Hydrogenosomes in the rumen fungus *Neocallimastix patriciarum*

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Sedimentable hydrogenase activity was demonstrated in cell-free extracts from both zoospores and vegetative growth of the anaerobic rumen fungus *Neocallimastix patriciarum*. Electron micrographs of the fraction enriched in hydrogenase activity contained finely granular microbody-like organelles, about 0.5 μm in diameter and having an equilibrium density of about 1.2 $\text{g}\cdot\text{ml}^{-1}$ in sucrose, 1.12 $\text{g}\cdot\text{ml}^{-1}$ in Percoll and 1.27–1.28 $\text{g}\cdot\text{ml}^{-1}$ in Metrizamide. These organelles, which are sedimentable at $10^5\text{ g}\cdot\text{min}$, bear no similarity to mitochondria, but are morphologically similar to hydrogen-evolving organelles possessed by certain anaerobic protozoa and termed ‘hydrogenosomes’. Other typical hydrogenosomal enzymes, namely ‘malic’ enzyme, pyruvate:ferredoxin oxidoreductase and NADPH:ferredoxin oxidoreductase, were enriched in the same particle fraction as hydrogenase. The synthesis of pyruvate:ferredoxin oxidoreductase was found to be suppressed when the organism was cultured under an atmosphere of CO_2 , and an alternative pathway is proposed for growth under these conditions.

INTRODUCTION

During anaerobic fermentation, products of both higher and lower oxidation states than the substrate are formed (Wood, 1961). In several species of protozoa, e.g. trichomonads and rumen ciliates, the reducing equivalents generated during fermentation are eliminated in the form of molecular hydrogen. These protozoa possess a specialized redox organelle termed the ‘hydrogenosome’, which is characterized by the possession of pyruvate:ferredoxin oxidoreductase and hydrogenase (Čerkasovová *et al.*, 1973; Lindmark & Müller, 1973, 1974; Lindmark *et al.*, 1975; Yarlett *et al.*, 1981, 1983, 1984; Snyers *et al.*, 1982). In some other protozoal species hydrogenase is absent, e.g. *Entamoeba histolytica* and *Giardia lamblia*, and in these anaerobes reducing equivalents are removed by the reduction of acetaldehyde to ethanol by cytosolic alcohol dehydrogenase (Lo & Reeves, 1978; Lindmark, 1980).

Hydrogenosomes from all the species of protozoa so far examined have a similar structure. They are vesicles 0.5–2.0 μm in diameter, sometimes elongated, with a fine granular matrix and often containing a denser core (Müller, 1980).

The rumen anaerobic fungus *Neocallimastix patriciarum* has a two-stage life-cycle, alternating between the motile zoospore stage and a non-motile vegetative reproductive stage (Orpin, 1975). Axenic cultures of this phycomycete produce hydrogen during the fermentation of carbohydrates (Orpin, 1978; Bauchop & Mountfort, 1981), but the mechanism of hydrogen production has not been established and the presence of specialized redox organelles has not been reported.

As judged by electron microscopy, *Neocallimastix* does not contain structures recognizable as mitochondria (Munn *et al.*, 1981; Heath *et al.*, 1983), although zoospores do contain microbody-like organelles which

are morphologically similar to protozoal hydrogenosomes. We show here that similar organelles are also present in the vegetative growth. Examination of the mechanism of hydrogen production revealed the presence of sedimentable hydrogenase, pyruvate:ferredoxin oxidoreductase, NADPH:ferredoxin oxidoreductase and ‘malic’ enzyme activities, which are associated with the microbody-like structures, making this the first report of a hydrogenosome-like organelle in a species of fungus.

The synthesis of pyruvate:ferredoxin oxidoreductase was suppressed by growth under an atmosphere of CO_2 ; an alternative pathway is proposed for growth under these conditions.

EXPERIMENTAL

Organism

The strain of *Neocallimastix* used has previously been designated ‘*N. frontalis*’, but it does not show the bipartite distribution of cellular organelles (Fig. 1a) nor the equatorial constriction of the zoospores typical of the type strain of *N. frontalis* described by Heath *et al.* (1983). This isolate has been named ‘*N. patriciarum*’ (Orpin & Munn, 1985).

