Influence of CO₂ and low concentrations of O₂ on fermentative metabolism of the rumen ciliate *Dasytricha ruminantium*

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The effects of ruminal concentrations of CO₂ and O₂ on glucose-stimulated and endogenous fermentation of the rumen isotrichid ciliate *Dasytricha ruminantium* were investigated. Principal metabolic products were lactic, butyric and acetic acids, H₂ and CO₂. Traces of propionic acid were also detected; formic acid present in the incubation supernatants was found to be a fermentation product of the bacteria closely associated with this rumen ciliate. ¹³C NMR spectroscopy revealed alanine as a minor product of glucose fermentation by *D. ruminantium*. Glucose uptake and metabolite formation rates were influenced by the headspace gas composition during the protozoal incubations. The uptake of exogenously supplied D-glucose was most rapid in the presence of O₂ concentrations typical of those detected *in situ* (i.e. 1–3 µM). A typical ruminal gas composition (high CO₂, low O₂) led to increased butyrate and acetate formation compared to results obtained using O₂-free N₂. At a partial pressure of 66 kPa CO₂ in N₂, increased cytosolic flux to butyrate was observed. At low O₂ concentrations (1–3 µM dissolved in the protozoal suspension) in the absence of CO₂, increased acetate and CO₂ formation were observed and *D. ruminantium* utilized lactate in the absence of extracellular glucose. The presence of both O₂ and CO₂ in the incubation headspaces resulted in partial inhibition of H₂ production by *D. ruminantium*. Results suggest that at the O₂ and CO₂ concentrations that prevail *in situ*, the contribution made by *D. ruminantium* to the formation of ruminal volatile fatty acids is greater than previously reported, as earlier measurements were made under anaerobic conditions.

Introduction

The isotrichid holotrich ciliate *Dasytricha ruminantium* utilizes glucose and certain other soluble carbohydrates present in rumen fluid (Howard, 1959; Williams, 1986). The principal metabolic products of carbohydrate fermentation are lactate, acetate, butyrate, H₂ and CO₂ (Williams & Harfoot, 1976; Van Hoven & Prins, 1977; Williams, 1979). Traces of propionic and formic acids have also been reported (Heald & Oxford, 1953; Gutierrez, 1955; Williams & Harfoot, 1976; Van Hoven & Prins, 1977). An intracellular storage polysaccharide, amylopectin, is also synthesized and subsequently supports endogenous fermentation during periods of extracellular substrate depletion (Williams, 1986). The pathways leading to the formation of these principal

metabolites have been partially characterized (Yarlett *et al.*, 1981, 1982, 1985).

D. ruminantium lacks mitochondria, but possesses specialized redox organelles, the hydrogenosomes. In these organelles, pyruvate oxidation yields acetate, H₂ and CO₂ (Yarlett *et al.*, 1981, 1982). This organism is susceptible to oxygen when exposed to air, but it has a high affinity for O₂ at low concentrations (Lloyd *et al.*, 1982; Hillman *et al.*, 1985*b*): the hydrogenosome has been implicated as a site of O₂ consumption in both *Tritrichomonas foetus* (Čerkasov *et al.*, 1978; Müller & Lindmark, 1978) and *D. ruminantium* (Yarlett *et al.*, 1982). H₂ evolution is, however, markedly influenced by dissolved O₂; O₂ concentrations > 2.8 µmol l⁻¹ result in irreversible inactivation of the hydrogenase system (Yarlett *et al.*, 1983; Hillman *et al.*, 1985*b*). In the rumen, the protozoal population actively scavenges dissolved O₂ (Ellis *et al.*, 1989*a, b*) and has a protective role *in situ*,

Abbreviation: VFA, volatile fatty acids.

removing O₂ from the environment of more susceptible organisms (e.g. methanogens) (Hillman *et al.*, 1988).

