

Carbohydrate and Amino Acid Fermentation in the Free-Living Primitive Protozoon *Hexamita* sp.

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***Hexamita* sp. is an amitochondriate free-living diplomonad which inhabits O₂-limited environments, such as the deep waters and sediments of lakes and marine basins. ¹³C nuclear magnetic resonance spectroscopy reveals ethanol, lactate, acetate, and alanine as products of glucose fermentation under microaerobic conditions (23 to 34 μM O₂). Propionic acid and butyric acid were also detected and are believed to be the result of fermentation of alternative substrates. Production of organic acids was greatest under microaerobic conditions (15 μM O₂) and decreased under anaerobic (<0.25 μM O₂) and aerobic (200 to 250 μM O₂) conditions. Microaerobic incubation resulted in the production of high levels of oxidized end products (70% acetate) compared to that produced under anoxic conditions (20% acetate). In addition, data suggest that *Hexamita* cells contain the arginine dihydrolase pathway, generating energy from the catabolism of arginine to citrulline, ornithine, NH₄⁺, and CO₂. The rate of arginine catabolism was higher under anoxic conditions than under microaerobic conditions. *Hexamita* cells were able to grow in the absence of a carbohydrate source, albeit with a lower growth rate and yield.**

The free-living anaerobic flagellate *Hexamita* sp. is an unusual protozoon lacking both mitochondria and Golgi apparatus (2). Sequences of complete small-subunit rRNA coding regions place it as one of the deepest-branching eukaryotes (3, 20, 41). In the genus *Hexamita*, there exist both free-living and parasitic species. All species are believed to be anaerobic or microaerobic (depending on definition), with the free-living species reported only in reducing environments such as stagnant waters, wastewater treatment plants, and anoxic marine basins (7, 14, 30). However, in all of these environments, it is unlikely that permanent anoxia can be guaranteed and thus *Hexamita* undoubtedly experiences periodic fluctuations of O₂ tension. Recently, this free-living species of *Hexamita* has been shown to lack detectable cytochromes, but it nevertheless actively consumes O₂ both endogenously and in the presence of several substrates with an O₂ K_m of 13 μM (1). In addition, *Hexamita* was observed to withstand high O₂ tensions (up to 100 μM) by the adoption of several antioxidant defense strategies (1), making this organism a microaerobe rather than an anaerobe.

Metabolic studies on free-living anaerobic protozoa have been hampered by the limited number of organisms growing in axenic cultures; to date, only the ciliate *Trimyema compressum* has been studied in any depth (15, 17, 45, 46). Hence, detailed metabolic studies of anaerobic protozoa have been confined to rumen-dwelling and parasitic protozoa (6, 29, 44). These studies have revealed that these organisms, unlike their aerobic counterparts, do not generate energy by oxidative phosphorylation but rather have developed extended glycolytic metabolic profiles and derive their ATP from substrate-level phosphorylation.

Anaerobic protozoa are sensitive to the ambient O₂ tension. Like *Hexamita* sp., many have a high affinity for O₂ as well as high O₂ consumption rates (12, 23, 25, 31). It is not clear as yet whether this affinity is part of a protective strategy against O₂ toxicity or whether O₂ is beneficial. Trace amounts of O₂ have been demonstrated to enhance growth and yield of *Trichomonas vaginalis* (32) and *Giardia lamblia* (34) and to influence the flux of metabolic products of these and many other protozoa such as those found in the rumen (16, 39, 44).

Studies of the catabolism of amino acids by anaerobic protozoa have also been limited to a few anaerobic protozoa (22, 26, 37). In the natural environment, the uptake of amino acids, both free and as released from proteinase activity, may be a primary source of energy. With the aid of ¹³C nuclear magnetic resonance (¹³C NMR), high-performance liquid chromatography (HPLC), and mass spectrometry, it has been the aim of this study to elucidate the primary products of glucose and amino acid fermentation of *Hexamita*. In addition, the influence of different O₂ tensions on the fermentative metabolism of this primitive flagellate has been investigated.