Culture technique

N. patriciarum from the ovine rumen (Orpin, 1975), was cultured as described for *Piromonas communis* (Kemp *et al.*, 1984), except that maltose (5 g/l) replaced cellobiose, and filter-sterilized ampicillin (100 mg/l) and chloramphenicol (50 mg/l) were added after autoclaving. Bicarbonate was omitted from cultures grown under an atmosphere of N_2 . Vegetative growth was harvested in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI, U.S.A.) by filtration through four layers of muslin and washed with the following salts solution (g/l):

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Fig. 1. Electron micrographs of sections of *N. patriciarum*

(a) Whole zoospore, and (b), at higher magnification, a region situated below the flagella showing the hydrogenosome-like vesicles. In vegetative growth (c,d) the hydrogenosome-like vesicles are present in small groups distributed apparently at random throughout the length of the rhizoids (they also occur in the sporangia). The scale bars represent 1 μm in (a) and (c) and 0.5 μm in (b) and (d).

K_2HPO_4 , 0.45; KH_2PO_4 , 0.45; $(\text{NH}_4)_2\text{SO}_4$, 0.9; NaCl, 0.9; MgSO_4 , 0.09; CaCl_2 , 0.09; cysteine hydrochloride, 1.0; NaHCO_3 , 6.0; saturated with CO_2 at room temperature. Zoospores were recovered by centrifugation of the filtrate at 2500 g -min at room temperature and washed twice by centrifugation under the same conditions in salts solution.

Disruption and centrifugation

The washed cell pellets were disrupted inside an anaerobic chamber using a Teflon/glass Potter homogenizer [Jencons (Scientific) Ltd., Leighton Buzzard, Beds.,

U.K.] rotating at 120 rev./min and 10 strokes/min for 3 min, on ice. The disruption buffer consisted of (g/l): sucrose, 85.6; EDTA, 0.27; KCl, 1.49; KH_2PO_4 , 1.36; MgCl_2 , 1.0; Tris/HCl (pH 7.4), 1.21. The homogenate was centrifuged at 2600 g -min (P1) in the anaerobic chamber to remove unbroken cells, membrane fragments and nuclei, and the supernatant carefully decanted and subjected to differential centrifugation at 4 °C yielding particulate fractions sedimenting at 10⁵ g -min (P2) and 4.1 $\times$ 10⁵ g -min (P3) using a Sorval RC2B centrifuge (du Pont Biomedical Products, Stevenage, Herts., U.K.); a 6.5 $\times$ 10⁶ g -min (P4) particulate fraction and a non-

sedimentable fraction, using a Beckman model L5-65 ultracentrifuge with a SW25 rotor (Beckman RIIc Ltd., High Wycombe, Bucks., U.K.).

Density-gradient centrifugation

Density gradients were prepared from 15 and 65% (w/w) sucrose in 10 mM-Tris/HCl buffer, pH 7.4, containing 0.7 mM-EDTA and 30 mM-2-mercaptoethanol. Fraction P2 was carefully layered on top of the gradient and centrifuged in a 3 × 25 ml swing-out rotor (Beckman L5-65 ultracentrifuge) at 4 °C for 1 h at 10000 rev./min (6.5×10^6 g·min). Fractions (about 2 ml) from the gradient were collected inside the anaerobic chamber after puncturing the base of the tube. The density of the fractions was measured with a refractometer (Bellingham and Stanley Ltd., London N.19, U.K.). Self-generating gradients were prepared with 67.5% (w/v) Percoll [Pharmacia (G.B.) Ltd., Uxbridge, Middx., U.K.], containing 0.25 M-sucrose and 10 mM-Tris/HCl, pH 7.4. Fraction P2 was carefully layered on top of the Percoll and centrifuged in a 6 × 12 ml swing-out rotor (Beckman L5-65 ultracentrifuge) at 4 °C for 45 min at 18500 rev./min (2×10^6 g·min). Fractions (about 1 ml) from the gradient were collected in 1 ml graduated disposable pipettes (Sarstedt) inside the anaerobic chamber. The density of the fractions was measured by using density marker beads.

Metrizamide [2-(3-acetamido-5-*N*-methylacetamide-2,4,6-tri-iodobenzamide)-2-deoxy-D-glucose; analytical grade; Nyegaard and Co., Birmingham, U.K.] was adjusted to pH 7.4 with 0.01 M-NaOH. Gradients were prepared by layering portions of dilutions of a stock solution (1.55 g/ml) to give a density range from 1.15 to 1.55 g/ml. Fraction P2 was either layered on top of the gradient or mixed with metrizamide to a density of 1.30 g/ml and layered on a cushion of metrizamide (1.55 g/ml) at the bottom of the tube.

