Hydrogenosomes in the rumen entodiniomorphid ciliate *Polyplastron multivesiculatum*

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The rumen entodiniomorphid ciliate protozoan *Polyplastron multivesiculatum* was shown, by biochemical and electron microscopic techniques, to possess hydrogenosomes. After differential centrifugation of whole cell homogenates the hydrogenosomal marker enzymes pyruvate:ferredoxin oxidoreductase and hydrogenase were recovered predominantly (61% and 70% of activity respectively) in the large granular fractions that were sedimented by centrifugation for 10^4 g-min (fraction P1) and 10^5 g-min (fraction P2). These subcellular fractions contained membrane-bound organelles that were approximately 0.4–0.6 µm in diameter and which had a mean equilibrium density of 1.22–1.24 g ml⁻¹ after isopycnic centrifugation in sucrose gradients. Malate dehydrogenase (decarboxylating) activity, however, was predominantly non-sedimentable after centrifugation for 6×10^6 g-min. Numerous hydrogenosome-like organelles were present in the ectoplasm and endoplasm of the cell. Hydrogenase activity was demonstrated and localized in the protozoan cell using a novel staining procedure with distyryl nitroblue tetrazolium chloride (DSNBT).

Introduction

Protozoa that inhabit environments typified by low oxygen tensions frequently do not possess mitochondria. The principal site of energy metabolism in such eukaryotes is cytosolic, but in the trichomonad flagellates and some rumen and sapropelic ciliates the enzymic conversion of pyruvate to acetate and hydrogen is localized in a specialized subcellular redox organelle, the hydrogenosome (Müller, 1980, 1988; Zwart *et al.*, 1988). The hydrogenosomes in these protozoa are structurally similar and are membrane-bound spherical or elongated microbodies, 0.5–2.0 µm in diameter, with a granular matrix. Intact hydrogenosomes produce hydrogen (Steinbüchel & Müller, 1986) and are able to utilize oxygen when a suitable substrate is available (Müller, 1988); protozoa possessing hydrogenosomes exhibit characteristics of aerotolerance at physiological oxygen concentrations.

In the rumen ecosystem the two most important groups of protozoa, from the sub-class Trichostomatia, are the holotrich and entodiniomorphid ciliates (Williams & Coleman, 1988). The holotrich protozoa *Dasytri-*

cha ruminantium, *Isotricha prostoma* and *I. intestinalis* from the family Isotrichidae have been shown to possess hydrogenosomes (Yarlett *et al.*, 1981, 1983a) and exhibit respiratory activity (Yarlett *et al.*, 1982; Hillman *et al.*, 1985). Although ciliates from the entodiniomorphid group (family Ophryoscolecidae) usually predominate in the rumen evidence for the occurrence of hydrogenosomes in the individual genera is less conclusive, as biochemical and ultrastructural observations have not been correlated. Organelles morphologically and biochemically similar to hydrogenosomes were present in the large granule fraction prepared from cell homogenates of both *Epidinium ecaudatum caudatum* and *Eudiplodinium maggii* (Yarlett *et al.*, 1984). Hydrogenosomes were also separated from the homogenate prepared from a holotrich-containing mixed entodiniomorphid cell suspension (Snyers *et al.*, 1982) and hydrogenosome-like microbodies were also detected in entodiniomorphid protozoa by electron microscopy (Delfosse-Debuscher, 1977; Constantinescu & Dragos, 1984). Additional indirect evidence for the occurrence of hydrogenosomes is afforded by the similarities of the oxygen affinities of the entodiniomorphid ciliates *Eu. maggii* and *Polyplastron multivesiculatum* to those of the hydrogenosome-containing isotrichid ciliates *D. ruminantium* and *Isotricha* spp. (Ellis *et al.*, 1989a, b).

Abbreviations: BSPT, 2-(2'-benzothiazolyl)-5-styryl-3-(4'-phthalhydrazidyl)-tetrazolium chloride; DSNBT, distyryl nitroblue tetrazolium chloride; PFO, pyruvate:ferredoxin oxidoreductase.

Cytochemical staining reactions have been used successfully for the specific detection of hydrogenosomes and subcellular localization of hydrogenase activity in *Trichomonas vaginalis* and two species of sapropelic ciliates (Zwart *et al.*, 1988). The cytochemical staining procedure was dependent on the hydrogenase-mediated reduction of the tetrazolium salt 2-(2'-benzothiazolyl)-5-styryl-3-(4'-phthalhydrazidyl)-tetrazolium chloride (BSPT). The subcellular location of hydrogenosomes in intact cells is thus possible by the specific cytochemical detection of hydrogenase activity.