MATERIALS AND METHODS

Isolation and culture. *Hexamita* sp. was isolated by Jaroslav Kulda from a Czechoslovakian lake. Axenic cultures were established by treatment with ciprofloxacin (5 μg ml⁻¹) and colistin sulfate (100 μg ml⁻¹). The culture medium contained 2% (wt/vol) Trypticase (BBL), 1% (wt/vol) yeast extract (Oxoid), 0.5% (wt/vol) maltose, 0.1% L-cysteine, 10 mM K phosphate buffer, 10% (vol/vol) fetal calf serum (heat inactivated), and gentamycin sulfate (50 μg ml⁻¹) grown at pH 7.2 and 25°C. For experimentation, cultures were grown to late exponential phase (ca. 6.5 × 10⁵ cells ml⁻¹), harvested by centrifugation at 650 × g (5 min), and washed twice in 100 mM K phosphate buffer (pH 7.2) sparged with N₂. Organisms were counted with a hemocytometer.

Incubation of *Hexamita* cells at desired O₂ tensions. Cell suspensions (5 ml) in 100 mM K phosphate buffer were incubated (25°C) in a stainless-steel open O₂ electrode system fitted with a Teflon membrane-covered O₂ electrode (Radiometer A/S, Copenhagen, Denmark) (9). With the aid of a digital gas mixer (8), gas mixtures of O₂ in N₂, humidified by passage through moist cotton wool, were passed over the surface of the stirred liquid vortex (stirring at 790 rpm), enabling the O₂ tension to be maintained at desired levels. Addition of substrates (e.g., glucose) and removal of metabolites for quantification were made through the

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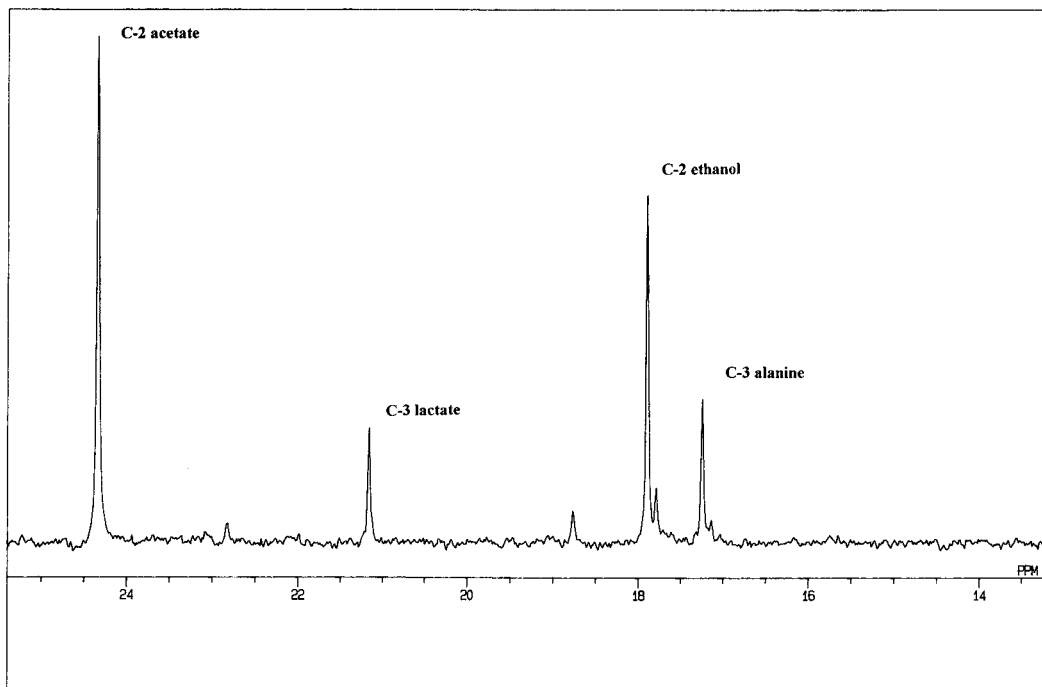


