fig. 1b). The cells were frequently irregular in shape soon after release from the sporangium (Fig. 5d), but most became regular within 2 to 3 min. Locomotion was effected by the rapid beating of up to 14 flagella which formed a single locomotory organelle, curved in three dimensions, up to  $36.6 \mu\text{m}$  long and  $2.5 \mu\text{m}$  thick. In live preparations the individual flagella adhered closely (Fig. 1a); in fixed preparations the flagella separated (Fig. 1b). The fresh cells were actively motile; the maximum linear speed recorded was  $5 \text{ mm}/\text{min}^1$ , timed over a  $200 \mu\text{m}$  grid of a haemocytometer chamber (Thoma ruling). Linear motility was rare, the usual movement being erratic and gyratory. Occasionally the flagellates would cease this erratic movement, the flagellum became inert, and the cell moved amoeboidly for 10 to 15 min, after which the cell became rounded and active movement by means of the



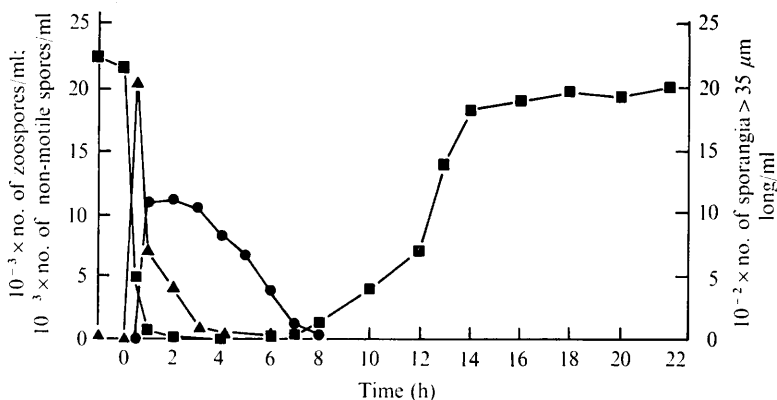


Fig. 14. Typical population density curves for various phases in the life history of *N. frontalis*. ■, Sporangia > 35  $\mu\text{m}$  long; ●, non-motile, non-flagellated cells and sporangia < 35  $\mu\text{m}$  long; ▲, motile neocallimastix cells. Inducer from 300 g crushed oats was added to the rumen at zero time to synchronize the growth of the neocallimastix.

flagella recurred. The amoeboid movement is depicted in Fig. 9, and was quite rapid. Considerable differences in cell shape were visible at 1 min intervals; these differences were not due to the rotation of the cell about its longitudinal axis since the position of the locomotory organelle remained approximately constant. Pseudopodia are clearly visible in Fig. 9*d*. Whilst the locomotory organelle was inert, it became disorganized and lost its three-dimensional structure, and the flagella frequently formed a parallel array (Fig. 10), up to 7  $\mu\text{m}$  wide. The individual flagella were not clearly differentiated.

Within 30 min to 1 h after liberation from the sporangium after growth *in vivo*, and 2 to 4 h after liberation from the sporangium after growth *in vitro*, the motility of the flagellates decreased and the locomotory organelle became inert and split into smaller flagellar bundles each consisting of 2 to 5 flagella (Fig. 11). In rumen fluid these flagella became detached from the cell, which had assumed a more oval and refractile state (Fig. 12). In cultures *in vitro*, the flagella frequently remained attached to the non-motile cell, which germinated with the outgrowth of a single rhizoid. The rhizoid originated from the half of the cell opposite the insertion of the flagella, and was polar or lateral in position (Fig. 13).

In experiments *in vivo* using an extract of 300 g oats, 100% rupture of the sporangia could be induced, resulting in the synchronization of flagellate production. Population density curves for the non-flagellated stage showed that a peak occurred soon after that of the motile stage, and decreased as the population of the larger sporangia increased (Fig. 14). Unfortunately it was not possible to follow the germination *in vivo* because of the abundance of particulate material present.

