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Influence of CO_2 and Low Concentrations of O_2 on Fermentative Metabolism of the Ruminal Ciliate *Polyplastron multivesiculatum*

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The effects of ruminal concentrations of CO_2 and oxygen on the end products of endogenous metabolism and fermentation of D-glucose by the ruminal entodiniomorphid ciliate *Polyplastron multivesiculatum* were investigated. The principal metabolic products were butyric, acetic, and lactic acids, H_2, and CO_2. ^{13}C nuclear magnetic resonance spectroscopy identified glycerol as a previously unknown major product of D-[1-^{13}C]glucose fermentation by this protozoan. Metabolite formation rates were clearly influenced by the headspace gas composition. In the presence of 1 to 3 μM O_2, acetate, H_2, and CO_2 formation was partially depressed. A gas headspace with a high CO_2 content (66 kPa) was found to suppress hydrogenosomal pathways and to favor butyrate accumulation. Cytochromes were not detected (<2 pmol/mg of protein) in *P. multivesiculatum*; protozoal suspensions, however, consumed O_2 for up to 3 h at 1 kPa of O_2. Under gas phases of >2.6 kPa of O_2, the organisms rapidly became vacuolate and the cilia became inactive. The results suggest that fermentative pathways in *P. multivesiculatum* are influenced by the O_2 and CO_2 concentrations that prevail in situ in the rumen.

Under certain dietary conditions, ruminal ciliate protozoa account for approximately half of the ruminal biomass and therefore substantially contribute to total volatile fatty acid production in situ (42). Two distinct groups of ciliate protozoa exist in the rumen: the entodiniomorphid ciliates, which are principally regarded as particulate feeders (10), and the holotrich ciliates, which utilize certain soluble carbohydrates (21, 41). The large entodiniomorphid ciliate *Polyplastron multivesiculatum* is actively cellulolytic (1, 2, 8), amylolytic (1, 9), and hemicellulolytic (43) and utilizes exogenous glucose (12, 17).

Glucose is the major monosaccharide liberated during the digestion of plant cellular material and starch in the rumen (6) and is the predominant substrate from which microbial populations form volatile fatty acids (22). However, little is known about the intermediary metabolic pathways in ruminal entodiniomorphid ciliates. Some of the glucose fermentation products are acetate, butyrate, H_2, and CO_2; lower concentrations of lactate, formate, and propionate have also been detected (10).

P. multivesiculatum possesses hydrogenosomes (36). These membrane-bounded subcellular organelles are also present in trichomonads (23), certain other ruminal ciliates (44–46), and the rumen fungus *Neocallimastix patriciarum* (49). Oxidation of pyruvate proceeds in these organelles, yielding acetate, H_2, and CO_2. *P. multivesiculatum* has a high affinity for O_2 at low concentrations; however, H_2 evolution is reversibly inhibited by dissolved O_2 at tensions of <0.25 μM (17). In other organisms, hydrogenosomes act as centers for O_2 consumption (3, 34, 47) and thus confer some aerotolerance to these organisms. In this study, we investigated the respiratory pigments in whole cells of *P. multivesiculatum* and examined the survival of this ruminal ciliate under different O_2 tensions.

The rumen provides an environment in which *P. multivesiculatum* experiences low, fluctuating concentrations of O_2 (0.25 to 3 μM) (19, 37) and high concentrations of CO_2 (160 to 3,800 μM dissolved in ruminal liquor, representing 65% [vol/vol] of the ruminal gas headspace) (19, 33). The effects of these gases at physiological concentrations on the fermentation of glucose by this organism have not been determined, as previous studies have used gas headspaces of 5 kPa of CO_2 in O_2-free N_2 (12).

In this study, ^{13}C nuclear magnetic resonance spectroscopy (NMR), high-performance liquid chromatography (HPLC), and membrane-inlet mass spectrometry were used to identify and quantify the end products of glucose fermentation by *P. multivesiculatum* in vitro. We determined the effects of O_2 and CO_2, at concentrations typical of conditions that prevail in the rumen, on the fermentative metabolism of this ruminal ciliate and report for the first time that glycerol is an important fermentation product of *P. multivesiculatum*.

MATERIALS AND METHODS

Isolation of the organism. *P. multivesiculatum* was isolated from the ruminal contents of a sheep that had been defaunated and reinoculated with *P. multivesiculatum* and *Entodinium* spp. The animal received a diet of concentrates and chopped hay. Water was given ad lib. Isolation of *P. multivesiculatum* from ruminal fluid was carried out as previously described (17). Washed suspensions of *P. multivesiculatum* were sedimented by centrifugation at 500 × *g* for 2 min at room temperature and resuspended in 2 ml of buffer D (7) under N_2. Contamination of the preparation by *Entodinium* spp. was negligible (<1%). Enumeration of protozoa was performed with an improved Neubauer Hawksley hemacytometer (Weber Scientific International Ltd., Sussex, United Kingdom).