FIG. 1. Proton-decoupled ^{13}C NMR spectra of *Hexamita* supernatant. Washed cells were incubated (25°C) for 6 h under a dissolved- O_2 tension of 23 to $34\ \mu\text{M}$ with $\text{D}-[1-^{13}\text{C}]\text{glucose}$ ($30\ \text{mM}$). Chemical shifts in parts per million were as follows: C-1 glucose α peak, 93.2; C-1 glucose β peak, 97.0; C-2 acetate, 24.5; C-3 lactate, 21.2; C-2 ethanol, 18.0; C-3 alanine, 17.3.

gas exit port. The O_2 concentration of air-saturated buffer at 25°C was taken to be $253\ \mu\text{M}$ (43). Each incubation was done in triplicate.

NMR spectroscopy measurements. Products of glucose fermentation were identified by incubating organisms in the open O_2 electrode system with $30\ \text{mM}$ $\text{D}-[1-^{13}\text{C}]\text{glucose}$. Proton-decoupled ^{13}C NMR spectra were recorded at 67.5 MHz on a JEOL EX270 spectrometer equipped with a 5-mm multinuclear probe. Free induction decay was measured for a total of 32,000 data points covering a spectral width of 200 ppm with pulses of $7.4\ \mu\text{s}$ (70°) at 29-s intervals. $^2\text{H}_2\text{O}$ was used as the internal lock. Chemical shifts, in parts per million, were measured with respect to the $\beta\text{C}-1$ resonance in the added D -glucose (97.0 ppm) (27).

Quantification of organic acids. At specific time intervals, samples of cell suspension were removed from the open O_2 electrode system and centrifuged immediately. Soluble metabolites present in the supernatant were identified and quantified by use of a HPLC coupled to a variable-wavelength UV detector (for examples, see references 13 and 34). Samples ($20\ \mu\text{l}$) were separated by injection through a fermentation monitoring column packed with hydrogen sulfonated divinyl benzene-styrene copolymer resin (Bio-Rad) with a $1\ \text{mM}$ H_2SO_4 mobile phase flowing at $0.6\ \text{ml}\ \text{min}^{-1}$. Eluent streams were monitored at 210 nm and recorded with a potentiometric chart recorder. Metabolites were identified and quantified by using known standards.

Membrane inlet mass spectrometry. Dissolved-gas concentrations were monitored with a HAL series quadrupole gas analyzer (Hiden Analytical) linked to a temperature-controlled (25°C) incubation vessel (2 ml) by a stainless-steel probe (1.5-mm outside diameter; 0.5-mm inside diameter) with a 1-mm-diameter inlet covered by a silicone membrane (10, 24). Partial pressures of O_2 in the mobile phase were controlled with a digital gas mixer. Endogenous and substrate-supported CO_2 production rates ($m/z = 44$) were calibrated against those of standard solutions of NaHCO_3 .

Amino acid analysis. Sulfosalicylic acid (10% [wt/vol]) was added to samples (1:1) for 1 h. Solutions were then centrifuged, and the supernatant was filtered (0.22- μm pore size). Amino acids were identified and quantified by ion-exchange chromatography with a Biochrom 20 amino acid analyzer (Pharmacia).

RESULTS

Identification of fermentation products by NMR. Proton-decoupled ^{13}C NMR spectra of *Hexamita* incubated under microaerobic conditions (23 to $34\ \mu\text{M}$ O_2) with $\text{D}-[1-^{13}\text{C}]\text{glucose}$ ($30\ \text{mM}$) revealed that the primary products were acetate, ethanol, lactate, and alanine (Fig. 1). HPLC analysis also con-

firmed acetate and lactate ($<0.1\ \text{mM}$) as products of glucose fermentation; however, in addition, propionate and *n*-butyrate (and *iso*-valerate, $<0.1\ \text{mM}$) were also detected. The rate of acetate production was greatest under microaerobic conditions (Fig. 2a) compared to that under anaerobic (Fig. 2b) or aerobic (Fig. 2c) conditions. Since propionate and butyrate were not detected as products from labelled glucose by ^{13}C NMR, it is suggested that these are the products of endogenous substrate fermentation.