Enzyme assays

One enzyme unit is defined as 1 μ mol of substrate transformed/min. Hydrogenase (EC 1.18.3.1), 'malic' enzyme (EC 1.1.1.40), pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1), and malate dehydrogenase (oxalacetate reductase) (EC 1.1.1.37) activities were assayed by the method of Lindmark & Müller (1973, 1974), formate dehydrogenase (EC 1.2.1.2) as described by Friedrich *et al.* (1979), and lactate dehydrogenase (EC 1.1.1.27) as described in the Boehringer (1970) catalogue. NADH oxidase (EC 1.6.99.3) was determined spectrophotometrically at 340 nm by monitoring the initial oxidation rate of NADH (0.2 μ mol) in 300 μ mol of potassium phosphate, pH 7.0, plus 20 μ g of catalase in a final volume of 2.4 ml. The amount of NADH oxidized was calculated by using an ϵ_{340} value of $6.2 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for NADH. Alcohol dehydrogenase (EC 1.1.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), pyruvate decarboxylase (EC 4.1.1.1) and pyruvate kinase (EC 2.7.1.40) were assayed as described by Bergmeyer *et al.* (1974). Hexokinase (EC 2.7.1.1), phosphoenolpyruvate carboxykinase (EC 4.1.1.32) and enolase (EC 4.3.1.11) were assayed by the methods described by Darrow & Colowick (1962), Vennesland (1962) and Westhead (1970) respectively. D-Amino acid oxidase (EC 1.4.3.3) was assayed spectrophotometrically by the method of Wellner & Lichtenberg (1971). Aldehyde dehydrogenase (NAD⁺) (EC 1.2.1.3) was measured by replacing ethanol with acetate in the

Table 1. Activity of *N. patriciarum* hydrogenase with various electron acceptors

Electron acceptor	Concn.	Activity (nmol·min ⁻¹ ·mg of protein ⁻¹)
Methyl Viologen	10 mM	178
FMN	0.5 mM	59
FAD	0.5 mM	100
Spinach ferredoxin	1 mg/ml	50
Metronidazole	1 mM	87

alcohol dehydrogenase assay. NADPH:ferredoxin oxidoreductase (EC 1.18.1.2) was measured spectrophotometrically in anaerobic cuvettes, the incubation mix containing 20 mM-Methyl Viologen, 100 mM-potassium phosphate, pH 7.4, 0.1 mg of spinach ferredoxin, 5 mM-NADPH, 250 mM-2-mercaptoethanol, 0.1% Triton X-100, and a gas phase of N₂. The absorption coefficient for Methyl Viologen was taken as $6.3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 600 nm. A second method used measured NADP⁺ (0.4 mM) reduction with 0.1 mg of ferredoxin, 100 mM-potassium phosphate, pH 7.4, 0.1% Triton X-100 and 250 mM-2-mercaptoethanol in anaerobic cuvettes under a gas phase of H₂. The absorption coefficient for NADP at 340 nm is $6.2 \text{ M}^{-1} \cdot \text{cm}^{-1}$. NADH:ferredoxin oxidoreductase was measured as for NADPH:ferredoxin oxidoreductase, replacing NADPH with NADH. Phosphatases were assayed by determining the rate of *p*-nitrophenol release from 0.015 M-*p*-nitrophenol phosphate in 0.1 M-acetate buffer, pH 4.2 (acid phosphatase) or 0.1 M-Tris/HCl, pH 8.0 (alkaline phosphatase) at 39 °C. The *p*-nitrophenol was measured spectrophotometrically at 420 nm in alkaline solution (Barrett & Heath, 1977). With the exception of hydrogenase, all enzymes were assayed in the presence of 0.1% (v/v) Triton X-100. Protein was measured by using an automated Lowry (Lowry *et al.*, 1951) technique (Technicon Instruments, Chertsey, Surrey, U.K.), with bovine serum albumin as standard.