***P. multivesiculatum* incubations under defined gas phases.** Samples (2 ml) were adjusted to pH 7.0 and transferred to incubation vessels maintained at 39°C. Separate incubations were carried out with four different headspace compositions:

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101 kPa (100%) of N_2; a partial pressure of O_2 in N_2, usually 7.5 kPa of O_2 (35% air–65% N_2 [vol/vol]), to yield 1 to 3 μM O_2 in the protozoal suspension; 66 kPa of CO_2 in N_2; and a gaseous mixture of 66 kPa of CO_2 and 7.5 kPa of O_2 (65% CO_2–35% air [vol/vol]) to yield 1 to 3 μM O_2 in the protozoal suspension. Each incubation was performed in triplicate. The substrate used was D-glucose (30 mM final concentration). Organisms were incubated for 2 h. Samples (200 μl) were withdrawn every 30 min and centrifuged for 10 min at 5,100 × *g* and room temperature. Supernatants were filtered to remove protein with a molecular filter (molecular weight cutoff, 10,000) (micropartition system; Amicon Ltd.) and stored frozen at −20°C. Endogenous metabolite formation was determined by an identical procedure, except that no D-glucose was added. Samples for NMR were obtained in a similar manner, except that D-[1-^{13}C]glucose (30 mM final concentration) was added. Samples (0.5 ml) were withdrawn after 2 and 4 h and centrifuged at 5,100 × *g* for 10 min. Supernatants were decanted, subjected to extraction with 60% perchloric acid on ice for 10 to 15 min, neutralized with 4 M NaOH, centrifuged at 5,100 × *g* for 10 min, and stored at −20°C.

Bacterial incubations. Controls were set up with extracts from protozoa that might have contained bacteria. Washed protozoa were maintained at 4°C under N_2 and disrupted by ultrasound treatment with an MSE Soniprep 150 (Fisons Plc., Loughborough, United Kingdom) at a frequency of 20 kHz, in cycles of 15 s (with 15-s intervals), and at an amplitude of 6 to 12 μm for 6 min. The absence of intact protozoa after this treatment was confirmed by microscopic examinations. The crude homogenate (10 ml) was centrifuged at 500 × *g* for 10 s at 25°C, and the supernatant was decanted and centrifuged at 7,000 × *g* for 10 min. The pellet was washed twice in buffer D by recentrifugation at 7,000 × *g* for 10 min each time. The pellet contained amylopectin and intact, motile bacteria (observed by microscopic examinations) that were present in the original protozoal suspension (12). The pellet was resuspended under anaerobic conditions to the original homogenate volume in buffer D. Duplicate incubations were performed in the presence and absence of D-glucose (30 mM final concentration) under all the gas phases used for the protozoal suspension.

Mass spectrometry. Dissolved-gas concentrations were monitored with a HAL series quadrupole gas analyzer (Hiden Analytical Ltd., Warrington, United Kingdom) 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 (26). The probe monitored gases dissolved in the 2-ml reaction vessel under a mobile gas phase (14, 28); mixing was carried out by bubbling at a constant flow rate (10 ml/min) (26). The partial pressures of O_2 and CO_2 in the gases were controlled with a digital gas mixer (15). The concentrations of dissolved gases at 39°C and pH 7.0 were taken as follows: H_2, 740 μM; O_2 in air, 215 μM; and CO_2, 5,258 μM (40). Rates of gas production were calculated as previously described (28).

NMR measurements. Proton-decoupled ^{13}C NMR spectra were recorded at 22.49 MHz on a JEOL FX90Q spectrometer equipped with a 10-mm multinuclear probe. Free induction decays were acquired in 8,000 data points covering a spectral width of 120 ppm (2,700 Hz) with pulses of 13 μs (78°) at 10-s intervals. Each free induction decay was multiplied by an exponential function that produced a line broadening of 1.03 Hz before transformation. D_2O was used as the solvent and internal lock. Chemical shifts, in parts per million, were measured with respect to the βC_1 resonance in the added D-glucose (97.0 ppm) (31).

Quantitation of metabolites. Soluble metabolites present in the supernatant were separated and quantified by HPLC with a variable-wavelength UV detector (HPLC Technology Ltd., Macclesfield, United Kingdom). Separation was achieved by injecting 20-μl volumes onto a fermentation monitoring column (Bio-Rad Laboratories Ltd., Watford, United Kingdom). Eluant streams were monitored at 210 nm with a readout on a potentiometric chart recorder. The eluant flow rate was 0.8 ml/min, and the sustained pressure was approximately 800 lb/in^2. Metabolites were identified and quantified with internal standards and calibration curves of pure compounds. The glucose concentration was determined with a coupled hexokinase–glucose-6-phosphate dehydrogenase assay kit (Sigma Diagnostics, Poole, Dorset, United Kingdom). L-Lactic acid was measured with a coupled L-lactate dehydrogenase–glutamate pyruvate aminotransferase assay kit, and glycerol was similarly measured with a coupled glycerol kinase–pyruvate kinase–L-lactate dehydrogenase assay kit (both obtained from B.C.L., Sussex, United Kingdom).