*The sporangium.* The size of sporangia producing the flagellates *in vivo* varied from 21 × 9  $\mu\text{m}$  to 74 × 52  $\mu\text{m}$  (Fig. 3). Some of the sporangia possessed a single rhizoid of up to 140 × 8.5  $\mu\text{m}$ , which was frequently branched (Fig. 3*a*). Up to 80% of the sporangia present in filtered rumen fluid lacked rhizoids (Fig. 3*c*) but the larger the sporangium, the greater was the likelihood of its possessing one. Similarly, up to 50% of sporangia possessed a short rhizoid (Fig. 3*b*). It is probable that many of the rhizoids were broken off during the filtration through muslin, either entirely (as suggested by the squared-off appearance of the narrower end of the sporangium in Fig. 3*c*, which may have been the point of attachment of a rhizoid) or partially (as in Fig. 3*b*, where the thick portion nearest the

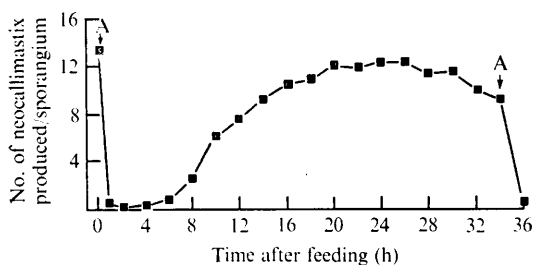


Fig. 15

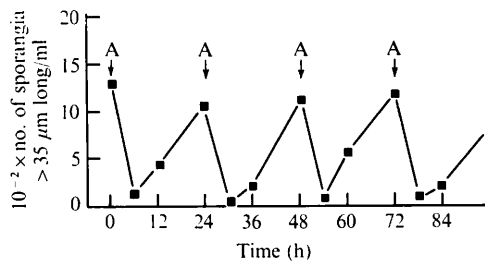


Fig. 16

Fig. 15. Typical curve showing the production of neocallimastix flagellates *in vitro* related to time after feeding of the host at points A. Samples were removed at intervals and assayed for neocallimastix flagellate production *in vitro*.

Fig. 16. Population density of sporangia > 35 μm long over 4 days. The sheep was fed at points A.

sporangium still remained). The presence of large food particles in samples of rumen fluid before filtration, prevented the examination of a sufficiently thin film to observe the rhizoids. At no time were the rhizoids seen to penetrate plant material in the rumen fluid, or to be used for the anchorage of the sporangium.

*In vitro*, the sporangia were capable of growing to larger dimensions, up to 155 × 83 μm, with rhizoids of up to 1380 μm in length. These rhizoids were repeatedly branched, but in no instance was more than one sporangium observed on each rhizoid system (Fig. 7b).

The number of neocallimastix flagellates produced per sporangium *in vivo* ranged from 2 to 38; they could be produced by sporangia only at least 6 h after the induction of synchronous growth by the addition of sufficient inducer to the rumen to promote 100% maturation of the sporangia. The average maximum number produced per sporangium in the rumen of a sheep fed once daily was 12; the number produced increased with time after feeding to reach a maximum after 20 to 30 h. After 30 h, the number decreased (Fig. 15). Population density curves for the sporangia larger than 35 μm long over 4 days are given in Fig. 16. After culture *in vitro*, when the sporangia were generally larger than found *in vivo*, a larger number of flagellates may be generated from each sporangium; up to 92 were recorded as issuing from one sporangium under these conditions.

*Development of the sporangia.* Since the non-motile neocallimastix cells were visible in rumen fluid taken at any time more than 30 min after feeding, and did not disappear before sporangium production, it is postulated that the non-motile cell develops into the larger sporangium and grows the rhizoid, as occurs *in vitro*; if the non-motile cell developed into the rhizoid first, with subsequent growth of the sporangium, a period would exist under conditions of synchronous growth when no sporangia at all would be visible. This did not occur; sporangia were always present.

#### *Conditions for neocallimastix production in samples of rumen fluid*

*Effect of concentration of inducer.* Experiments conducted *in vitro* showed that the disappearance of all the sporangia and a maximum increase in flagellate population density could be achieved by the addition of extract of inducer from 30 mg dry wt of oats/ml fluid. In later experiments, except where the concentration of the inducer was critical, an excess of the inducer prepared from 0.1 g dry wt of oats/ml FRF was used.

*Effect of storage of FRF.* Storage of FRF at 39 °C resulted in a rapid decrease in the ability of added inducer to stimulate the development of the sporangia and liberation

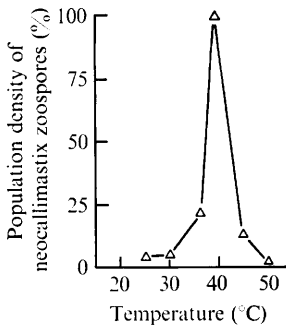


Fig. 17

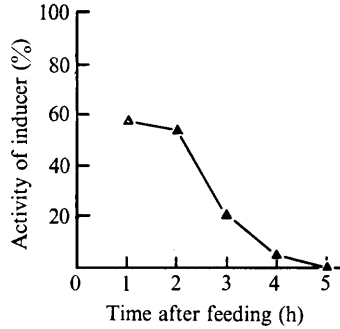


Fig. 18

Fig. 17. Effect of temperature on the production of neocallimastix flagellates *in vitro*. Results are expressed as a percentage of those liberated at 39 °C.