This paper details the use of an alternative cytochemical staining procedure for the detection and localization of hydrogenase activity in the cell ultrastructure, and confirms, by biochemical and cytochemical techniques, the occurrence of hydrogenosomes in the rumen entodiniomorphid ciliate *Polyplastron multivesiculatum*.

Methods

Isolation of *P. multivesiculatum*. The ciliate was recovered directly from ovine rumen contents withdrawn 2 h after the ration (hay 250 g, goat mix 250 g) had been consumed. The protozoal population had been established in the rumen of a defaunated (ciliate-protista-free) animal and comprised only *P. multivesiculatum* and small *Entodinium* spp. The larger *P. multivesiculatum* was readily separated from the entodinia without cross-contamination by differential filtration through defined porosity polyester textiles as described previously (Lockwood *et al.*, 1988). After collection on a 45 µm porosity filter cloth, which did not retain entodinia and bacteria, the *P. multivesiculatum* preparation was thoroughly washed with buffered salts solution D (Coleman, 1978) to ensure removal of free bacteria and to minimize contamination by adherent bacteria. Anaerobiosis was maintained by gassing the surfaces of the filters continuously with oxygen-free nitrogen during the isolation procedures. The salts solution had been prepared in distilled water deoxygenated by autoclaving (121 °C for 60 min) and continuous gassing with nitrogen during the cooling period. The salts solution was prewarmed to 39 °C before use.

Cell disruption and fractionation. The washed cell suspension (20 ml) containing approximately 10^6 – 10^7 cells ml⁻¹ was disrupted under continuous gassing with oxygen-free nitrogen by homogenization in a glass homogenizer with a constant-torque motor-driven (100 r.p.m.) Teflon pestle (approx. 150 µm clearance) at 20 strokes min⁻¹ for 2.5 min. An anaerobic buffer, pH 7.4, was prepared in deoxygenated distilled water and comprised 0.25 M-sucrose, 0.72 mM-EDTA, 10 mM-Tris/HCl and 50 mM-2-mercaptoethanol. The whole homogenate was centrifuged for 3000 g-min at 4 °C under oxygen-free nitrogen to remove unbroken cells. The supernatant cell-free homogenate was decanted at 25 °C inside a Whitley Mark 3 anaerobic cabinet (Don Whitley Scientific) with a gas atmosphere of 3% (v/v) hydrogen in carbon dioxide. The supernatant was fractionated by differential centrifugation in sealed tubes with an oxygen-free gas phase at 4 °C to give fractions sedimentable at 10⁴ g-min (P1), 10⁵ g-min (P2), 4 × 10⁵ g-min (P3), 6 × 10⁶ g-min (P4) and a nonsedimentable [soluble (S)] fraction (Yarlett *et al.*, 1981). All transfers and post-centrifugation manipulations were done in the anaerobic cabinet.

Isopycnic density separation. Gradients were prepared in deoxygenated distilled water from 20% and 65% (w/w) sucrose containing 0.72 mM-EDTA, 10 mM-Tris/HCl, 50 mM-2-mercaptoethanol, pH 7.4;

all solutions and gradients were prepared in the anaerobic cabinet. The 10⁴ g-min (P1) and 10⁵ g-min (P2) fractions (2 ml) were layered on the gradients in the cabinet and centrifuged in a 3 × 25 ml swing-out rotor at 10⁵ g for 60 min at 4 °C. Fractions (2 ml) were collected in the anaerobic cabinet by pumping from the base of the tube using fine-bore stainless steel tubing. The density of the sucrose was measured using a refractometer. Fractionation experiments were replicated at least three times to confirm the enzyme distribution profiles.

Enzyme assays. Malate dehydrogenase (decarboxylating; EC 1.1.1.40), pyruvate:ferredoxin oxidoreductase (PFO; EC 1.2.7.1) and hydrogenase (EC 1.18.3.1) were assayed as described by Lindmark & Müller (1973, 1974) and Steinbüchel & Müller (1986). The extinction coefficient of methyl viologen at 600 nm was taken as 1.3×10^4 M⁻¹ cm⁻¹ (Thornley, 1974). One enzyme unit is defined as the amount of enzyme required to transform 1 µmol of substrate min⁻¹. Protein was determined by the method of Bradford (1976).