Electron microscopy

Suspensions of washed organisms and materials in fractions were fixed overnight at 4 °C in 2% (w/v) glutaraldehyde in 80 mM-phosphate buffer, pH 7.4, containing 1.4 mM-CaCl₂. Samples were processed by the method of Munn *et al.* (1981).

RESULTS

Hydrogenase activity was demonstrated in cell-free extracts obtained from vegetative growth and zoospores of the rumen phycomycete *N. patriciarum*. Hydrogenase activity was demonstrated with Methyl Viologen, FMN⁺, FAD⁺, spinach ferredoxin or metronidazole (1-hydroxyethyl 2-methyl-5-nitroimidazole) as the terminal electron acceptor (Table 1). The specific activity of this enzyme in homogenates obtained from both the vegetative and zoospore stage was several-fold greater in the large granular fraction (P2), which was sedimented at 10^5 g·min (Figs. 2a and 3a), than in the cell-free extract.

'Malic' enzyme, demonstrated by its dependence on bivalent cations, was present in cell-free extracts from

Fig. 2. Distribution of enzymes in fractions obtained by differential centrifugation of a cell-free extract of *N. patriciarum* vegetative growth

Organisms were cultured under an N_2 atmosphere. Relative specific activities (the ratio of specific activities in fractions to those in the cell-free extract) were plotted against cumulative percentage of protein recovered in each fraction. The centrifugal field increases from left to right. The far-right-hand bar represents the fraction containing material non-sedimentable at 6.5×10^6 g-min. Percentage recoveries (based on enzyme units in the cell-free extract) and specific activities (nmol/min per mg of protein) for peak fractions were (a) hydrogenase, 83%, 397 (P2); (b) pyruvate:ferredoxin oxidoreductase, 87%, 109 (P2); (c) NADPH:ferredoxin oxidoreductase, 90%, 138 (P2); (d) 'malic' enzyme, 92%, 486 (P2); (e) lactate dehydrogenase, 113%, 39 (S); (f) phosphoenolpyruvate carboxykinase, 91%, 230 (S); (g) NADH oxidase, 106%, 33 (S); (h) acid phosphatase, 109%, 73 (P3); (i) alkaline phosphatase, 138%, 118 (P3); (j) enolase, 71%, 61 (S); (k) hexokinase, 122%, 201 (S); (l) malate dehydrogenase, 115%, 2088 (S). The amount of protein recovered was 96%. Material from the shaded area was subjected to isopycnic density centrifugation (Figs. 4, 5 and 6).

vegetative growth and zoospores (Tables 2 and 3), and its specific activity was enriched in the P2 fraction which sedimented at 10^5 g-min (Fig. 2d).

When the organisms were grown under standard

conditions with HCO_3^- and a gas phase of CO_2 , pyruvate ferredoxin: oxidoreductase activity could not be detected in cell-free extracts within the pH range 5.0–8.0, when Methyl Viologen, FAD^+ or FMN^+ were used as electron

Fig. 3. Distribution of enzymes in fractions obtained by differential centrifugation of a cell-free extract of *N. patriciarum* zoospores

Organisms were cultured under a CO_2 atmosphere. Conditions are described in Fig. 2. Percentage recoveries and specific activities for peak fractions were: (a) hydrogenase, 74%, 694 (PI); (b) 'malic' enzyme, 85%, 285 (PI); (c) lactate dehydrogenase, 99%, 52 (S); and protein, 81%. The shaded area sedimented at 10^5 g·min and corresponds to the shaded area of Fig. 2.

acceptors (limits of detection were 1.6, 4.8 and 2.5 munits respectively). There was always a slight endogenous rate of reduction with either FAD^+ or FMN^+ as electron acceptor (2.2 and 1.5 munits/mg of protein respectively), but this was not increased by the addition of pyruvate. However, pyruvate:ferredoxin oxidoreductase activity could readily be demonstrated in cell-free extracts of cultures grown in the absence of HCO_3^- and under a gas phase of N_2 (Table 3), and this activity was enriched in the same sedimentable fraction as hydrogenase, NADPH:ferredoxin oxidoreductase and 'malic' enzyme. The activity of hydrogenase and NADPH:ferredoxin oxidoreductase are 3–5-fold higher in samples grown with HCO_3^- and under a CO_2 atmosphere. 'Malic' enzyme was not significantly affected by changing the growth conditions. The activities of the non-sedimentable enzymes malate dehydrogenase and phosphoenolpyruvate carboxykinase were doubled by growth under a N_2 atmosphere.