Oxygen electrode measurements. The survival of *P. multivesiculatum* maintained under defined O_2 partial pressures was measured at 39°C with a 25-μm-thick Teflon membrane-covered oxygen electrode as previously described (17). The solubility of O_2 in air at 39°C was assumed to be 215 μM (40).

Difference spectra. Difference spectra (reduced minus oxidized) of whole cells of *P. multivesiculatum* were obtained to investigate respiratory pigments. Measurements were carried out at 77 K in a cell with a 2-mm path length with a Hitachi-Perkin Elmer 557 double-beam spectrophotometer scanning from 400 to 650 nm at a speed of 30 nm min^{-1}. Sodium dithionite was the reductant used; aeration and ammonium persulfate were used to oxidize samples.

Sources of chemicals. D-[1-^{13}C]glucose (99%) was purchased from Aldrich Chemical Co. (Dorset, United Kingdom). Sulfuric acid used for HPLC was Aristar grade (BDH Ltd., Poole, Dorset, United Kingdom). All other chemicals were of analytical grade and were obtained from BDH.

RESULTS

NMR identification of products. An initial identification of all of the products released by *P. multivesiculatum* during the catabolism of D-glucose was carried out after incubation of the organism with D-[1-^{13}C]glucose under various gas phases.

^{13}C NMR spectra obtained after 4 h of incubation under 101 kPa of N_2 revealed butyrate, acetate, and lactate to be the principal fermentation end products (Fig. 1a). Labeled glycerol was detectable in the supernatants obtained from 4-h incubations after exposure to 1 to 3 μM O_2 dissolved in the protozoal suspension (Fig. 1b). The product profile under 66 kPa of CO_2 in N_2 was similar, but the glycerol signal intensity was reduced (Fig. 1c).

Labeled glycerol was never detectable in the supernatants obtained from bacterial incubations with D-[1-^{13}C]glucose; this observation confirms the protozoal origin of the metabolite. No anomeric specificity for D-[1-^{13}C]glucose utilization by *P. multivesiculatum* was detected for any of the incubations. D-[1-^{13}C]glucose was always detectable after 4 h of incubation irrespective of the gas phase used.

Metabolite formation during incubations with D-glucose.