Electron microscopy. Whole cells and subcellular fractions were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M-sodium cacodylate buffer, pH 7.4, for 30 min. The fixed material was washed by resuspension in 0.1 M-cacodylate buffer, pH 7.4, for periods of 5 min, recovered by centrifugation (2 min, 11000 g) and post-fixed in 2% (w/v) osmium tetroxide in 0.1 M-cacodylate buffer, pH 7.4, for 30 min. Fixed preparations were dehydrated in an ethanol series and embedded in Spurr resin. Sections were stained with uranyl acetate (2%, w/v, in 70% v/v, ethanol) and lead citrate (0.3% in 0.1 M-NaOH) and examined in a Philips 400T electron microscope.

Cytochemistry. Unfixed cells were incubated anaerobically at 29 °C for 60 min in 0.2 M-phosphate buffer (pH 7.4) containing 0.25 M-sucrose and distyryl nitroblue tetrazolium chloride (DSNBT, 0.25 mg ml⁻¹). The incubation medium had been pre-saturated with hydrogen and the head space was also filled with hydrogen gas. The tetrazolium salt was rapidly converted to the deep purple formazan. Control preparations were prepared from cells that had been incubated in oxygen-saturated medium to inactivate the hydrogenase activity in the cells (Yarlett *et al.*, 1983b; Hillman *et al.*, 1985). Following incubation, the cells were rinsed three times in 0.2 M-potassium phosphate buffer, pH 7.4, containing 0.25 M-sucrose to remove any unreacted tetrazolium. The cells were subsequently fixed in 1.5% (v/v) glutaraldehyde in 0.2 M-phosphate buffer, pH 7.4, for 30 min, washed (3 × 5 min) in the phosphate buffer, post-fixed in 1% (w/v) osmium tetroxide for 30 min in the same buffer, washed (3 × 5 min) in phosphate buffer, dehydrated in an ethanol series and embedded in Spurr resin. Sections were examined either unstained or stained with uranyl acetate and lead citrate as described above, and examined in a Philips 400T electron microscope.

Results

*Subcellular distribution of hydrogenosomal enzymes in *P. multivesiculatum**

PFO and hydrogenase activities were only demonstrable when very strict anaerobic precautions and conditions were used. Failure to maintain anaerobiosis resulted in a complete loss of hydrogenase and PFO activities.

The reproducible distribution profiles of the hydrogenosomal marker enzymes following differential centrifugation of cell-free homogenates are summarized in Fig. 1.

Fig. 1. Distribution of enzymes in fractions obtained by differential centrifugation of cell-free extracts of *P. multivesiculatum*. Relative specific activities (the ratios of specific activities in fractions to those in cell-free extract) were plotted against cumulative percentage of protein recovered in each fraction. The specific activities [$\text{mU (mg protein)}^{-1}$] in the original whole homogenates and the recoveries were: (a) hydrogenase, 1115 (82%); (b) PFO, 897 (80%); (c) malate dehydrogenase (decarboxylating), 0.1 (58%).

PFO and hydrogenase were predominantly associated with the large granule fractions P1 and P2, sedimenting after centrifugation at 10^4 g-min and 10^5 g-min respectively. In the representative experiment presented approximately 61% (P1, 36.8%; P2, 24.6%) of PFO and 70% (P1, 43.4%; P2, 26.4%) of hydrogenase activity were recovered in these large granular fractions. Hydrogenase activity was not present in fractions P3 and P4 while PFO could not be detected in P4. Malate dehydrogenase (decarboxylating), however, was predominantly non-sedimentable after centrifugation for $6 \times 10^6 \text{ g-min}$; 90% of recovered activity was present in the soluble fraction (S) and was not detectable in fraction P4. The sedimented fractions were not washed prior to assay and the presence of this enzyme in fractions P1–P3 is probably due to minor contamination with material from the soluble fraction. The distribution profile was consistent in replicate experiments.

Electron microscopic examination of thin cell sections revealed that numerous hydrogenosome-like organelles ($0.4\text{--}0.6 \mu\text{m}$ in diameter) were present in both the ectoplasm and the endoplasm (Fig. 2*a,b*). These ovoid-shaped organelles contained a granular matrix but apparently did not possess a denser core. Smaller pleiomorphic organelles which contained fine granular contents were found exclusively in the endoplasm (Fig. 2*b*). Electron-opaque regions were often present in these pleiomorphic organelles. The endoplasm was also rich in ribosomes and membrane aggregations that may have been derived from Golgi bodies (Fig. 2*b*).