The relative proportions of these organic acids were greatly influenced by the dissolved- O_2 tension. After 2 h, under microaerobic conditions, acetate accounted for 70% of the total organic acids produced. This value diminished under aerobic conditions to 55% and decreased further under anaerobic conditions to 20%. Butyrate and propionate (35 and 45%, respectively) accounted for the major portion of the organic acids produced during anoxia.

Influence of O_2 on the production of CO_2 with various substrates. CO_2 production by *Hexamita* cells was shown to be influenced by O_2 tension (Table 1). Pyruvate-, arginine-, and ethanol-supported CO_2 production rates were greatest under microaerobic conditions, and in all cases, a high O_2 tension inhibited CO_2 production by approximately one-third. The high rate of production of CO_2 from arginine suggests that this amino acid is rapidly catabolized.

Amino acid consumption by *Hexamita*. The amino acid composition of the culture media of *Hexamita* was analyzed before and after 6 days of growth, when stationary phase of growth was reached (Table 2). Confirmation of data from the ^{13}C NMR measurements was obtained; alanine was again shown to be generated. Noticeably, asparagine was taken up, with almost the same amount of aspartic acid produced. The production of CO_2 from arginine indicated that arginine may be used as a substrate. Its uptake from the medium with the concomitant

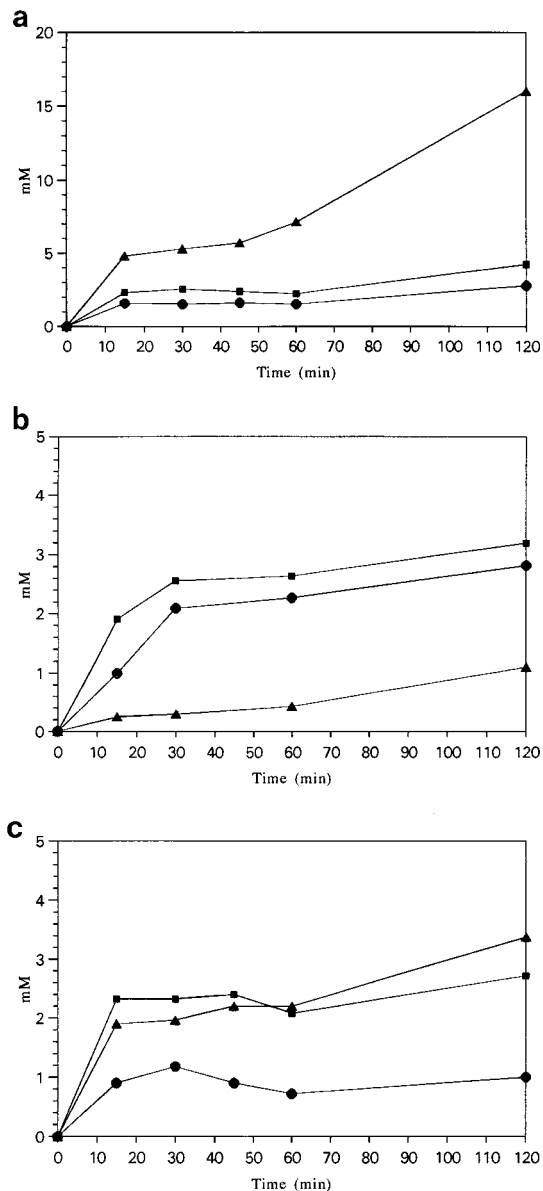


FIG. 2. Production of principle organic acids by washed cell suspension of *Hexamita* (1.95×10^5 cells ml^{-1}) at 25°C and pH 7.2 with added glucose (30 mM) under microaerobic ($15 \mu\text{M O}_2$) (a), anaerobic ($<0.25 \mu\text{M O}_2$) (b), and aerobic (200 to $253 \mu\text{M O}_2$) (c) conditions. Products were detected by HPLC. Symbols: ▲, acetate; ■, propionate; ●, butyrate. Values are the means of the results of two separate experiments differing by $<6\%$.

production of ornithine (and to a lesser extent citrulline) suggests the presence of the arginine dihydrolase pathway. Incubation of the *Hexamita* cell suspension with arginine resulted again in the uptake of arginine and the production of ornithine and citrulline (Table 3). The rate of arginine consumption was greater under anaerobic conditions than microaerobic conditions. The other amino acids were probably released from the intracellular amino acid pool. Alanine was again generated, its production being greatest under anoxia.