The rumen provides an environment in which *D. ruminantium* experiences low, fluctuating concentrations of O₂ (0.25–3 µM, Scott *et al.*, 1983; Hillman *et al.*, 1985a) and high concentrations of CO₂ [160–3800 µM dissolved in rumen liquor (Hillman *et al.*, 1985a) and forming 65% (v/v) of the ruminal gaseous headspace (McArthur & Miltimore, 1961)]. The effects of these gases at physiological concentrations on the fermentation of soluble carbohydrates by this organism have not been determined, as previous studies *in vitro* have used O₂-free N₂ (Williams & Harfoot, 1976) or CO₂ (Van Hoven & Prins, 1977).

In this study, ¹³C NMR, HPLC and membrane-inlet mass spectrometry have been used to identify and quantify the end products of glucose fermentation by *D. ruminantium in vitro*. We have determined the effects of O₂ and CO₂, at concentrations typical of conditions that prevail in the rumen, on the fermentative metabolism of this ruminal ciliate.

Methods

Isolation of organism. *D. ruminantium* was isolated from rumen contents of a sheep that had been defaunated and reinoculated with *D. ruminantium* and *Entodinium* spp. The animal received a diet of concentrates and chopped hay. Water was given *ad lib*. Samples of rumen fluid (500 ml) were withdrawn 1 h after feeding, strained and diluted twofold with holotrich isolation buffer (Williams & Harfoot, 1976) from which Na₂S had been omitted. The buffer was rendered O₂-free by using distilled water that had been boiled and cooled with N₂ gassing. Samples were transported to the laboratory in a portable incubator and maintained at 39 °C in sealed, N₂-gassed insulated containers. The sample temperatures never fell below 37 °C, and the pH remained between 6.7 and 7.0 during transportation. Subsequently, samples were strained to remove residual plant material, diluted twofold with buffer, and placed into N₂-gassed conical flasks. The samples were incubated in a water-bath at 39 °C until contaminating small plant particles formed a scum layer and could be removed.

D. ruminantium preparations were collected on defined aperture textiles (20 µm pore size) (Simon Engineering) following prefiltration through a filter cloth (80 µm pore size), then thoroughly washed with buffer on the 20 µm pore-size filter to remove free and adherent bacteria and any remaining *Entodinium* spp. Anaerobiosis was maintained during the isolation procedures by thorough gassing with N₂. Washed suspensions of *D. ruminantium* were sedimented by centrifugation at 500 g for 2 min at room temperature and resuspended in 2 ml of buffer under N₂. Contamination of the preparation by *Entodinium* spp. was negligible (<1%). Protozoa were counted using a haemocytometer.

Protozoal incubations and dissolved gas analyses. Samples (2 ml) were adjusted to pH 7.0 and transferred to an incubation vessel maintained at 39 °C. Separate incubations were carried out using four different headspace compositions: 101 kPa (100%) N₂; a partial pressure of O₂ in N₂, usually 7.5 kPa O₂ (35% air:65% N₂, v/v) to give a balance of 1–3 µM dissolved O₂ in the protozoal suspension; 66 kPa CO₂ in N₂; and a gaseous mixture of 66 kPa CO₂ and 7.5 kPa O₂ (65% CO₂:35% air, v/v) to give 1–3 µM-O₂ and 3000–3400 µM-CO₂ in the protozoal

suspension. Each incubation was done in triplicate. Dissolved gas concentrations were monitored using the HAL series quadrupole gas analyser (Hidden Analytical) which was linked to the incubation vessel by a quartz probe sealed at one end with a 100 µm diameter inlet covered by a 15-µm-thick silicone membrane (Lloyd *et al.*, 1987). The probe monitored gases dissolved in the 2 ml reaction vessel under a mobile gas phase (Degn *et al.*, 1985; Lloyd & Scott, 1985); mixing was by bubbling at a constant flow rate (10 ml min⁻¹) (Lloyd *et al.*, 1987). The partial pressures of O₂ and CO₂ in the gas were controlled using a digital gas mixer (Degn & Lundsgaard, 1980). The concentrations of dissolved gases at 39 °C and pH 7.0 were taken as: H₂, 740 µM; O₂ in air, 215 µM; CO₂, 5258 µM (Wilhelm *et al.*, 1977). Rates of gas production were calculated as described previously (Lloyd & Scott, 1985).