The subcellular fractions obtained by differential centrifugation of the cell homogenate were also examined by electron microscopy. Fraction P1 contained hydrogenosome-like organelles, membrane-lined vesicles, small pleiomorphic granular organelles, amylopec-

tin and cell debris including cilia (Fig. 2*c*). Bacteria that had been ingested by the protozoa and released by homogenization were also present. Fraction P2 also contained numerous hydrogenosome-like organelles, smaller granular organelles and membrane-bound vesicles (Fig. 2*d*). This subcellular fraction was not contaminated with amylopectin and bacteria (Fig. 2*d*). Fraction P3 predominantly contained membrane-lined vesicles, small pleiomorphic organelles and ribosomes (Fig. 2*e*). Fraction P4 was predominantly ribosomal in nature although small vesicles (approximately $0.1 \mu\text{m}$ in diameter) were observed (Fig. 2*f*).

Density gradient separation of large granule fractions

After isopycnic centrifugation of the 10^4 g-min (P1) fraction, protein and the hydrogenosomal marker enzymes PFO and hydrogenase were retained in the sucrose gradient, predominantly in fractions having equilibrium densities in the range $1.22\text{--}1.26 \text{ g ml}^{-1}$ (Fig. 3). In the representative fractionation presented these fractions contained 43.2%, 23.4% and 25% of the protein, PFO and hydrogenase activities, respectively, that were recovered from the gradient. Malate dehydrogenase (decarboxylating) was not detectable in any fraction from the gradient. A pellet of material formed at the base of the tube (i.e. density $> 1.3 \text{ g ml}^{-1}$) but this did not contain substantial protein or enzyme activity, and was comprised principally of amylopectin granules and intact bacteria (Fig. 4*b*).

After sucrose density gradient centrifugation of the 10^5 g-min (P2) fraction the hydrogenosomal enzymes were found in association with subcellular structures that had mean equilibrium densities of $1.20\text{--}1.24 \text{ g ml}^{-1}$ (Fig. 3). These two fractions contained 39.9% and 41.3% of the

Fig. 2. Electron micrographs of *P. multivesiculatum* and of derived subcellular fractions. Marker bar (applies to parts a-f), 0.5 μm . (a) Ectoplasm of intact organism showing: A, amylopectin; H, hydrogenosomes. (b) Endoplasm of intact organism showing: G, membrane aggregations that resemble Golgi apparatus; H, hydrogenosomes; P, pleiomorphic organelles with less dense regions (arrowhead); ribosomes (arrow). (c) Fraction P1 (10^4 g-min), containing: H, hydrogenosomes; P, pleiomorphic organelles; V, membrane-lined vesicles; C, cilia. (d) Fraction P2 (10^5 g-min) containing: H, hydrogenosomes; V, membrane-lined vesicles; C, cilia. (e) Fraction P3 (4×10^5 g-min) containing: R, ribosomes; V, membrane-lined vesicles; P, pleiomorphic organelles. (f) Fraction P4 (6×10^6 g-min) containing: R, ribosomes; V, membrane-lined vesicles.

Fig. 3. Typical distribution of hydrogenosomal enzymes after isopycnic centrifugation in sucrose gradients of fractions sedimented by centrifuging for 10^4 g-min (P1) and 10^5 g-min (P2). Recoveries were as follows. P1: (a) hydrogenase, 51%; (b) PFO, 125%; (c) protein, 65%. P2: (d) hydrogenase, 51%; (e) PFO, 142%; (f) protein, 77%. Frequencies were calculated by the method of Beaufay & Amar-Costesec (1976).

recovered PFO and hydrogenase activities respectively. The distribution profiles of the P1 and P2 large granule fractions were similar (Fig. 3).

Electron microscopic examination of fractions from the gradient that contained the highest hydrogenase and PFO activity confirmed the presence of hydrogenosome-like granular organelles (Fig. 4a, c). Some other

subcellular structures (e.g. membrane-bound vesicles, pleiomorphic structures) were also present in the hydrogenosome-enriched fractions. However, the larger cell debris, amylopectin and the intracellular bacteria that were present in the 10^4 g-min fraction formed a compact pellet at the base of the tube after centrifugation (Fig. 4b).

