The Role of Haems and Related Compounds in the Nutrition and Zoosporogenesis of the Rumen Chytridiomycete Neocallimastix frontalis H8

By COLIN G. ORPIN* AND YVONNE GREENWOOD
Department of Biochemistry, Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, UK

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The haem requirement for growth of the rumen chytridiomycete Neocallimastix frontalis H8 was satisfied by haem, haemin, haematin, mesoporphyrin IX, coproporphyrins I and III, uroporphyrins I and III, but not deuteroporphyrin IX. Porphyrin degradation products and precursors of porphyrin synthesis did not support growth. Protoporphyrin IX and haematin were ineffective. Haem could be provided by catalase and peroxidase, but not by cytochrome c. Zoosporogenesis was induced in N. frontalis H8 in rumen fluid principally by haem, haemin and haematin, and to a smaller extent by catalase, peroxidase and protoporphyrin IX. Haem precursors and degradation products were ineffective. Chlorophylls a and b and their ruminal degradation products, plant fraction 1 protein and chelated iron neither stimulated growth nor induced zoosporogenesis. Haem induced partial zoosporogenesis in haem-limited cultures and suspensions of sporangia grown in vitro.

INTRODUCTION

The anaerobic rumen chytridiomycete Neocallimastix frontalis has a life-cycle that consists of an alternation of generations between the cellulolytic vegetative reproductive stage, which occurs principally on the digesta particles (Orpin, 1975; Orpin & Letcher, 1979), and the motile flagellated zoospore stage, which occurs principally in the rumen liquor. The vegetative stage is eucarpic and monocentric with endogenous development. The rhizoid is much-branched and ramifies extensively within the digesta fragments (Orpin, 1977a). Detached sporangia occur in the rumen liquor, probably broken from rhizoids during the mastication of infected digesta during rumination. Orpin (1978a) reported that one isolate of N. frontalis (strain H8) grew in a defined medium, provided haemin was included.

In sheep fed once daily, a vast increase (normally up to 40-fold, but sometimes more) in the population density of the polyflagellated fungal zoospores may be observed in rumen contents soon after the animal has eaten (Orpin, 1975). This increase is due to the induction of zoosporogenesis and the release of zoospores from the sporangia present in the rumen liquor and from some of those present on the digesta particles. The liberated zoospores locate a freshly ingested plant particle by chemotaxis to soluble carbohydrates diffusing from the particle (Orpin & Bountiff, 1978), where they encyst and germinate (Orpin, 1977a). Labile plant components with a wide distribution in the plant kingdom induced zoosporogenesis with subsequent zoospore release (Orpin, 1977b) and haemin stimulated zoosporogenesis in vitro (Orpin, 1978b). This paper reports an examination of the role of haems and related compounds in the nutrition and induction of zoosporogenesis in N. frontalis H8.

METHODS

Organism. The organism was Neocallimastix frontalis strain H8 isolated by the method of Orpin (1975). The cultures were developed from a single vegetative growth picked by micromanipulator from a sloppy agar culture.

Culture methods. Stock cultures of N. frontalis were maintained both in the complex medium of Orpin & Letcher

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(1979) and in the defined medium of Roché et al. (1973) modified according to Orpin (1978a), using cellobiose as carbon source. Cultures were maintained by daily sub-culture, using a 10% inoculum into fresh medium. Haem-limited cultures were grown in the defined medium, but with only 5 μg haemin ml⁻¹.

Estimation of growth. Growth of N. frontalis was determined in 10 ml cultures, grown in pre-weighed culture tubes, by centrifuging the culture at 500 g for 5 min, washing the pellet twice by centrifugation in distilled water (5 ml) and drying at 106 °C overnight. The tubes were cooled over P₂O₅ in a desiccator and weighed, and the growth was determined by difference. Each result is the mean of four experiments.

Glassware. All glassware was boiled in Decon 90 for 15 min, soaked in 1 M-HCl for 24 h, rinsed in distilled water and heated at 450 °C for 4 h in a muffle furnace. Only new butyl rubber septa were used in the anaerobic culture tubes for assays and growth of the organism. These septa were boiled in Decon 90, treated with HCl and rinsed as above before use.