After recording endogenous steady-state levels of dissolved CO₂ and H₂ under a predetermined gas phase, substrates were added. Substrates used were D-glucose (30 mM final concentration) or sodium DL-lactate (5 mM final concentration) or both D-glucose (30 mM final concentration) and sodium DL-lactate (5 mM final concentration). Organisms were incubated for 2 h. Samples (200 µl) were withdrawn every 30 min and centrifuged for 10 min at 5100 g at room temperature. Supernatants were then filtered to remove protein using a molecular filter (mol. mass cut-off 10 kDa) (Amicon) and stored frozen at -20 °C. Endogenous end-metabolite formation was determined by an identical procedure except that no substrate was added. Samples for NMR were obtained in a similar manner except that D-[1-¹³C]glucose (30 mM final concentration) was added. Samples (0.5 ml) were withdrawn after 2 and 4 h, respectively and centrifuged at 5100 g for 10 min. Supernatants were decanted and extracted with perchloric acid (60%, v/v) on ice for 10–15 min, neutralized with 4 M-NaOH and centrifuged at 5100 g for 10 min. Supernatants were then stored at -20 °C.

Bacterial incubations. Controls were set up using extracts from the protozoa that might contain bacteria. Washed protozoa were maintained at 4 °C under N₂ and disrupted by treatment with ultrasound using an MSE Soniprep 150 at a frequency of 20 kHz, in cycles of 15 s, with 15 s intervals, at an amplitude of 6–12 µm for 6 min. The absence of intact protozoa after these treatments was confirmed by microscopic examinations. The crude homogenate (10 ml) was centrifuged at 7000 g for 10 min. The pellet was washed twice in buffer by recentrifugation at 7000 g for 10 min. The pellet contained intact, motile bacteria (observed by microscopic examination) that were present in the original protozoal suspension. The pellet was resuspended in buffer under anaerobic conditions to its original volume.

NMR measurements. ¹³C NMR proton decoupled spectra were recorded at 22.49 MHz on a JEOL FX90Q spectrometer equipped with a 10 mm multinuclear probe. Free induction decays (FID) were acquired into 8000 data points covering a spectral width of 120 p.p.m. (2700 Hz) using pulses of 13 µs (78°) at 10 s intervals. Each FID was multiplied by an exponential function that produced a line broadening of 1.03 Hz before transformation. D₂O was used as solvent and internal lock. Chemical shifts, in p.p.m., were measured with respect to the βC-1 resonance in the added D-glucose (97.0 p.p.m.) (London, 1988).

Quantification of metabolites. Soluble metabolites present in the supernatant were separated and quantified using reverse phase HPLC coupled to a variable wavelength UV detector (HPLC Technology Ltd). Separation was achieved by injecting 20 µl volumes into a resin-based fermentation monitoring column (Bio-Rad), via a guard column packed with the same material. Samples of supernatant were suitably diluted in the solvent which formed the mobile phase (1 mM-H₂SO₄). Eluent streams were monitored at 210 nm with readout on a potentiometric chart recorder. The eluent flow rate was 0.8 ml min⁻¹ and the sustained pressure was approximately 800 p.s.i. Metabolites were identified and quantified by using internal standards to plot

calibration curves of pure compounds. Glucose concentration was determined using a commercially available coupled hexokinase:glucose-6-phosphate dehydrogenase assay kit (Sigma).

Sources of chemicals. D-[1-¹³C]glucose (99%) was purchased from Aldrich. For HPLC, H₂SO₄ was Aristar grade (BDH) all other chemicals were of analytical grade obtained from BDH.