Fig. 4. Electron micrographs of subcellular structures present in fractions from the sucrose density gradients that had the highest hydrogenase and PFO activities. (a) Particles sedimented in aqueous sucrose to an equilibrium density of $1.22\text{--}1.26\text{ g ml}^{-1}$ from subcellular fraction P1: H, hydrogenosomes; C, cilia; D, cell debris; marker bar, $0.3\text{ }\mu\text{m}$. (b) Pellet formed at the base of P1 sucrose gradient containing: A, amylopectin; B, bacteria; C, cell debris; marker bar, $0.5\text{ }\mu\text{m}$. (c) Particles sedimented from subcellular fraction P2: H, hydrogenosomes; V, membrane-lined vesicles; marker bar, $0.3\text{ }\mu\text{m}$.

Cytochemical localization of hydrogenase in *P. multivesiculatum*

When unfixed *P. multivesiculatum* cells were incubated anaerobically with DSNBT (0.25 mg ml^{-1}) in hydrogen-saturated 0.2 M -phosphate buffer, pH 7.4, dark purple formazan deposits developed rapidly throughout the

whole cell. After osmification, a clearly visible osmium-black reaction product was present in close association with the periphery of the granular hydrogenosome-like organelles located in the ectoplasm and endoplasm (Fig. 5a).

Electron-dense deposits were present within the matrix of these organelles only after staining with uranyl

Fig. 5. (a) Section of DSNBT-reacted intact cell without post-reaction treatment with uranyl acetate and lead citrate showing electron-dense formazan deposits (arrowhead) exclusively associated with the periphery of the hydrogenosomes. Ec, ectoplasm; En, endoplasm; marker bar, 2 μm . (b) Hydrogenosomes with associated formazan deposit (f). Electron-dense deposits (arrowhead) were visible only after staining with uranyl acetate and lead citrate. Marker bar, 0.3 μm . (c) Section of control cells, incubated with DSNBT in oxygenated buffer, showing hydrogenosomes limited by a double membrane (Hm). Electron dense deposits (arrowhead) were visible only after post-reaction staining with uranyl acetate and lead citrate. Marker bar, 0.3 μm .

acetate and lead citrate (Fig. 5b). In control cells incubated with DSNBT in oxygenated buffer, the membranes of the organelles were unstained with osmium-black, although the matrix did contain some

electron-dense deposits after staining with uranyl acetate and lead citrate (Fig. 5c).

The organelles appeared to be bounded by a double membrane (Fig. 5c). In untreated cells the limits of these

organelles were not clear (Fig. 2a, b), and were obscured by osmium-black deposits in cells stained for hydrogenosomes (Fig. 5b).

When the incubations were done under a nitrogen atmosphere in the absence of exogenously supplied hydrogen both the reaction rate and the intensity of the formazan deposits within the cell were markedly reduced. No formazan deposits were formed when the hydrogenase was inactivated by pre-exposure to oxygen, confirming hydrogenase involvement in the formation of reaction product.

Discussion

The rumen entodiniomorphid ciliate *Polyplastron multivesiculatum* is a hydrogenogen with a high affinity for oxygen utilization at low oxygen tensions (Ellis *et al.*, 1989b). Rumen holotrich ciliates which possess hydrogenosomes exhibit similar characteristics (Williams, 1986; Lloyd *et al.*, 1989). The key enzymes involved in the conversion of pyruvate to hydrogen in *P. multivesiculatum* are PFO and hydrogenase. The hydrogen-evolving system in this ciliate was shown by Ellis *et al.* (1989b) to be inactivated by oxygen or a product of oxygen reduction, and this study has confirmed that both PFO and hydrogenase activities are inhibited by oxygen. Activities could be demonstrated only when rigorous anaerobic precautions were used during the cell preparation, fractionation and assay procedures. The enzymes were found in association with subcellular structures that were morphologically similar to organelles that have been identified as hydrogenosomes in other ruminal ciliates, ruminal chytridiomycete fungi and the trichomonad flagellates (Müller, 1980; Snyers *et al.*, 1982; Yarlett *et al.*, 1981, 1983a, 1984, 1986).