Growth of *Hexamita* in the absence of maltose. Maltose is the carbohydrate source in the growth medium of *Hexamita*. However, in the absence of maltose (ca. 1 mM glucose may be present in the serum), *Hexamita* cells were observed to grow,

TABLE 1. Influence of O_2 on CO_2 production by *Hexamita* with various substrates

Substrate ^a	Dissolved O_2 (μM)	CO_2 production ($\mu\text{mol min}^{-1} 10^6$ cells $^{-1}$) ^b	% Change in CO_2 production ^c
Pyruvate	75	1.07 ± 0.11	
	15	1.61 ± 0.08	34
Arginine	75	2.14 ± 0.13	
	15	3.22 ± 0.22	34
Ethanol	75	0.21 ± 0.02	
	15	0.34 ± 0.02	38

^a Substrates were used at a 30 mM concentration.

^b Mean \pm standard error from three experiments.

^c Compared to production at $75 \mu\text{M O}_2$.

albeit at a lower growth rate and with a lower yield (Fig. 3), showing that alternative sources of energy had been utilized for growth.

DISCUSSION

Hexamita is shown to possess a fermentative metabolism; the principle products of glucose fermentation are acetate, ethanol, lactic acid, alanine, and CO_2 . Other end metabolites include butyrate and propionate, which are not produced from glucose and therefore are products of an alternative substrate fermentation (amino acids and/or fatty acids).

Changes in O_2 concentration led to a marked alteration in the carbon balance of the metabolism. The production of organic acids and CO_2 was greatest under microaerobic conditions in comparison with that under aerobic and anaerobic conditions. The closely related diplomonad *Giardia lamblia* has

TABLE 2. Amino acid composition of *Hexamita* culture medium^a

Amino acid	Concn (mM) in medium		Concn change (mM) from day 0 to 6
	Day 0	Day 6	
Alanine	7.01	8.39	+1.38
Arginine	2.44	1.71	-0.73
Asparagine	1.5	0.24	-1.26
Aspartic acid	2.34	3.64	+1.3
Citrulline	0.42	0.58	+0.16
Cystine	0.37	0.37	NC ^b
Glutamic acid	5.55	5.33	-0.22
Glutamine	<0.01	<0.01	NC
Glycine	1.89	1.91	+0.02
Histidine	1.21	1.44	+0.23
Isoleucine	3.26	3.51	+0.25
Leucine	9.84	10.19	+0.35
Lysine	5.45	6.01	+0.56
Methionine	1.72	1.9	+0.18
Hydroxyproline	<0.01	<0.01	NC
Ornithine	0.72	1.75	+1.03
Phenylalanine	3.91	4.05	+0.14
Proline	1.51	1.99	+0.48
Serine	2.61	2.52	-0.09
Taurine	<0.01	0.19	+0.19
Threonine	2.68	2.75	+0.07
Tyrosine	1.16	1.33	+0.17
Valine	4.58	4.59	+0.01

^a Values are the means of results of two separate experiments differing by $<6\%$.

^b NC, no change.

TABLE 3. Amino acid analysis of supernatant following incubation of *Hexamita* with arginine (15 mM) under microaerobic and anaerobic conditions^a