Neither particulate nor soluble forms of formate dehydrogenase were detected (limits of detection 0.76 and 1.6 munits respectively). NADH oxidase was detected in cell-free extracts of vegetative growth (Table 3) and was found to be enriched in the non-sedimentable fraction (Fig. 2g). Lactate dehydrogenase, present in cell-free extracts from both stages of growth (Table 3), was also found to be non-sedimentable at 6.5×10^6 g·min (Figs. 2e and 3c). The hydrolases acid phosphatase and alkaline phosphatase were detected in cell-free extracts of vegetative growth (Table 3) and were characteristically enriched in the 4.1×10^5 g·min fraction (Figs. 2h and 2i).

The activity of both hydrogenase and 'malic' enzyme from vegetative growth with $\text{HCO}_3^-/\text{CO}_2$ in the culture media showed a mean equilibrium density of 1.20 g·ml $^{-1}$ in sucrose (Fig. 4).

Further characterization of the organelle was undertaken by use of other density-gradient materials. Fig. 5 shows the enzyme distribution pattern after centrifugation in Percoll of the 10^5 g·min fraction from vegetative cells grown in the absence of HCO_3^- and under an N_2 atmosphere. The enzymes hydrogenase, 'malic' enzyme, pyruvate:ferredoxin oxidoreductase and NADPH:ferredoxin oxidoreductase showed a mean equilibrium

Table 2. Inhibitory effect of EDTA on 'malic' enzyme by chelation of bivalent cations

Inhibition could be relieved by addition of Mn^{2+} ions.

EDTA (nmol)	Enzyme activity (units/ml)	Inhibition (%)
—	13	—
67	7	46
133	3	79
200	0.2	99

density of 1.12 g·ml $^{-1}$. The purification of *N. patriciarum* hydrogenosomes on a Metrizamide gradient is shown in Figs. 6(a) and 6(b); the low viscosity and osmolarity of this medium minimizes the possibility of particles becoming partially dehydrated, which does happen in very concentrated solutions (Rickwood, 1979). The profile of hydrogenase, 'malic' enzyme, pyruvate:ferredoxin oxidoreductase and NADPH:ferredoxin oxidoreductase obtained when the 2×10^5 g·min fraction was loaded at the top of the gradient gave a single peak at 1.28 g·ml $^{-1}$ (Fig. 6a). Making the loading material iso-osmotic with Metrizamide at 1.30 g·ml $^{-1}$ and layering this on to a 1.55 g·ml $^{-1}$ cushion at the bottom of the gradient (Fig. 6b) resulted in bimodal distribution of activity for the marker enzymes (hydrogenase, 'malic' enzyme, pyruvate:ferredoxin oxidoreductase and NADPH:ferredoxin oxidoreductase) at 1.27 g·ml $^{-1}$ and 1.31 g·ml $^{-1}$, the latter peak being suggestive of organelle damage due to hydrostatic pressure, a feature observed with peroxisomes in high concentrations of Metrizamide (Wattiaux *et al.*, 1978).

Spectrophotometric examination of homogenized vegetative growth at room temperature revealed no absorption in the region 450–650 nm, indicating the absence of typical cytochromes (limits of detectability for cytochrome *c* were less than 14 pmol·cm $^{-1}$ ·mg of protein $^{-1}$). An absorption band at 400–450 nm was present which suggested the presence of an iron–sulphur protein.

Table 3. Activities of enzymes in cell-free extracts of *N. patriciarum*

Specific activities are expressed as munits (nmol/min) per mg of protein ($\pm$ S.D.) for the appropriate number of experiments (in parentheses) where stated. Enzymes were assayed at their pH optima. Abbreviations used: N.D., not detectable within the limits of the assay conditions; N.A., not assayed.