Chemicals. Coproporphyrin I, coproporphyrin III, uroporphyrin I and uroporphyrin III were prepared from their tetramethyl and octamethyl derivatives (C.P. Laboratories Ltd) by acid hydrolysis (Falk, 1964); haem (ferroprotohaem IX) was prepared by the reduction of haemins (protohaem IX) (Falk, 1964); phylloerythrins a and b and phaeophytin were isolated from hay (Dawson & Hemington, 1974); plant fraction 1 protein (ribulose bisphosphate carboxylase, EC 4.1.1.39) was a gift from Dr G. P. Hazlewood of this institute. Haemin was prepared from cytochrome c by the method of Paul (1960). Other porphyrin derivatives, precursors of porphyrin synthesis, degradation products of haem, haem-containing proteins, bovine serum albumin fraction V and chlorophylls a and b were purchased from Sigma. Porphyrins and haems were dissolved in distilled water. Phaeophytin, chlorophylls a and b and phylloerythrins a and b were dissolved in 95% (v/v) ethanol. Bilirubin and biliverdin were dissolved in 1 M-NaOH and porphobilinogen in 1 M-KOH. The ethanolic solutions and the alkaline porphobilinogen were considered sterile as prepared, the others were sterilized by filtration through Millipore filters with a 0.22 μm pore size.

Sheep. The sheep used as a source of rumen fluid were Clun Forest wethers, each fitted with a permanent rumen cannula of 2.5 cm internal bore. The animals were fed 1 kg chopped hay and 100 g rolled oats once daily, and maintained in isolation from other ruminants. The rumen fluid volume of each animal was measured in vivo using Cr-EDTA (Binnerts et al., 1968). The animals were freed of ciliate protozoa by using diocetyl sodium sulphosuccinate (Orpin, 1977c) and freed of anaerobic fungi by two successive treatments with cycloheximide (1 μg per ml of rumen fluid), the second 4 d after the first. The sheep were isolated from other ruminants and, after 7 d free of rumen fungi, each was inoculated with a 500 ml culture of N. frontalis H8 grown in vitro (Orpin & Letcher, 1979). The animals remained free of contaminating rumen fungi for two months.

Rumen fluid. Rumen fluid for use in culture media was obtained by aspiration via the rumen cannula, strained through two layers of muslin, centrifuged at 38500 g for 30 min, and autoclaved (110 °C, 20 min) before incorporation into the culture medium.

Preparation of oats extract. An extract of oats, prepared and used as described by Orpin (1977b), was used as a positive control to induce zoosporogenesis from sporangia in rumen fluid, and is the 'inducer preparation' used in this paper.

Assay for zoosporogenesis in the rumen. Samples of rumen fluid were filtered through two layers of muslin. The population density of free zoospores of N. frontalis was determined by phase-contrast microscopy (Orpin, 1977b) before, and at different time intervals after, the addition of the compound under examination. Possible inducers were tested at a concentration of 10 μg per ml of rumen fluid, added to the rumen in 100 ml of salts solution (Orpin, 1972). After each negative experiment, a positive control experiment was done using an extract of 100 g oats added to the rumen contents (Orpin, 1977b). Each compound shown to induce zoosporogenesis was then tested over a range of 0–20 μg per ml of rumen fluid.

Assay for zoosporogenesis in rumen fluid in vitro. For assaying zoospore production in response to possible inducers of zoosporogenesis in vitro, rumen fluid was obtained by aspiration 18–20 h after feeding, from at least 10 different positions within the rumen, bulked and sub-sampled. The rumen fluid was immediately filtered through two layers of muslin. Although the filtrate was free of large plant fragments, sufficient sporangia were present in the filtrate to assay the effectiveness of possible inducers of sporogenesis. Samples (9 ml) of filtered rumen fluid were placed in Hungate-type anaerobic culture tubes under CO₂. The test compound in 0.1 ml of appropriate solvent was added to give the desired concentration and the tubes were incubated at 39 °C. The population density of the zoospores was determined before and at known time intervals up to 60 min after the start of each experiment, as described by Orpin (1977b). Initial assays were done with a concentration of 10 μg of test compound ml⁻¹; haemin was tested over a range of concentrations from 0–30 μg ml⁻¹.