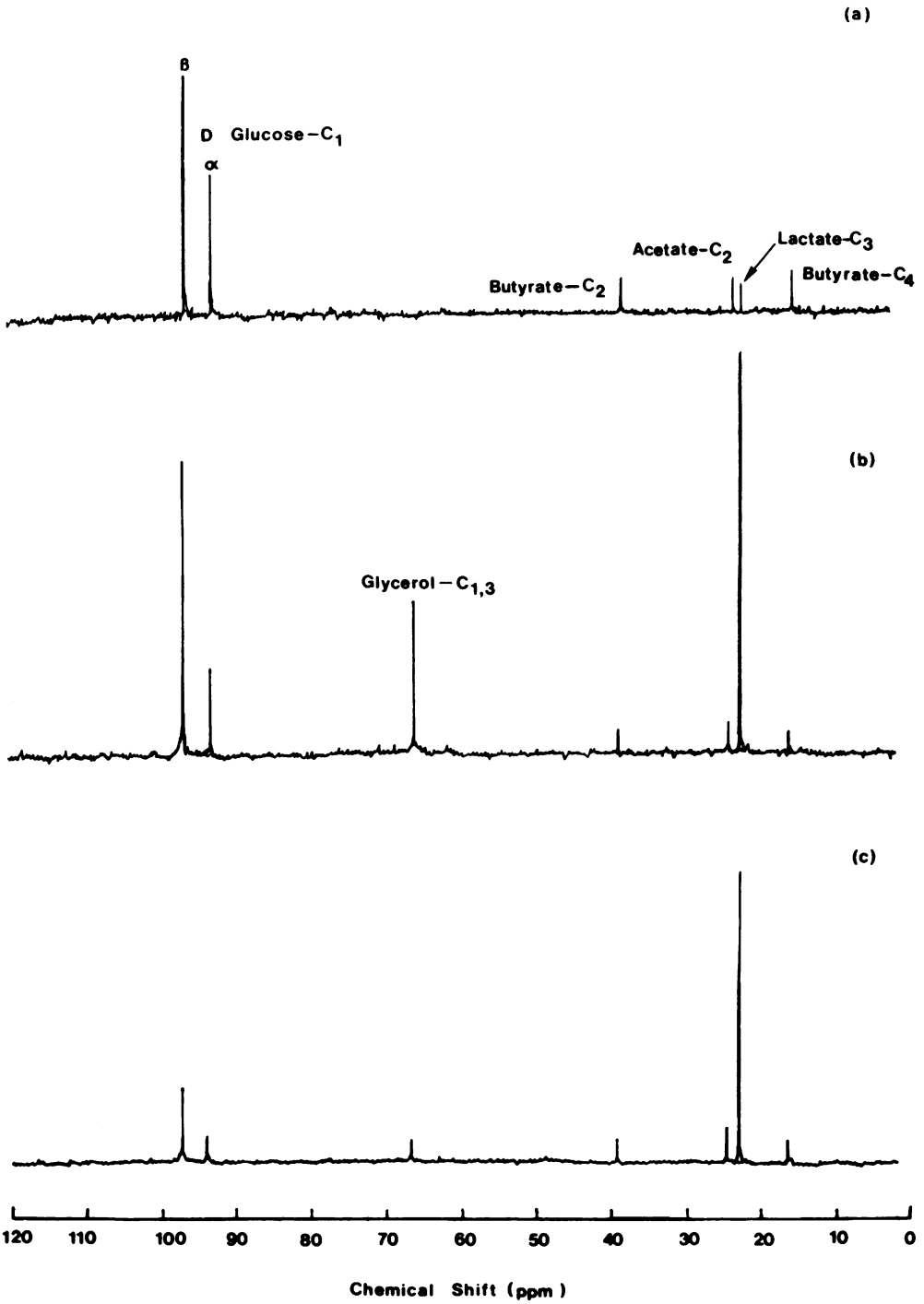

FIG. 1. Proton-decoupled ^{13}C NMR spectra of *P. multivesiculatum* (1.5×10^6/ml) supernatants after incubation with 30 mM D-[1-^{13}C] glucose for 4 h. Washed organisms were incubated at 39°C in buffer D at pH 7.0 in the presence of 101 kPa of N_2 (a), 7.5 kPa of O_2 in N_2 (1 to 3 μM O_2 in the protozoal suspension) (b), and 66 kPa of CO_2 in N_2 (c). Chemical shifts (in parts per million) were as follows: C_1 glucose α peak, 93.1; C_1 glucose β peak, 97.0; $C_{1,3}$ glycerol, 66.0; C_2 butyrate, 38.1; C_2 acetate, 22.7; C_3 lactate, 21.1; and C_4 butyrate, 13.9.

The principal products formed from glucose (30 mM) by *P. multivesiculatum* under 101 kPa of N_2 were acetate, butyrate, and lactate (Fig. 2a). The concentration of glycerol in the supernatant after 2 h was 1.1 mM. Glucose was initially utilized at a rate of 95 nmol/min per 10^5 organisms; after 2 h, 10.5 mM D-glucose was still present extracellularly.

The introduction of low O_2 concentrations (1 to 3 μM in the protozoal suspension) resulted in a 40% reduction in acetate accumulation in the supernatant; lactate and butyrate were formed at rates similar to those observed under 101 kPa of N_2 (Fig. 2b). However, the extracellular glycerol concentration was increased threefold in the 2-h supernatant