Stage of growth	Enzyme	Specific activity in cell-free extracts (munits/mg of protein)	
		Growth under CO ₂	Growth under N ₂
Vegetative	Hydrogenase	788 $\pm$ 74 (3)	178 $\pm$ 12 (3)
	'Malic' enzyme	213 $\pm$ 25 (3)	267 $\pm$ 29 (3)
	Pyruvate:ferredoxin oxidoreductase	N.D.	53 $\pm$ 7 (3)
	NADPH:ferredoxin oxidoreductase	196 $\pm$ 8 (3)	68 $\pm$ 7 (3)
	NADH:ferredoxin oxidoreductase	480 $\pm$ 23 (3)	N.A.
	NADH oxidase	20 $\pm$ 4 (2)	N.A.
	Lactate dehydrogenase	23 $\pm$ 7 (2)	21 $\pm$ 4 (2)
	Malate dehydrogenase	551 $\pm$ 39 (2)	1067 $\pm$ 58 (2)
	NADP:isocitrate dehydrogenase	142 $\pm$ 18 (2)	107 $\pm$ 21 (2)
	Alcohol dehydrogenase	N.D.	23
	Glucose-6-phosphate dehydrogenase	N.D.	N.D.
	Pyruvate kinase	9	N.A.
	Acetaldehyde dehydrogenase (NAD ⁺)	N.D.	N.A.
	Phosphoenolpyruvate carboxykinase	53 $\pm$ 6 (2)	124 $\pm$ 14 (3)
	Hexokinase	96	107
	Enolase	50	62
	3-Phosphoglycerate kinase	69	74
	D-Amino acid oxidase	N.D.	N.A.
	Pyruvate decarboxylase	34	27
	Formate dehydrogenase	N.D.	N.D.
	Formate hydrogen-lyase	N.D.	N.D.
	Acid phosphatase	47 $\pm$ 2 (2)	59
	Alkaline phosphatase	37 $\pm$ 3 (2)	41
Zoospores	Hydrogenase	322 $\pm$ 3 (2)	N.A.
	'Malic' enzyme	118 $\pm$ 22 (2)	N.A.
	Lactate dehydrogenase	30 $\pm$ 4 (2)	N.A.
	Pyruvate:ferredoxin oxidoreductase	N.D.	N.A.

Electron microscopy

Electron-microscopic examination of thin sections of zoospores (Fig. 1a) of *N. patriciarum* showed the presence of many intracellular vesicles at the flagella pole.

These were previously designated 'amorphous globules' (Munn *et al.*, 1981), and are circular (0.2–0.5 μ m diam.) or elongate in section, and filled with a fine granular, amorphous, matrix of variable electron density. Similar, but sometimes larger, vesicles were observed in the rhizoids of the vegetative stage (Figs. 1c and 1d) and in the sporangia. The 10⁵ g-min (P2) fraction with the highest level of hydrogenase, 'malic' enzyme, pyruvate:ferredoxin oxidoreductase and NADPH:ferredoxin oxidoreductase activities contained a mixture of fragments of cell organelles and intact vesicles resembling the amorphous globules. These vesicles, 0.25–0.50 μ m in diameter (Fig. 7a), were not found in fractions P3 or P4 (Figs. 7b and 7c) in which hydrogenase, 'malic' enzyme, pyruvate:ferredoxin oxidoreductase and NADPH:ferredoxin oxidoreductase activities were low (Fig. 2). Some of the vesicles had regions of increased electron density, and some had projections which gave the structure a 'dumb-bell' appearance (Fig. 7a). Some, like the amorphous globules seen in zoospores, contained an internal membrane. Fragments of cilia, pieces of membrane and small electron-dense particles were also present in the same fraction.

DISCUSSION

N. patriciarum produces acetate, lactate, CO₂, H₂ and only traces of ethanol and formate during the degradation of plant cell components (Orpin, 1978); in contrast *N. frontalis* produces formate and ethanol as major fermentation products (Bauchop & Mountfort, 1981). Two pathways of H₂ formation are known; both involve pyruvate as intermediate, but they differ in the mechanism of end-product formation. In one pathway, used by clostridia (Gray & Gest, 1965) and protozoa (Bauchop, 1971; Yarlett *et al.*, 1981), electrons for proton reduction are derived from the conversion of pyruvate into acetyl-CoA by pyruvate:ferredoxin oxidoreductase. In the second pathway, which occurs in the Enterobacteriaceae (Gray & Gest, 1965), electrons for proton reduction are derived from formate dehydrogenase. The absence of detectable formate dehydrogenase and formate hydrogen-lyase in extracts of *N. patriciarum* indicates the absence of the later pathway of H₂ evolution. However, hydrogenase, 'malic' enzyme and NADPH:ferredoxin oxidoreductase were detected under all growth conditions, and differential fractionation of homogenates showed that these activities were associated with the (P2) particulate fraction. This is similar to the distribution of enzyme activities found in hydrogenosomes from some anaerobic protozoa (Müller, 1980; Yarlett *et al.*, 1981; Snyers *et al.*, 1982). When the organism was grown under