Results

Identification of products by NMR

An initial identification of all of the products released by *D. ruminantium* during catabolism of D-glucose was carried out after incubating the organism with D-[1-¹³C]glucose under various gas phases. ¹³C NMR spectra of the supernatants obtained after 4 h incubations under 101 kPa N₂ revealed lactate, acetate and butyrate to be the principal fermentation end-products, as previously reported (Williams & Harfoot, 1976; Van Hoven & Prins 1977). D-[1-¹³C]Glucose was still present extracellularly after 4 h (Fig. 1*a*). Alanine was detected extracellularly as a minor product of glucose catabolism by this organism. Labelled alanine was never detectable in bacterial incubations with D-[1-¹³C]glucose, confirming the protozoal origin of the amino acid. Spectra obtained from the 2 h incubation after exposure to low O₂ concentrations revealed similar signals to those observed after 4 h under 101 kPa N₂ (Fig. 1*b*); however, the relative signal intensities changed dramatically in the 4 h incubation with low O₂ concentrations (Fig. 1*c*). Glucose was not detected extracellularly after 4 h. Alanine showed progressive accumulation; thus it was undetectable after 2 h, but present after 4 h: butyrate was increased and the lactate signal intensity was greatly diminished. ¹³C NMR spectra obtained after incubations under 66 kPa CO₂ in N₂ revealed similar metabolite signals as observed with the 101 kPa N₂ gas phase, except that alanine was not detected (Fig. 1*d*). No anomeric specificity for D-[1-¹³C]glucose utilization by *D. ruminantium* was detected for any of the incubations.

Volatile fatty acid (VFA) and lactate production during incubations with D-glucose

Incubations of *D. ruminantium* with 30 mM-D-glucose under 101 kPa N₂ revealed lactate to be the major end-product of fermentation (Fig. 2*a*). Acetate and butyrate were produced in equimolar portions but lactate was produced at a concentration 10-fold greater than that of each VFA. Glucose was initially utilized at a steady rate (55 nmol min⁻¹ per 10⁵ organisms), but the extracellular concentration never decreased below 3.5 mM. The introduction of low O₂ concentrations resulted in

Fig. 1. Proton-decoupled ¹³C NMR spectra of *D. ruminantium* supernatants taken after incubation for the time stated with 30 mM-D-[1-¹³C]glucose. Washed organisms were incubated at 39 °C in buffer at pH 7.0 in the presence of: (a) 101 kPa N₂, 4 h incubation (6.5 × 10⁵ organisms ml⁻¹); (b) 7.5 kPa O₂ in N₂ (1–3 μM-O₂ in the protozoal suspension), 2 h incubation (6.5 × 10⁵ organisms ml⁻¹); (c) as (b), except 4 h incubation; (d) 66 kPa CO₂ in N₂ (1.4 × 10⁶ organisms ml⁻¹), 4 h incubation. The final pH values of the supernatants after 4 h incubations were as follows: (a) 6.6, (c) 6.1, (d) 6.5. Chemical shifts in p.p.m. are: C-1 glucose α-peak, 93.1, β-peak, 97.0; C-2 butyrate, 38.1; C-2 acetate, 22.7; C-3 lactate, 21.1; C-3 alanine, 18.0; C-4 butyrate, 13.9.

dramatic changes in metabolite formation. Glucose was consumed at almost twice the rate observed in the absence of O₂ (101 nmol min⁻¹ per 10⁵ organisms) and was barely detectable in the incubation supernatants after 30 min (Fig. 2*b*). Rapid accumulation of extracellular lactate was observed which decreased by 50% over the following 90 min. The rate of acetate production was

Fig. 2. Consumption of glucose and production of principal metabolic products formed by *D. ruminantium* (9×10^5 ml $^{-1}$) incubated with 30 mM-D-glucose at 39 °C and pH 7.0, under: (a) 101 kPa N₂, (b) 7.5 kPa O₂ in N₂ (1–3 µM-O₂ in the protozoal suspension), (c) 66 kPa CO₂ in N₂, (d) 66 kPa CO₂/7.5 kPa O₂ (1–3 µM-O₂ in the protozoal suspension). Products were analysed by HPLC and are as follows: D-glucose (○), lactate (●), acetate (▲) and butyrate (■).