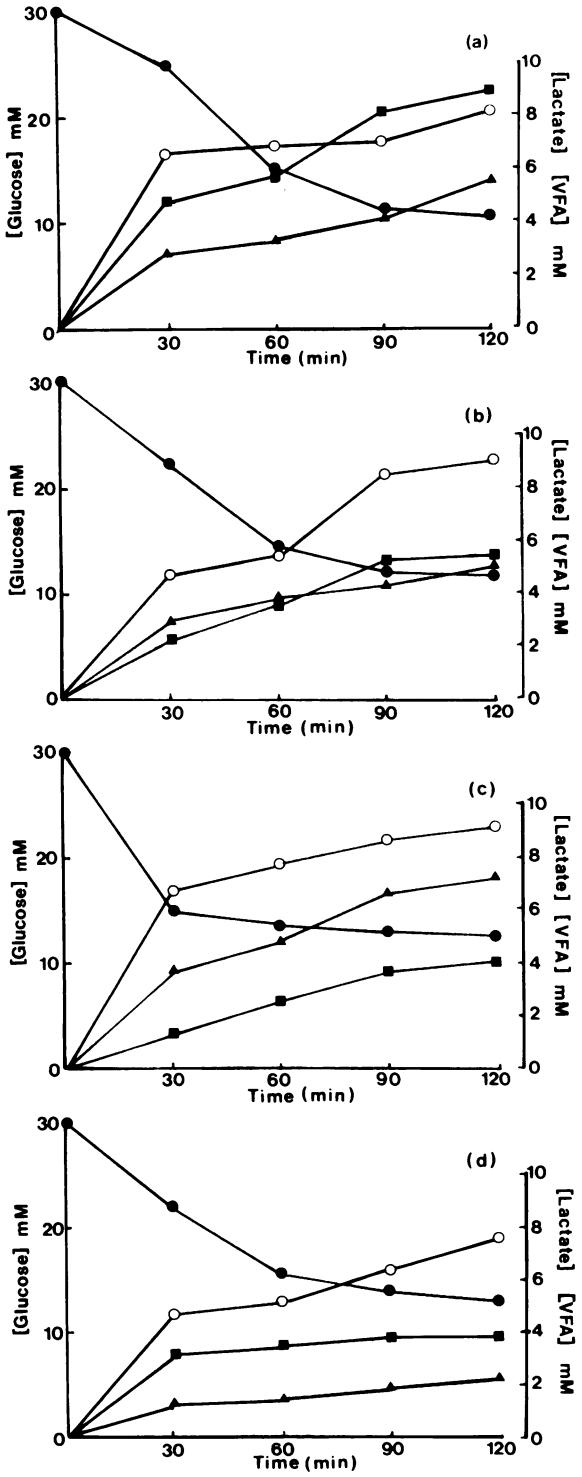

FIG. 2. Consumption of glucose and production of principal metabolic products by *P. multivesiculatum* (1.9×10^5/ml) incubated with 30 mM D-glucose at 39°C and pH 7.0 under 101 kPa of N_2 (a), 7.5 kPa of O_2 in N_2 (1 to 3 μM O_2 in the protozoal suspension) (b), 66 kPa of CO_2 in N_2 (c), and 66 kPa of CO_2 and 7.5 kPa of O_2 (1 to 3 μM O_2 in the protozoal suspension) (d). Products were analyzed by HPLC and were as follows: D-glucose (●), lactate (▲), acetate (■), and butyrate (○). VFA, Volatile fatty acids.

to 3.04 mM. Glucose was consumed at an initial rate of 133 nmol/min per 10^5 organisms, a rate 50% greater than that observed under the former gas phase.

Under a gas phase of 66 kPa of CO_2 in N_2, D-glucose was consumed by *P. multivesiculatum* at an initial rate of 285 nmol/min per 10^5 organisms. Lactate and butyrate formation rates were greater than those observed under 101 kPa of N_2 or low O_2 concentrations in N_2; acetate accumulation was 45% that obtained during incubation under 101 kPa of N_2 (Fig. 2c). Glycerol was present in the 2-h supernatant at a concentration of 0.91 mM.

Incubation under conditions typical of those that prevail in situ in the rumen (66 kPa of CO_2 and 34 kPa of air, yielding 1 to 3 μM O_2 in the protozoal suspension) yielded glucose uptake at a rate of 143 nmol/min per 10^5 organisms, a rate similar to that observed under low O_2 concentrations in N_2. Lactate and acetate formation rates were, however, the lowest obtained. Butyrate accumulation in the supernatant was similar to that observed under 101 kPa of N_2 (Fig. 2d). Glycerol was present in the 2-h supernatant at a concentration of 2.5 mM, a concentration comparable to that observed under low O_2 concentrations in N_2.

Extracellular D-glucose concentrations never fell below 12.5 mM for any of the incubations. Lactic acid detected in the supernatants was found to consist predominantly (86%) of the L-isomer, as indicated by a comparison of enzyme assay results and HPLC quantitation.

Endogenous metabolism of *P. multivesiculatum*. Endogenous metabolites produced by *P. multivesiculatum* are formed by intracellular amylopectin degradation. The changes observed in the principal metabolite concentrations in the incubation supernatants under the four gas phases studied are presented in Fig. 3a to d. The major product of endogenous metabolism in all cases was found to be butyrate, which was also the principal volatile fatty acid formed during equivalent incubations with D-glucose. Butyrate was formed most rapidly and accumulated to the highest concentrations during incubations under low O_2 concentrations in N_2 or 66 kPa of CO_2 in N_2. Under a low-O_2, high-CO_2 gas phase, the extracellular butyrate concentration was 60% that observed during equivalent incubations with exogenously added D-glucose. Endogenous lactate formation was highest under a low-O_2, high-CO_2 gas phase, typical of conditions in the rumen, and the L-isomer was predominant (85%) in all incubations.

Glycerol was detectable under all gas phases; the highest concentrations were present when low O_2 concentrations were included in the gas phase. Incubations under low O_2 concentrations in N_2 and a low-O_2, high-CO_2 gas phase yielded glycerol concentrations after 1 h of 0.94 and 0.81 mM, respectively. These were approximately 100% higher than the concentrations detected after incubations under 101 kPa of N_2 (0.38 mM) and 66 kPa of CO_2 in N_2 (0.42 mM).

H_2 and CO_2 production by *P. multivesiculatum*. Rates of H_2 and CO_2 production by *P. multivesiculatum* were determined simultaneously by dissolved-gas analysis with membrane-inlet mass spectrometry. Rates obtained during incubations under each of the four gas phases are presented for both glucose-stimulated and endogenous fermentations in Table 1. Under a gas phase of 101 kPa of N_2, H_2 and CO_2 were produced in equimolar concentrations. H_2 production was inhibited by approximately 50% when low O_2 concentrations were introduced into the gas phase, and CO_2 evolution was inhibited by 34%. The lowest H_2 production rates were observed under the low-O_2, high-CO_2 gas phase.