Fig. 18. The presence of inducer in the rumen after feeding. Samples (50 ml) of rumen fluid were taken at timed intervals after feeding, cooled to 4 °C, strained through one layer of muslin, and the particulate material retained was washed with 50 ml water. The inducer was extracted from the large particulate fraction as described in the text and assayed *in vitro* for the production of neocallimastix; its activity is expressed as a percentage of the neocallimastix produced by the inducer extracted from 0.1 g dry wt oats, when incubated with another sample of the same rumen fluid. Average of four experiments.

of flagellates. Flagellates produced by the same concentration of inducer after storage of the FRF for various periods of time were (giving the results as flagellate population density as a percentage of the number liberated at zero time under standard conditions): 0 min, 100%; 30 min, 107%; 60 min, 55%; 90 min, 39%; 120 min, 8%; 240 min, 2%. Increasing the concentration of inducer by 400% did not result in increased flagellate production. The activity of the inducer was checked at each stage against fresh FRF and found not to be diminished.

*Effect of temperature.* Incubation of FRF with inducer at temperatures ranging from 25 to 45 °C showed a marked peak in the production of neocallimastix flagellates at 39 °C (Fig. 17). Production at higher or lower temperatures was considerably reduced and was independent of the time of incubation – increasing the time to 1 h did not result in the liberation of more flagellates.

*Effect of gas phase.* Sporangia liberated neocallimastix flagellates in large numbers only in the presence of CO<sub>2</sub>. The results for the different gas phases were as follows (gas used, followed by production of flagellates as a percentage of those produced under standard conditions): CO<sub>2</sub>, 103; 5% CO<sub>2</sub>+95% N<sub>2</sub>, 38; N<sub>2</sub>, 15; air, 0. Afterwards, CO<sub>2</sub> was bubbled for 2 min through the tubes containing 5% CO<sub>2</sub>+95% N<sub>2</sub>, N<sub>2</sub> alone, and air; the tubes were then stoppered and incubated for a further 20 min, and the neocallimastix flagellates counted. In the same units, total flagellate production was then: 5% CO<sub>2</sub>+95% N<sub>2</sub>, 90; N<sub>2</sub>, 84; air, 0.

Thus the development of the sporangium to produce flagellates was totally inhibited by oxygen; with nitrogen however, although flagellate production was prevented the sporangia were not killed, since development occurred after the N<sub>2</sub> was flushed out with CO<sub>2</sub> and incubation continued. The reversal of N<sub>2</sub> inhibition by CO<sub>2</sub>, together with the lower rate of flagellate production in the presence of 5% CO<sub>2</sub>+95% N<sub>2</sub>, indicated that CO<sub>2</sub> is required in high concentration for reproduction to take place efficiently. The non-reversibility of oxygen inhibition indicated that oxygen is toxic to the vegetative stage.

*Effect of pH.* Results obtained from these experiments were: pH 5.5, 56; pH 6.0, 85; pH 6.5, 96; pH 7.0, 92; pH 7.5, 88; pH 8.0, 45. Thus within the pH range 6.0 to 7.5, alteration of pH had little effect on the rate of production.

*Effect of streptomycin and actidione.* Neither of these inhibitors of protein synthesis had any effect on the rate of flagellate production from sporangia. Actidione, however, completely inhibited the growth *in vitro* at 50 µg/ml.

*Effect of polymixin B and cytochalasin B.* Both these compounds inhibited neocallimastix production completely. The results for polymixin B were (concentration in µg/ml, followed by production of neocallimastix as a percentage of control): 50, 48; 100, 0; 200, 0. In the same units, results for cytochalasin B were: 50, 5; 100, 0; 200, 0.

*Presence of inducer in the rumen.* The inducer was found to be present in the rumen fluid CFP fraction until about 5 h after feeding (Fig. 18).