double that obtained with 101 kPa N₂, whereas butyrate formation rates remained unchanged. Under a gas phase consisting of 66 kPa CO₂ in N₂, D-glucose was consumed by *D. ruminantium* at an initial rate of 76 nmol min $^{-1}$ per 10⁵ organisms (Fig. 2c). This was 38% higher than that obtained using O₂-free N₂, but 25% lower than glucose uptake rates in the presence of O₂. Elevated butyrate production rates were observed; acetate was formed at a similar rate to that observed during incubations under 101 kPa N₂. A combination of low dissolved O₂ and high CO₂ concentrations, typical of conditions that prevail *in situ* in the rumen, produced a glucose uptake rate of

Table 1. Metabolites formed by *D. ruminantium* during 2 h incubations with various substrates at 39 °C

Values presented are means $\pm$ SD obtained from three measurements using HPLC.

Gas phase	Substrate	Rate of metabolite production (+) or utilization (–) (nmol min $^{-1}$ per 10 ⁵ organisms)		
		Lactate	Acetate	Butyrate
101 kPa N ₂	None	+7.2 $\pm$ 1.6	+3.8 $\pm$ 1.0	+4.4 $\pm$ 0.8
101 kPa N ₂	Sodium lactate (5 mM)	+2.4 $\pm$ 0.5	+1.3 $\pm$ 0.2	1.8 $\pm$ 0.5
7.5 kPa O ₂ in N ₂ *	Sodium lactate (5 mM)	–4.3 $\pm$ 1.0	+2.8 $\pm$ 0.5	+4.0 $\pm$ 0.9
7.5 kPa O ₂ in N ₂ *	D-Glucose (30 mM)	–49.3 $\pm$ 5.5†	+8.6 $\pm$ 2.4	+8.6 $\pm$ 1.8
7.5 kPa O ₂ in N ₂ *	Sodium lactate (5 mM) + D-glucose (30 mM)	–15.1 $\pm$ 3.1†	+4.0 $\pm$ 0.5	+4.2 $\pm$ 1.5

* Partial pressure of O₂ in N₂ required to give 1–3 µM-O₂ dissolved in the protozoal suspension.

† After depletion of D-glucose from supernatant (approx. 30 min).

85 nmol min $^{-1}$ per 10⁵ organisms. This was slightly higher (11%) than uptake rates under 66 kPa CO₂ in N₂, but 35% greater than the uptake rate under 101 kPa N₂. Lactate production was 25% lower than rates obtained using 101 kPa N₂. Equimolar butyrate and acetate were formed at 25% higher concentrations than those obtained with 101 kPa N₂ (Fig. 2d).

Lactate utilization

Further investigations of lactate uptake by *D. ruminantium* (Fig. 2b) were carried out. Incubations with extracellularly added sodium DL-lactate (5 mM) under 101 kPa N₂ in the absence of D-glucose showed no consumption of extracellular lactate although production of acetate, lactate and butyrate was detectable (Table 1). However, their formation rates were 30–40% lower than those found after similar incubations in the absence of any substrate. Incubation of *D. ruminantium* without D-glucose but with extracellularly added lactate (5 mM) in the presence of 1–3 µM-O₂ gave a slow consumption of lactate. Organisms incubated at 1–3 µM-O₂ in the presence of both D-glucose (30 mM) and lactate (5 mM) preferentially consumed D-glucose prior to lactate utilization. This was similar to results obtained previously with *D. ruminantium* incubated with D-glucose (30 mM) and low dissolved O₂ concentrations in the absence of extracellular lactate (Fig. 2b). In both cases acetate formation increased. No change in the rate of butyrate production was detected.

Fig. 3. Metabolites formed by *D. ruminantium* during endogenous fermentation, in the absence of extracellularly added D-glucose (9×10^5 organisms ml^{-1}). Incubations were performed at 39°C and pH 7.0 under: (a) 101 kPa N_2 , (b) 7.5 kPa O_2 in N_2 ($1-3 \mu\text{M}$ O_2 dissolved in the protozoal suspension), (c) 66 kPa CO_2 in N_2 , (d) 66 kPa $\text{CO}_2/7.5$ kPa O_2 ($1-3 \mu\text{M}$ O_2 dissolved in the protozoal suspension). Products were analysed by HPLC and are as follows: lactate (●), acetate (▲) and butyrate (■).