Other metabolites formed by *P. multivesiculatum*. Low

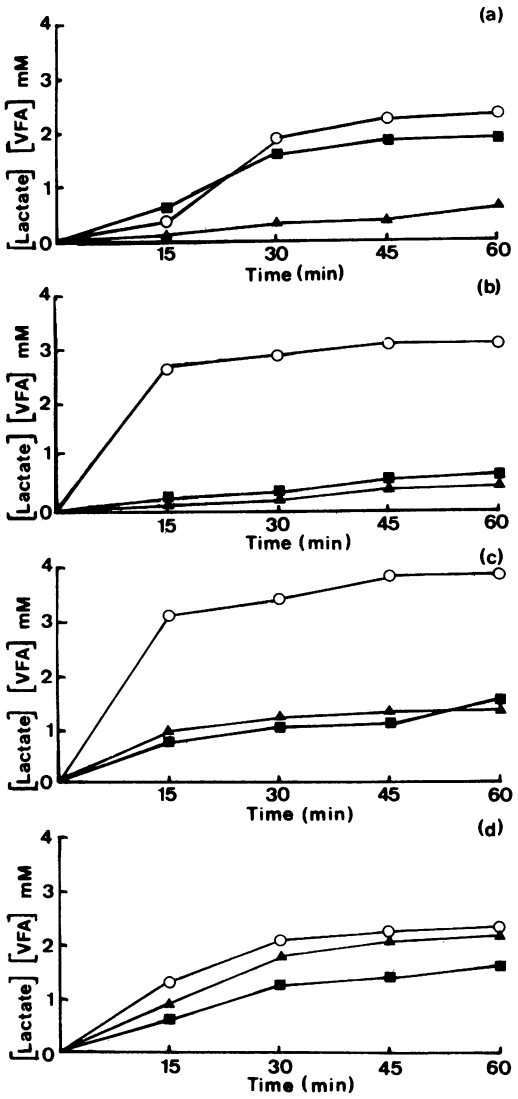

FIG. 3. Metabolites formed by *P. multivesiculatum* during endogenous fermentation in the absence of extracellularly added D-glucose (10^5 organisms per ml). Incubations were performed at 39°C and pH 7.0 under 101 kPa of N_2 (a), 7.5 kPa of O_2 in N_2 (1 to 3 μM O_2 in the protozoal suspension) (b), 66 kPa of CO_2 in N_2 (c), and 66 kPa of CO_2 and 7.5 kPa of O_2 (1 to 3 μM O_2 in the protozoal suspension) (d). Products were analyzed by HPLC and were as follows: lactate (▲), acetate (■), and butyrate (○). VFA, Volatile fatty acids.

concentrations of propionic, succinic, pyruvic, isobutyric, and formic acids were detected in all incubation supernatants (concentrations of <1 mM after 2 h of incubation with D-glucose). L-Glutamine was also detectable in trace amounts. Formate concentrations in protozoal incubation supernatants were similar to those detected in corresponding suspensions of bacteria which had been isolated from the protozoa. Bacterial suspensions produced about 5% the principal volatile fatty acid and lactic acid concentrations detected in corresponding protozoal incubation supernatants.

Survival of *P. multivesiculatum* at different O_2 tensions. The effects of oxygen exposure on O_2 consumption by *P. multivesiculatum* were investigated during incubations under various O_2 tensions (1 to 21 kPa of O_2). At O_2 concentrations above 2.6 kPa, the consumption of O_2 decreased rapidly to an undetectable rate (Fig. 4a to d). When the O_2 concentration was maintained at 1 kPa, O_2 uptake was stable for 3 h before steadily declining. *P. multivesiculatum* remained nonvacuolate and sustained ciliary movement for up to 4 h under 1 kPa of O_2.

TABLE 1. Mass spectroscopic analyses of dissolved-gas production by *P. multivesiculatum* during glucose-stimulated and endogenous fermentations under different gas phases

Gas phase	Evolution (μM/min/10^5 organisms) of:			
	Hydrogen		Carbon dioxide	
	Endogenous	Glucose stimulated	Endogenous	Glucose stimulated
101 kPa of N_2	7.5 ± 2.4	12.9 ± 3.1	3.8 ± 1.2	13.5 ± 3.5
7.5 kPa of O_2[a] in N_2	4.2 ± 1.0	6.4 ± 2.9	2.1 ± 0.8	8.7 ± 2.0
66 kPa of CO_2 in N_2	4.9 ± 2.0	9.2 ± 3.5	ND[b]	ND
7.5 kPa of O_2[a] and 66 kPa of CO_2	3.9 ± 1.0	5.7 ± 2.5	ND	ND

[a] Partial pressure of O_2 required to yield 1 to 3 μM O_2 dissolved in the protozoal suspension.
[b] ND, CO_2 evolution was not measured because of the presence of high extracellular concentrations of dissolved CO_2.