Preparation of suspensions of sporangia grown in vitro. A well-grown haem-limited culture (100 ml) 24 h old, was homogenized in a Potter homogeniser for 2 min at 10 strokes min⁻¹ at 39 °C anaerobically in a Coy Anaerobe Chamber with a 95% N₂ + 5% H₂ gas phase and the partial homogenate was filtered through four layers of muslin, which retained intact vegetative growth. The sporangia broken from the rhizoids were sedimented by centrifuging at 150 g for 5 min at 39 °C, and were washed twice by centrifugation. Washed sporangia were
suspended in culture medium devoid of haemin, and kept at 39 °C for 1 h. The putative inducer of zoosporogenesis was then added at a haem concentration of 10 μg ml⁻¹. The population density of intact sporangia was determined as described above for flagellates, before and after known time intervals of up to 1 h of incubation at 39 °C under CO₂.

RESULTS AND DISCUSSION

Effect of haems and related compounds on growth of N. frontalis

Iron protohaems. Haem, haemin and haematin (ferriprotohaem IX) supported growth of N. frontalis, and gave similar growth responses to increasing concentrations, each compound stimulating growth at low concentrations and maximum growth occurring at concentrations exceeding 6 μg ml⁻¹. Growth response curves for haem and haemin are shown in Fig. 1(a). In the culture medium (pH 6.7; \( E_0 = -240 \text{ mV} \)) both haemin and haematin would be reduced to haem; this reaction occurs at \( E_0 < -170 \text{ mV} \) at acid pH (Falk, 1964). To determine if the chelated iron rather than the porphyrin was responsible for growth stimulation, iron citrate and Fe-EDTA complexes were added separately to the basal medium in concentrations of iron up to 40 μg ml⁻¹, with no consequent stimulation of growth, showing that the iron-haems probably did not stimulate growth by providing chelated iron to the organism.

Haemoproteins. Growth occurred in the presence of catalase, peroxidase (Fig. 1b) and haemoglobin, total growth yields being similar to that given by haemin, when the haemoproteins were supplied to provide haem concentrations in the range 1–16 μg ml⁻¹. The rates of growth were less than that with the haems (Fig. 1c). Cytochrome c did not stimulate growth.

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![Fig. 1](image)

**Fig. 1.** Effect of haems and haem-containing compounds on the growth *in vitro* of *N. frontalis* H8 in the defined medium. Growth response to different concentrations of haem in (a) haemin (●) and haem (■) and (b) catalase (▲) and peroxidase (△) after 28 h. (c) Growth curve in the presence of 10 μg haem ml⁻¹ in haemin (●), catalase (▲) and haemoglobin (○). (d) Growth response to different concentrations of haem in cytochrome c (▽) and haem prepared from cytochrome c (□) after 28 h. All incubations were at 39 °C and CO₂; the results are means of three experiments, bars represent 1 SD.
growth, but haem prepared from cytochrome c did (Fig. 1d). This suggests that whilst N. frontalis could remove the haem from the other haemoproteins, it was unable to break the protein–haem bonding in cytochrome c. This is in agreement with the known high stability of this compound (Paul, 1960). Plant fraction I protein and bovine serum albumin fraction V, containing no haem, did not stimulate growth, confirming the dependence of the organism on the haem moiety rather than on the protein moiety of haemoproteins.

Haem precursors, degradation products and porphyrins. Growth was supported by protoporphyrin IX, mesoporphyrin IX, haematoporphyrin, coproporphyrin I, coproporphyrin III, uroporphyrin I and uroporphyrin III, but not by deuteroporphyrin IX (Fig. 2a). No growth occurred with the precursors of porphyrin synthesis, porphobilinogen and δ-aminolaevulinic acid, or with the porphyrin degradation products bilirubin and biliverdin.