Endogenous metabolism of *D. ruminantium*

The endogenous metabolites produced by *D. ruminantium* are formed by intracellular amylopectin degradation (Williams & Harfoot, 1976; Van Hoven & Prins, 1977). The changes observed in principal metabolite concentrations under the four gas phases studied are presented in Fig. 3(a-d). Highest metabolite concentrations were obtained under 101 kPa N_2 . Lactate was the major product of endogenous metabolism in all cases.

Table 2. Mass spectrometric analyses of dissolved gas production by *D. ruminantium* during glucose-stimulated and endogenous fermentation, under different gas phases

Gas formation rates were measured in a 2 ml incubation vessel containing 9×10^5 organisms ml^{-1} . Glucose was added at a final concentration of 30 mM and all experiments were done at 39°C . Values are means $\pm$ SD from three measurements. ND, Not determined; CO_2 evolution was not measured due to the presence of high extracellular concentrations of dissolved CO_2 .

Gas phase	Gas production rate ($\mu\text{M min}^{-1}$ per 10^5 organisms)			
	Hydrogen		Carbon dioxide	
	Endogenous	Glucose	Endogenous	Glucose
101 kPa N_2	4.5 ± 1.0	13.7 ± 2.5	2.1 ± 0.6	6.8 ± 1.5
7.5 kPa O_2 in N_2	2.7 ± 0.5	3.7 ± 0.8	2.0 ± 0.5	17.7 ± 1.5
66 kPa CO_2 in N_2	2.3 ± 0.5	7.2 ± 1.2	ND	ND
7.5 kPa $\text{O}_2/66$ kPa CO_2^*	2.4 ± 1.0	4.4 ± 0.9	ND	ND

* Partial pressure of O_2 required to give $1-3 \mu\text{M}$ O_2 dissolved in the protozoal suspension.

Acetate formation was reduced by 30% under a gas phase high in CO_2 by comparison with its production under 101 kPa N_2 . Endogenous fermentation under 101 kPa N_2 , or a combination of low O_2 and high CO_2 concentrations resulted in the production of approximately equimolar concentrations of acetate and butyrate.

H_2 and CO_2 production by *D. ruminantium*

Rates of H_2 and CO_2 production by *D. ruminantium* were determined simultaneously by dissolved gas analysis using membrane-inlet mass spectrometry. Rates obtained during incubations with each of the four gas phases are presented for both glucose-stimulated and endogenous fermentation (Table 2). In both cases, H_2 production was partially inhibited by the presence of low concentrations of O_2 . During glucose-stimulated fermentation under low O_2 , inhibition of H_2 production was coupled to a 2.5-fold increase in the rate of CO_2 evolution. Endogenous CO_2 evolution was unaffected by the presence of low dissolved O_2 concentrations. H_2 evolution was also inhibited during incubations with 66 kPa CO_2 in N_2 . Both glucose-stimulated and endogenous fermentation under this gas phase showed an approximate 50% inhibition of H_2 production compared to gas evolution rates measured under a 101 kPa N_2 gas phase.

Other metabolites formed by *D. ruminantium*

Small amounts of formate ($<10 \mu\text{M}$), propionate ($<100 \mu\text{M}$) and isobutyrate ($<25 \mu\text{M}$) were detected in all

incubation supernatants. The presence of alanine in the supernatants was also indicated, although concentrations were not measurable by the methods employed. Formate concentrations in the protozoal incubation supernatants were similar to those detected in corresponding incubations of bacteria which had been isolated from the protozoal suspensions. Bacterial suspensions produced <2% of the principal VFA and lactic acid concentrations detected in equivalent protozoal incubations.

Following all incubations the protozoa were observed microscopically and appeared intact with maintained ciliary movement.