Fig. 4. Distribution of hydrogenase and 'malic' enzyme after equilibrium density-gradient centrifugation in a sucrose gradient of the 10^5 g-min fraction

Organisms were grown under a CO_2 atmosphere. The direction of sedimentability was from left to right. The specific activities of enzymes (munits/mg of protein) at their maxima, and percentage recoveries (in parentheses), were as follows: hydrogenase, 8786 (63%); 'malic' enzyme, 2914 (72%). The recovery of protein was 106%.

Fig. 5. Distribution of hydrogenase, 'malic' enzyme, pyruvate:ferredoxin oxidoreductase and NADPH:ferredoxin oxidoreductase after equilibrium density-gradient centrifugation in Percoll of the 10^5 g-min fraction

Organisms were grown under an N_2 atmosphere. Direction of sedimentability was from left to right. The specific activities of enzymes (munits/mg of protein) at their maxima, and percentage recoveries (in parentheses), were as follows: hydrogenase, 1860 (67%); 'malic' enzyme, 2270 (71%); pyruvate:ferredoxin oxidoreductase, 930 (74%); NADPH:ferredoxin oxidoreductase, 781 (58%). The recovery of protein was 79%.

an atmosphere of N_2 and in the absence of HCO_3^- , it was shown that, in addition to the aforementioned enzymes, a sedimentable pyruvate:ferredoxin oxidoreductase was also detectable. The concentration of CO_2 in the rumen is about 65% (McArthur & Miltimore, 1961), hence it is likely that, under these conditions, pyruvate:ferredoxin oxidoreductase would be present in hydrogenosomes from *N. patriciarum*. The lowered activities of the iron-sulphur-containing enzymes NADPH:ferredoxin

Fig. 6. Distribution of hydrogenase, 'malic' enzyme, pyruvate:ferredoxin oxidoreductase and NADPH:ferredoxin oxidoreductase after equilibrium density-gradient centrifugation in a Metrizamide gradient of the 10^5 g-min fraction

Organisms were grown under an N_2 atmosphere. The direction of sedimentability was from left to right. The specific activities of enzymes (munits/mg of protein) at their maxima, and percentage recoveries (in parentheses), were as follows: (a) 10^5 g-min fraction loaded on top of gradient; hydrogenase, 2000 (66%); 'malic' enzyme, 2667 (95%); pyruvate:ferredoxin oxidoreductase, 616 (69%); NADPH:ferredoxin oxidoreductase, 933 (82%); protein recovery was 69%; (b) 10^5 g-min fraction made iso-osmotic with Metrizamide ($1.30 \text{ g} \cdot \text{ml}^{-1}$) and loaded on cushion ($1.55 \text{ g} \cdot \text{ml}^{-1}$) at the bottom of the gradient; hydrogenase, 1006 (65%); 'malic' enzyme 1646 (87%); pyruvate:ferredoxin oxidoreductase, 366 (76%); NADPH:ferredoxin oxidoreductase, 411 (79%); protein recovery was 72%.

oxidoreductase and hydrogenase after growth under a N_2 atmosphere may be due to the decrease in available iron attributable to its incorporation into the pyruvate:ferredoxin oxidoreductase that is synthesized under these growth conditions. The inclusion of iron in growth media has been shown to increase levels of these enzymes in *Trichomonas vaginalis* (Gorrell, 1985), *Clostridium formicoaceticum* (Ragsdale & Ljungdahl, 1984) and *Clostridium acetobutylicum* (Marczak *et al.*, 1985), indicating the limitation of iron in unsupplemented growth media.

The presence of certain glycolytic enzymes but the absence of detectable glucose-6-phosphate dehydrogenase suggests that glycolysis is the sole mechanism of glucose metabolism in *N. patriciarum*. The enzyme data enables

the proposal of Scheme 1 for the formation of acetate, lactate, ethanol, CO_2 and H_2 from glucose.