Cytochrome spectra. Difference spectra (dithionite reduced minus aerated oxidized) of whole cells of *P. multivesiculatum* were featureless over the range of 460 to 650 nm, indicating the absence of detectable cytochromes (limit of detection, <2 pmol of cytochrome *c* per mg of protein). Oxidation of the reference cuvette contents with ammonium persulfate also failed to reveal cytochromes.

DISCUSSION

The ruminal entodiniomorphid ciliate *P. multivesiculatum* principally forms butyrate, acetate, lactate, glycerol, H_2, and CO_2 both during endogenous amylopectin degradation and from the fermentation of exogenous D-glucose. The ratios in which these fermentation products are formed are clearly modulated by O_2 and CO_2 at concentrations typical of those encountered in situ in the rumen. The pathways leading to the formation of the main products of *P. multivesiculatum* fermentation await elucidation, although similar metabolites are formed by the ruminal isotrichid holotrich ciliate *Dasytricha ruminantium*, and the metabolic pathways in that case have been partially characterized (45, 47, 48).

The formation of glycerol as a significant metabolite is unusual among eukaryotes, and it was not previously known that a ruminal ciliate could provide glycerol to other ruminal microbes and/or the host animal. As glycerol is readily fermented to propionate by certain ruminal bacteria (e.g., *Selenomomas ruminantium*; 20, 39), the presence of a large population of *P. multivesiculatum* protozoa may indirectly lead to an elevated propionate output to the host, although the ciliate protozoa themselves are not major propionate producers. Anaerobic glycerol production previously has been shown in trypanosomatid (13, 16, 32) and trichomonad (5, 38) flagellates. *Trichomonas vaginalis* produces high concentrations of glycerol, as does *P. multivesiculatum*, under conditions of low O_2 concentrations (<0.25 μM) and in the presence of CO_2 (35, 38). Further work is required to determine the pathway(s) of glycerol production in *P. mul-*