The organelle in *P. multivesiculatum* was enriched from cell homogenates in the large granule fractions that were recovered by centrifugation for 10^4 and 10^5 g-min respectively. The membrane-bound hydrogenosome-like organelles were ovoid in shape, 0.4–0.6 µm in diameter, contained a granular matrix, and had an equilibrium density, after isopycnic centrifugation in sucrose gradients, in the range 1.20–1.26 g ml⁻¹. Hydrolytic enzymes in *P. multivesiculatum*, however, cosediment with acid phosphatase in less-dense pleiomorphic lysosome-like vesicles which had a mean equilibrium density of 1.17 g ml⁻¹ (Williams & Ellis, 1985). The hydrogenosomes isolated from entodiniomorphid ciliates (Snyers *et al.*, 1982), *Isotricha* spp. (Yarlett *et al.*, 1983a) and trichomonad flagellates (Müller, 1980) also have isopycnic densities in sucrose that are above 1.2 g ml⁻¹, while those isolated from the smaller holotrich ciliate, *Dasytricha ruminantium*, and the rumen chytridiomycete fungus

Neocallimastix patriciarum are similar, being 1.18 and 1.20 g ml⁻¹ respectively (Yarlett *et al.*, 1981, 1986).

There is both electron microscopic and biochemical evidence for the presence of hydrogenosomes in two other ruminal entodiniomorphid ciliates, *Eudiplodinium maggii* and *Epidinium ecaudatum caudatum* (Snyers *et al.*, 1982; Yarlett *et al.*, 1984). Ultrastructural studies of the blepharocorythid ciliate *Charonina ventriculi* have also revealed the presence of hydrogenosome-like microbodies (unpublished results). It is also probable that some or all of the entodiniomorphid protozoa *Ophryoscolex*, *Eremoplastron* and *Ostracodinium*, previously reported to lack hydrogenase but to possess PFO and decarboxylating malate dehydrogenase (Yarlett *et al.*, 1984) would, under the strict anaerobic conditions described here, be shown to possess all hydrogenosomal marker enzymes. The occurrence of hydrogenosomes within rumen ciliates is probably greater than is currently accepted.

The presence of hydrogenosomes within *P. multivesiculatum* was also confirmed by the cytochemical localization of hydrogenase. It was confirmed that the reaction was hydrogenase-mediated as the formazan reaction product was not detected in cells in which the hydrogenase activity had been inactivated by exposure to oxygen (Yarlett *et al.*, 1983b; Ellis *et al.*, 1989b). In addition, rate of product formation was markedly lowered when hydrogen in the headspace was replaced by nitrogen and was thus only available from endogenous reactions. The hydrogenosomes, which appeared to possess a double membrane, as suggested for hydrogenosomes in other eukaryotes (Müller, 1980), were present in both the ectoplasm and the endoplasm. The osmium-black reaction product was found in the periphery of these organelles. This suggested that some hydrogenase activity was membrane-associated, if not bound to or integrated within the membrane. The almost exclusive staining of the hydrogenosome membrane may, alternatively, be due to the lipophilic nature of DSNBT (Hanker, 1975) or its failure to cross the membrane. However, similar membrane staining was observed using the lipophobic BSPT with sapropelic ciliates (Zwart *et al.*, 1988) indicating that in some species, hydrogenase is membrane-associated. The presence of electron-dense material within the matrix of these organelles in preparations stained with uranyl acetate and lead citrate may be due to the reaction of lead citrate with the formazan deposit. This effect would increase electron opacity, as has been described for other formazans (Bradbury & Steward, 1964; di Fanchiotti *et al.*, 1971).

The cytochemical localization of hydrogenase described here should be regarded as a useful procedure for the identification of hydrogenosomes, not only in the rumen ciliates but also in other eukaryotes from anaerobic or low-oxygen environments, particularly

when large cell numbers needed for biochemical localization are not available.

Although malate dehydrogenase (decarboxylating) activity was detectable in the whole cell homogenate, the enzyme was not organelle-associated in *P. multivesiculatum*. Non-sedimentable (i.e. soluble) malate dehydrogenase (decarboxylating) has also been detected in the rumen ciliates *Dasytricha ruminantium*, *Isotricha prostoma* and *I. intestinalis* (Yarlett *et al.*, 1981, 1983a). However, this particular activity within the trichomonad flagellates (Müller, 1980), mixed rumen ophryoscolecids (Snyers *et al.*, 1982) and the rumen fungus *Neocallimastix patriciarum* (Yarlett *et al.*, 1986) is sedimentable. Further studies on the occurrence and distribution of this enzyme in other rumen ciliates are therefore warranted, as there would appear to be species differences in the complement of hydrogenosomal enzymes.

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