Haems are normally required by micro-organisms for the synthesis of haemoproteins. If this is true for N. frontalis, the absence of growth stimulation by likely porphyrin precursors suggests that porphyrin and haem synthesis do not occur in this organism. However, some capacity of N. frontalis to modify substituent groups in porphyrins is suggested by its ability to grow with uroporphyrins I and III, coproporphyrins I and III and mesoporphyrin IX. For synthesis of protoporphyrin IX from these compounds, conversion of acetic acid, propionic acid and ethyl side-chains to methyl, vinyl and vinyl side-chains respectively, and, for the type I isomers,
isomerization is necessary. This would be an unusual reaction, but unless conversion to porphobilinogen occurred, it is the only obvious explanation for growth of the organism on these type I compounds. Lack of growth on porphobilinogen does not rule this out, since the porphobilinogen may not be taken up by the cells from the medium. Bacteroides ruminicola can utilize coproporphyrinogen I and uroporphyrinogen I for growth (Caldwell et al., 1965), perhaps by a similar conversion and synthesis. We were unfortunately unable to test growth of *N. frontalis* on these compounds.

The inability of deuteroporphyrin IX to support growth may represent either an inability of the organism to add vinyl side-chains or an impermeability of the cells to deuteroporphyrin IX. Since growth occurred with protoporphyrin IX and haematoporphyrin IX it is probable that *N. frontalis* contains a ferrochelatase and inserts the iron into the porphyrin ring to synthesize haem, but it is apparently unable to synthesize the porphyrin ring system from precursors. Growth on haemoporphyrin indicates that *N. frontalis* can dehydrate the α-hydroxyethyl side chains of haematoporphyrin IX to vinyl residues to form protoporphyrin IX before the insertion of iron.

*Growth in the presence of other compounds.* The other major sources of porphyrin entering the rumen in the diet are chlorophylls *a* and *b*, containing chelated magnesium. Neither supported growth of *N. frontalis* in the absence of haem. The degradation products of chlorophylls *a* and *b*, phylloerythrins *a* and *b* and phaeophytin, which occur in rumen contents within 1 h of feeding (Dawson & Hemington, 1974), were also non-stimulatory. This indicates that haemoporphyrins and related protoporphyrins are the only porphyrins which occur in the diet of the animal that can be utilized by *N. frontalis*, and adds support to the suggestion that only a limited amount of available porphyrins can be modified by the organism.

**Effect of haems and related compounds on zoosporogenesis**

Since the provision of haem or certain porphyrins was essential for the growth of *N. frontalis* *in vitro*, the same range of compounds tested for growth stimulation was tested to see if zoosporogenesis was induced in rumen fluid *in vivo* or in rumen fluid incubated *in vitro*. Zoosporogenesis was induced *in vitro* by several of the compounds tested, but only haem, haemin, haematin and the haemoproteins catalase and peroxidase induced extensive zoospore production (Fig. 2b). Haem, haemin and haematin stimulated zoosporogenesis in the rumen with no delay, but not to the same extent as the inducer preparation from oats (Fig. 3a, b), while zoospore production after the addition of haemoproteins showed a distinct lag phase (Fig. 3b). The addition of cytochrome *c* resulted in only low numbers of zoospores being released into the rumen fluid, but the addition of haematin prepared from cytochrome *c* resulted in a level of zoospore production equivalent to that with haem.

Zoosporogenesis could not be induced *in vitro* by the addition of haems to normally grown cultures of *N. frontalis*. Cultures grown in limited haem concentrations (5 μg haemin ml⁻¹) did, however, show partial (<35% after 1 h) zoosporogenesis in response to haem added at 10 μg ml⁻¹, provided growth had ceased. Suspensions of sporangia prepared from haem-limited cultures showed a maximum of 48% zoosporogenesis after 25 min exposure to haem at 10 μg ml⁻¹, indicating that sporangia which become detached from the rhizoid (e.g. during rumination) may be more susceptible to the induction of zoosporogenesis *in vitro*. We have also observed that virtually all of the free sporangia in rumen fluid release zoospores after exposure to the oats extract or haem-containing porphyrins (Orpin, 1978b).