A factor other than inducer present in the diet was also active within the rumen in the absence of inducer. The dietary inducer disappeared from the rumen within 5 h and flagellates lost their motility within 1 h. If induction by a component in the diet was the only mechanism triggering flagellate differentiation and release, no flagellate production should have occurred when the inducer was absent. However, a few motile flagellates were always seen in the rumen at more than 5 h after feeding. This indicated that a second factor, independent of the time of feeding, was involved, which did not result in synchronized flagellate production. This second factor appeared to be operative in cultures *in vitro*, since no active dietary inducer was present in the medium.

#### DISCUSSION

The life history of the rumen organism *N. frontalis* consists of a cycle alternating between two phases, the motile flagellated phase, and the non-motile, vegetative, reproductive phase. The entire cycle occurs within the rumen and the organism could be cultured *in vitro*; the conditions under which maximum reproduction occurred (39 °C, pH 6.5, absence of oxygen, presence of CO<sub>2</sub>) indicated that neocallimastix is a true rumen organism since conditions within the rumen are essentially similar (Hungate, 1966). This is supported by the wide distribution of neocallimastix in ruminants (Braune, 1913; Das Gupta, 1935; Eadie, 1962; Warner, 1966). The possession of a rhizoid by the reproductive body shows that the organism may be fungal rather than protozoal; protozoal reproductive bodies do not normally possess rhizoids. Fungi possessing life histories of this type are found in the aquatic Phycomycetes (Sparrow, 1960), and the *N. frontalis* flagellates previously described as flagellate protozoa (Braune, 1913; Vavra & Joyon, 1966; Hungate, 1966) may therefore be zoospores of a phycomycete fungus. However, the neocallimastix flagellate possesses more flagella (up to 14) than the maximum of 2 previously recorded for aquatic phycomycete zoospores by Sparrow (1960), and more even than the 2 to 9 recorded for *Callimastix cyclopis*, a member of the Blastocladiales (Vavra & Joyon, 1966) which, too, was previously regarded as a flagellate protozoon. Further structural examination of both stages in the life history of *Neocallimastix frontalis* are necessary before any firm conclusions are drawn concerning its taxonomic position.

The dramatic increase in population density of neocallimastix flagellates in the rumen observed by Warner (1966) and Orpin (1974) occurred when the reproductive bodies (sporangia) were stimulated to produce and liberate flagellates in response to a component in the diet of the host. In the presence of the inducer, differentiation within the sporangium and release of the flagellates occurred rapidly (within 15 to 45 min) in fresh rumen fluid.

During this time no protein synthesis occurred, since development was not inhibited by actidione, although it inhibited growth in culture *in vitro*. Assuming that the actidione penetrated the sporangia, all the enzymes necessary for the differentiation and liberation of the flagellates must have been synthesized before the induction of differentiation. Development of the sporangium was inhibited by compounds known to interfere with membrane structure and function (polymixin B, cytochalasin B) although the sporangia remained intact.

The average maximum production of flagellates in samples of fresh rumen fluid was 12 per sporangium. This number appears high in terms of reproduction of rumen organisms, which usually divide only 2 to 4 times a day under stable population conditions, but since in a sheep fed once daily only a single life-cycle is possible, the production of 12 daughter cells should be compared to a rumen bacterium dividing 4 to 5 times a day resulting in 8 to 12 progeny. It was plain from the diurnal population curves that the population of the vegetative phase remained approximately constant in samples taken at the same time from day to day, and therefore at least 11 flagellates or vegetative units passed out of the rumen or were otherwise destroyed every day. Predation by the protozoan *Entodinium* sp. of non-motile flagellates occurred *in vitro*, and may account for some of the loss. The turnover time, based on the half-life of PEG in the rumen used for the diurnal population studies, was 0.48 days. This was sufficient to account for the loss of about 75% of the organisms, assuming they were predominantly associated with the free rumen fluid as the results of Orpin (1974) suggest, rather than with the mat of food floating on top of the fluid. The survival of only 8.3% is necessary to maintain the population density constant; predation by oligotrich protozoa may be significant *in vivo* and may account for at least part of the remaining 17% of the organisms.

Vavra & Joyon (1966), whilst discussing the taxonomic position of *N. frontalis*, stated that it reproduced by binary fission. This conclusion is incorrect and may have been prompted by the observation of a single cell bearing two tufts of flagella. It was suggested by Bovee (1961) that *C. cyclopis* could possibly be a larval form of a species of *Callimastix* (now *Neocallimastix*) occurring as the adult in a mollusc, herbivore, or other vertebrate host. In the case of *N. frontalis* in the sheep rumen, this suggestion also is clearly incorrect.

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