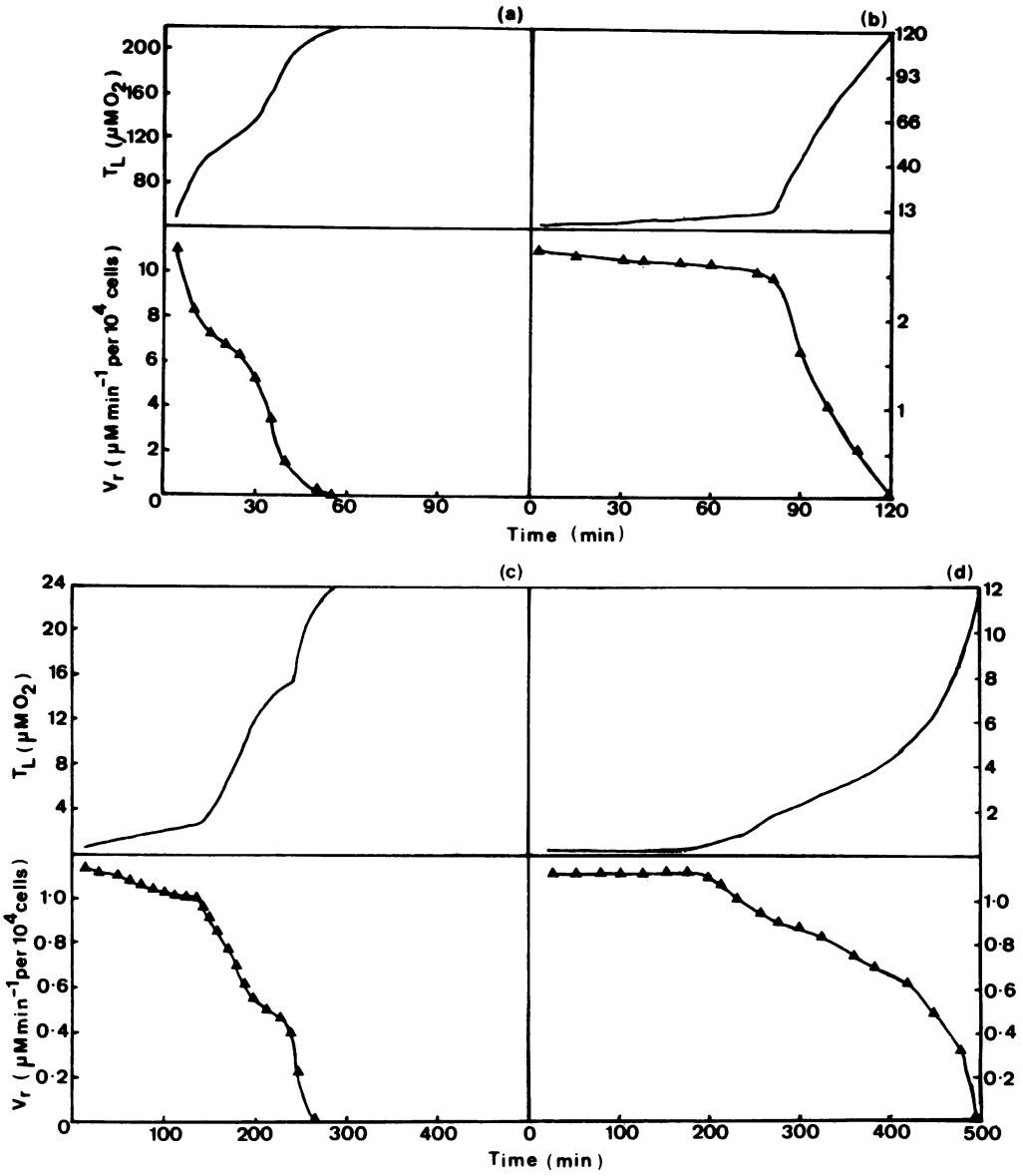

FIG. 4. Oxygen consumption of *P. multivesiculatum* in an open oxygen electrode system. *P. multivesiculatum* whole cells (4×10^5/ml) were suspended in buffer D at pH 7.0 in a total volume of 5.5 ml and maintained at 39°C. Gas mixtures were produced with a digital gas mixer. Partial pressures of O_2 were as follows: (a) 21 kPa; (b) 10 kPa; (c) 2.6 kPa; and (d) 1 kPa. V_r is the O_2 consumption rate; T_L is the O_2 tension in the liquid. The incubation vessel volume was 5.5 ml.

tivesiculatum; presumably it arises from dihydroxyacetone phosphate, with glycerol 3-phosphate as an intermediate.

Hydrogenosomal metabolism in *P. multivesiculatum* is sensitive to low levels of dissolved O_2 (1 to 3 μM), and acetate formation rates are partially inhibited by its presence. Sensitivity to low levels of dissolved O_2 is a common feature of hydrogenosome-containing eukaryotes. Oxygen competes with protons within the hydrogenosome by acting as an alternative terminal acceptor for electrons released during pyruvate oxidation (3, 4, 34). Inhibition of H_2 evolution occurs, corresponding to observations made with *P. multivesiculatum* both here and during a previous study (17). Difference spectra of whole cells of *P. multivesiculatum*, reported here for the first time, show that in common with other hydrogenosome-containing protozoa (27, 47), *P. multivesiculatum* lacks detectable cytochromes. The nature of the hydrogenosomal terminal oxidase is not known. Substrate-level phosphorylation is the only mechanism of energy generation in the hydrogenosome-containing eukaryotes, and at present there is no evidence to suggest that the consumption of O_2 by hydrogenosomes is linked to any other form of energy generation.

The hydrogenosomal enzymes hydrogenase and pyruvate synthase are extremely O_2 sensitive (36), and their compartmentation indicates a possible protective role for hydrogenosomes in organisms inhabiting environments in which they are periodically exposed to low levels of O_2 (25). *P. multivesiculatum* is able to survive for long periods at low O_2

concentrations typical of those which prevail in the rumen. At higher O_2 concentrations (>2.6 kPa), the organism becomes rapidly inactivated. Aerotolerance at low O_2 tensions is also a feature of the trichomonads (29, 30), the rumen fungus *N. patriciarum* (50), and certain other ruminal ciliates (17, 18, 47). Cytosolic oxidases acting on reduced pyridine nucleotides are detectable in the trichomonads (24) and the holotrich ciliate *D. ruminantium* (45) and may also be present in *P. multivesiculatum*, although this idea awaits confirmation. In the presence of physiological levels of O_2, these cytosolic O_2-scavenging systems would affect the redox balance of these organisms, which in turn may also influence the ratios of fermentation products formed.

Suppression of acetate formation and H_2 evolution under high CO_2 concentrations previously has been observed during growth of the hydrogenosome-containing flagellate *T. vaginalis* (35), in which high CO_2 concentrations favor biosynthetic pathways. In nonproliferating suspensions of *P. multivesiculatum*, high CO_2 concentrations favor butyrate and lactate formation. Previous studies with *P. multivesiculatum* grown in vitro showed improved growth rates when the organisms were grown in the presence of 5 kPa of CO_2 in N_2 (vol/vol) (11), and this finding, together with our findings, suggests that CO_2 may stimulate biosynthetic pathways. High extracellular concentrations of CO_2 also have been observed to affect hydrogenosomal metabolism in the ruminal fungus *N. patriciarum* (49). The combination of high CO_2 and low O_2 concentrations, resembling those which prevail in situ, had inhibitory effects on hydrogenosomal pathways during this study.

These results indicate that in *P. multivesiculatum*, fermentative pathways are influenced by the gaseous conditions prevailing in situ. Therefore, consideration of in situ conditions is crucial in the study of microorganisms from environments such as the rumen, for which the use of strictly anaerobic techniques may give irrelevant data. Studies with these microorganisms are most appropriately carried out under conditions which mimic those in situ.

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