Since haematin and haemin would be reduced to haem by the low redox potential of the rumen fluid it is highly probable that it was haem that induced zoosporogenesis. Microbial action on haemoproteins in the rumen could result in the release of the haem group, which would then also induce zoosporogenesis. The limited zoosporogenesis produced by cytochrome *c* was probably due to only a low level of microbial destruction and haem release from this very stable haemoprotein. Our inability to demonstrate 100% zoosporogenesis in sporangia grown *in vitro* may have been due to damage sustained by the sporangia during preparation of the suspension.

Although haems clearly played a role in inducing zoosporogenesis, zoosporogenesis in rumen...
Fig. 3. Effect of haem-containing compounds on zoosporogenesis in vivo. (a) Effect of concentration of haemin. Zoospore cell density was estimated before and 25 min after adding the haemin to the rumen and expressed as a percentage of the zoospore production in response to inducer preparation from 100 g dry wt oats, measured during the 3 d preceding the experiment; the results are means of experiments on three animals on consecutive days. (b) Zoospore production with time in response to 10 µg haem ml⁻¹ added to the rumen as haemin (●), catalase (▲) and cytochrome c (○), expressed as a percentage of that produced in response to inducer preparation from 100 g dry wt oats (●). The results with haemin and the inducer preparation are means of three experiments; bars represent 1 SD. The results with catalase and cytochrome c are from single experiments.

fluid was less than that shown in response to the inducer preparation from oats. It is possible that complete zoosporogenesis is brought about by haem acting in synergism with other components of the diet, or that the cells used in vitro were not in the correct physiological state.

Haems, in oxidized or reduced forms, and haemoproteins occur in virtually all vegetative plant tissues because of their role as enzyme prosthetic groups. Therefore, whenever the animal eats, these compounds enter the rumen fluid. In these experiments when the animals were fed once daily, haems entering the rumen triggered zoosporogenesis. In animals fed more frequently (for example, at pasture) this probably does not occur every time the animal eats. There is evidence (Orpin, 1977b) that the fungi must be at least 8 h old before zoosporogenesis is induced by dietary components, and zoosporogenesis may therefore be controlled by a more complex system.

At present we do not know the role of haems in the metabolism of N. frontalis. Spectrophotometric evidence suggests the lack of any cytochromes (C. G. Orpin, unpublished experiment), but haems may be required for peroxidases (Dixon & Webb, 1964).

Haemin is often incorporated into rumen bacteriological culture media, since it stimulates the growth of some species (Bryant & Robinson, 1962). For example Bacteroides ruminicola subsp. ruminicola requires haem for growth, and a variety of porphyrins and haems can satisfy this requirement (Caldwell et al., 1965). One strain of B. ruminicola subsp. brevis grew in the absence of tetrapyroles, providing δ-aminolaevulinic acid was present from which it could synthesize hames, but a source of haem or protoporphyrin IX was required by several other anaerobic Bacteroides spp. (Sperry et al., 1977). The requirement for haem is due to cytochrome-linked fermentation (White et al., 1962). Incorporation of haemin into non-selective culture media used for counting and culturing viable rumen bacteria results in higher viable counts, suggesting that haemin may be required by other ruminal species (Caldwell & Bryant, 1966).

Coleman & Reynolds (1982) showed stimulation of growth in vitro of the rumen ciliates Ophryoscolex caudatus, Epidinium ecaudatum and, under some conditions, Entodinium caudatum by the addition of haemin, but absolute dependence on haem was not demonstrated.

The majority of aerobic fungi do not appear to require haem, but Page (1952) demonstrated a haem requirement in Pilobolus spp. The requirement of the anaerobic N. frontalis for porphyrins
may reflect adaptation to the rumen environment where porphyrins are regularly supplied in the diet of the animals and haem synthesis is therefore wasteful.

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