Discussion

The glucose fermentation balance of *D. ruminantium* is modulated by ambient gas partial pressures. Under all incubation conditions employed this holotrich ciliate predominantly ferments glucose to lactate, acetate, butyrate, H₂ and CO₂, but the relative proportions in which these are produced are sensitive to O₂ and CO₂ at those concentrations typically observed *in situ* in the rumen (Hillman *et al.*, 1985a). Alanine, not previously recognized as a product of glucose catabolism by this organism, was detected by ¹³C NMR spectroscopy. Glycerol and ethanol, known products of the trichomonads (Müller, 1988), were not detected as metabolites of *D. ruminantium* fermentation by the methods employed.

Lactic acid is the major end-product of glucose-stimulated fermentation; this confirms previous studies (Williams & Harfoot, 1976; Van Hoven & Prins, 1977). In the rumen, lactic acid is a short-lived intermediate (Hungate, 1966) and its over-accumulation results in a condition potentially lethal to the host. The uptake of lactic acid from rumen fluid by the entodiniomorphid ciliates has been reported previously (Newbold *et al.*, 1987). However, lactate has previously been observed to have adverse effects on holotrichs (Prins & Van Hoven, 1977; Van Hoven & Prins, 1977; Williams & Morrison, 1982).

The presence of traces of O₂ (1–3 µM) profoundly affects the balance of metabolic fluxes in *D. ruminantium*. Lactate was consumed by *D. ruminantium* in the presence of low dissolved O₂ concentrations only when glucose was depleted from the supernatant. Increased CO₂ evolution suggests an increased flux of carbon through hydrogenosomal pathways; acetate production was correspondingly increased. Increased production of acetate in the presence of O₂ has also been reported to occur in *Isotricha* spp. (Prins & Prast, 1973). Acetate formation under strictly anaerobic conditions may be limited by the electron-accepting activity of protons in

the hydrogenase reaction. Oxygen competes with protons within the hydrogenosome by acting as a terminal acceptor for electrons released during pyruvate oxidation (Chapman *et al.*, 1986a). Increased carbon flux through the hydrogenosome, as observed with *D. ruminantium* in the presence of O₂, might be energetically favourable although no direct experimental evidence exists for increased ATP generation aerobically in this organism or in the trichomonads (Müller, 1988).

Certain hydrogenosomal enzymes are oxygen-sensitive (Yarlett *et al.*, 1981) and their compartmentation indicates a possible protective role for the organelle in organisms living in the presence of O₂ (Lloyd & Coombs, 1989). During periods between feeds the isotrichid holotrich ciliates sequester on the reticulum wall (Abe *et al.*, 1981; Dehority & Tirabasso, 1989; Ankrah *et al.*, 1990), where they would be exposed to the full flux of O₂ entering the rumen through the mucosal epithelium (Czerkawski, 1986). Conversion of lactate to pyruvate by *D. ruminantium* regenerates cytosolic NADH. This organism possesses functional cytosolic NADH oxidase and peroxidase activities (Yarlett *et al.*, 1981) which may also have a role in protecting O₂-sensitive systems from O₂ and its reduction products, and in keeping the organism in redox balance (Williams, 1986).

The inhibition of H₂ production by O₂ in *D. ruminantium* confirms previous studies (Yarlett *et al.*, 1983; Hillman *et al.*, 1985b). Both inhibitory and stimulatory effects of traces of O₂ on H₂ production have been observed in organisms containing hydrogenosomes (Lloyd *et al.*, 1987; Ellis *et al.*, 1989b; Paget & Lloyd, 1990). These results emphasize the complexities of the interactions of O₂ with the hydrogenosomal electron transport system.

Suppression of acetate formation and H₂ evolution under high CO₂ concentrations have been observed previously in the hydrogenosome-containing flagellate *Trichomonas vaginalis* (Paget & Lloyd, 1990), where high CO₂ favours biosynthetic pathways. In *D. ruminantium* high CO₂ favours butyrate formation. Cultivation of the rumen fungus *Neocallimastix patriciarum* under high CO₂ concentrations leads to suppression of pyruvate:ferredoxin oxidoreductase synthesis and therefore acetate formation (Yarlett *et al.*, 1986).