Hydrogenosomes from *N. patriciarum* have an isopycnic density in sucrose of $1.20 \text{ g} \cdot \text{ml}^{-1}$, a value similar to that of hydrogenosomes isolated from rumen protozoa (Yarlett *et al.*, 1981, 1983). Density gradients in Percoll or Metrizamide gave buoyant densities of 1.12 and $1.28 \text{ g} \cdot \text{ml}^{-1}$ respectively; the distribution profile of the organelle with these gradient materials was suggestive of less osmotic-pressure damage.

The glycolytic conversion of glucose into 3-phosphoglyceric acid by *Trypanosoma brucei* is associated with a microbody-like organelle termed the 'glycosome' (Opperdoes & Borst, 1977; Oduro, 1977; Taylor *et al.*, 1980).

Fig. 7. Electron micrographs of sections after differential fractionation

(a) Fraction P2: although it also contained fragments of flagella and membranes of unidentified origin, fraction P2 was the only one to contain hydrogenosome-like vesicles. Their contents usually appeared less electron-dense than those in zoospores and rhizoids. (b) Fraction P3: contained rough endoplasmic reticulum, some empty vesicle elements and polyribosomal-like particles. (c) Fraction P4: characterized by the presence of extensive arrays of sheets of membrane interspersed with aggregates of ribosome-like particles. The scale bars represent 0.5 μm .

Trypanosomes are completely dependent upon glycolysis for ATP synthesis (Bowman & Flynn, 1976), and it has been suggested (Oppenheimer & Borst, 1977) that compartmentation of glycolytic enzymes within the glycosome has evolved to allow high optimal concentrations of enzymes, substrates and cofactors to be maintained. The non-sedimentable nature of the glycosomal enzymes hexokinase and 3-phosphoglycerate kinase, and also the absence of a detectable D-amino acid oxidase and a

sedimentable NADH oxidase, excludes the possibility that the finely granular microbody-like organelles observed in *N. patriciarum* are either glycosomes or peroxisomes according to the accepted definitions, and therefore biochemically have similarities only to hydrogenosomes.

In the present study and in previous ultrastructural studies of the zoospore stage of *N. patriciarum* (Munn *et al.*, 1981; Heath *et al.*, 1983) the presence of microbody-

Scheme 1. Metabolic transformation of glucose to acetate, lactate and ethanol by *N. patriciarum*

Reactions within the box are sedimentable at 10^5 g-min. 1, Phosphoenolpyruvate carboxykinase; 2, malate dehydrogenase; 3, 'malic' enzyme; 4, NADPH:ferredoxin oxidoreductase; 5, hydrogenase; 6, pyruvate:ferredoxin oxidoreductase; 7, lactate dehydrogenase; 8, pyruvate decarboxylase; 9, alcohol dehydrogenase; 10, pyruvate kinase. Abbreviations: PEP, phosphoenol pyruvate; PFOR, pyruvate:ferredoxin oxidoreductase, Fd, ferredoxin. Broken lines indicate reactions suppressed by growth under high CO_2 concentrations.

like organelles with a finely granular matrix, slightly smaller in size but with an ultrastructure similar to that of protozoal hydrogenosomes are apparent. Other similarities with protozoal hydrogenosomes (Müller, 1980) are internal regions of greater electron density and the existence of protrusions from some of these structures giving them a 'dumb-bell' appearance.

Hydrogenosomes have been shown to be involved in energy generation (Müller, 1980) and have a close association with structures involved in cell motility. Trichomonad hydrogenosomes were originally termed 'paraxostylar' or 'paracostal' granules because of their close association with the axostyle or costa respectively (Brugerolle, 1971). In the rumen holotrichs *Dasytricha ruminantium* and *Isotricha* sp. (Yarlett *et al.*, 1981, 1983) hydrogenosomes are arranged on the inner side of the fibrous band that separates the ectoplasm and endoplasm, and are therefore situated near the periphery of the cell and near the ciliary apparatus. In common with these observations the hydrogenosome-like organelles in the motile zoospore stages of *N. patriciarum* occur in greatest numbers near the kinetosomes. In the non-motile vegetative stage they occur in the sporangium (E. A. Munn & C. G. Orpin, unpublished work), where the zoospores develop, and in the rhizoids.

The results of the present study show the occurrence of hydrogenosomes, as presently defined, in this anaerobic rumen fungus, and extends the diversity of organisms known to possess this organelle.

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