Possible control points in *D. ruminantium* fermentation are indicated in Fig. 4. In summary, by comparison with anaerobic metabolism under 101 kPa N₂, high CO₂ favours cytosolic carbon fluxes (i.e. production of lactate from pyruvate and butyrate from acetyl-CoA) whereas low O₂ concentrations favour those pathways located in the hydrogenosomes. These metabolic tendencies are qualitatively similar to those observed in *T. vaginalis* (Paget & Lloyd, 1990).

Traces of formate detected in this study could be

Fig. 4. Fermentation pathways in *D. ruminantium*. Pathways favoured by the presence of low dissolved O_2 ($1-3 \mu M$) are indicated by bold arrows ($\Rightarrow$). Pathways favoured by high CO_2 concentrations (66 kPa CO_2 in N_2 gas phase) are indicated by dashed arrows ($\dashrightarrow$). The box encloses those reactions proceeding in the hydrogenosome. Enzymes are as follows: (1) Pyruvate:acceptor oxidoreductase; (2) hydrogenase; (3) phosphate acetyltransferase; (4) acetate kinase; (5) β -hydroxybutyryl-CoA dehydrogenase; (6) crotonase; (7) crotonyl-CoA reductase; (8) phosphate butyryltransferase; (9) butyrate kinase; (10) lactate dehydrogenase; (11) malate dehydrogenase (decarboxylating). X is the uncharacterized low-redox-potential electron-transporting component (Yarlett *et al.*, 1981, 1982, 1985). The formation of acetyl phosphate from acetyl-CoA is based on physiological observations (Yarlett *et al.*, 1982) and awaits confirmation, as this pathway of acetate formation is considered unique amongst eukaryotes.

bacterial products and not products of protozoal metabolism as previously reported (Van Hoven & Prins, 1977). Attachment of bacteria to the cilia and pellicle of *D. ruminantium* (Imai & Ogimoto, 1978) and also their presence in digestive vacuoles (Gutierrez & Hungate, 1957; Stern *et al.*, 1977) are commonly observed. The possible metabolic interrelationships are uncertain although studies with rumen ciliates and methanogens suggest that these microbial groups interact strongly (Hillman *et al.*, 1988).

At the low population densities which typically survive the protozoal isolation procedure, contaminating and associated bacteria produced no detectable alanine. The C-3 atom of alanine is derived from C-1 of D-glucose during glucose catabolism by *D. ruminantium*. Alanine has been identified as an end-product of fermentation by parasitic protozoa (Chapman *et al.*, 1986b; Darling *et al.*, 1987; Edwards *et al.*, 1989; Paget *et al.*, 1990). The pathway leading to alanine formation is sensitive to O_2 in *Giardia lamblia* (Paget *et al.*, 1990). At the dissolved O_2 concentrations used in this study ($1-3 \mu M$) no apparent effect of O_2 on alanine formation was observed. Relatively little is known about the metabolism of amino

acids by *D. ruminantium*. Alanine secretion has been reported in *Isotricha* spp. (Harmeyer, 1971) and aminotransferase activities have been detected in extracts obtained from mixed protozoal preparations (Bhatia *et al.*, 1979). Further work is required to determine the pathway(s) of alanine production from D-glucose; presumably pyruvate is the direct precursor and the final step may involve either reductive amination or the participation of an aminotransferase (e.g. alanine:2-oxoglutarate aminotransferase).

Previous estimates (Van Hoven & Prins, 1977) of the contributions of *D. ruminantium* to the overall VFA output of the ruminant have been based on anaerobic measurements *in vitro* of endogenous metabolism using O_2 -free CO_2 atmospheres. These measurements take no account of the actual conditions prevailing *in situ* in the rumen. The present results therefore suggest that the importance of *D. ruminantium* may have been underestimated as the endogenous metabolite formation rate under O_2 -free CO_2 was used in the determination. During the fermentation of exogenous glucose by *D. ruminantium* under a gas headspace composition similar to conditions that prevail *in situ* in the rumen, acetate and

butyrate formation were stimulated by 7- and 8-fold, respectively, compared to their endogenous production under a high CO₂ in N₂ gas phase.

J. E. E. held a SERC (CASE) postgraduate studentship.

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