Amino acid	Change in concn (mM) ^b after:	
	Microaerobic incubation	Anaerobic incubation
Alanine	+0.35	+0.67
Arginine	-5.81	-8.11
Asparagine	+0.03	NC ^c
Aspartic acid	-0.06	+0.07
Citrulline	+4.16	+8.40
Cystine	-0.01	+0.01
Glutamic acid	+0.22	-0.50
Glutamine	+0.03	NC
Glycine	+0.16	+0.07
Histidine	+0.03	+0.04
Isoleucine	+0.12	+0.08
Leucine	+0.05	+0.13
Lysine	+0.12	+0.10
Methionine	+0.01	+0.05
Hydroxyproline	NC	NC
Ornithine	+0.16	+0.24
Phenylalanine	+0.04	+0.04
Proline	-0.07	+0.07
Serine	+0.12	+0.08
Taurine	-0.01	-0.01
Threonine	+0.14	+0.07
Tyrosine	-0.02	+0.08
Valine	+0.20	+0.25

^a A total of 1.37×10^7 cells ml⁻¹ were incubated for 4 h.

^b Values are the means of results of two separate experiments differing by <6%.

^c NC, no change.

been studied extensively and has also been shown to have a fermentative metabolism (18, 21, 42), generating acetate, ethanol, and CO₂ but, unlike *Hexamita*, not lactate. Similarly, all the anaerobic protozoa studied to date, e.g., the parasitic flagellates *Trichomonas vaginalis* (5, 28) and *Tritrichomonas foetus* (36), the rumen ciliates *Dasytricha ruminantium* (13, 40) and *Isotricha* sp. (35), and the free-living ciliate *Trimyema compressum* (15, 17, 46), metabolize glucose into a variety of organic acids.

The production of alanine was shown to be higher under anaerobic than microaerobic conditions; this is consistent with the results found for *Giardia*, where alanine is also produced (11) and is produced at increased rates under anoxia (33). In *Giardia*, alanine is thought to function as a major osmoregulator (19), and it is possible that it plays a similar role in *Hexamita*.

It is becoming increasingly apparent that glucose or other carbohydrates are not the sole energy sources of anaerobic protozoa (26, 38). Consistent with this view, *Hexamita* was shown to rapidly consume arginine, with the simultaneous production of CO₂, ornithine, and citrulline. Together with previous observations demonstrating arginine-supported respiration (1), these data suggest that a functional arginine dihydrolase pathway like that found in *Giardia* (37) and *Trichomonas vaginalis* (22) is present in *Hexamita*. The species of *Hexamita* we used is free-living, and thus it seems appropriate that it has alternative energy-yielding routes which best suit its mode of living. Certainly, it was shown that in the absence of an added carbohydrate source (Fig. 3), *Hexamita* was still able to grow, albeit with a lower growth rate and yield. Arginine uptake was highest under anaerobic conditions, which

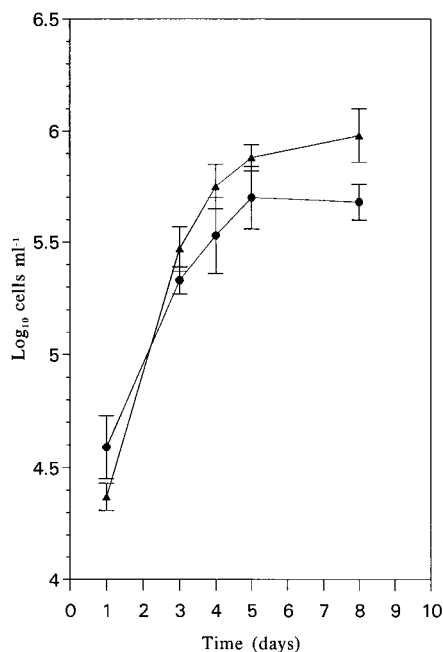


FIG. 3. Growth rate of *Hexamita* in culture medium with (▲) and without (●) added maltose (0.5% [wt/vol]). The experiment was performed in triplicate; error bars indicate \pm standard deviation.

suggests that this pathway may be linked to the redox state of the NAD(P)H pool.

During growth, *Hexamita* was shown to take up a relatively large amount of asparagine, with stoichiometric production of aspartate. In bacteria under conditions where amino acids are consumed as carbon sources, asparaginase activity resulting in the deamination of asparagine to aspartic acid is stimulated more than a hundredfold under anaerobic conditions (4). The results reported here suggest that *Hexamita* also possesses asparaginase activity operating as a means of deamination under anaerobiosis. The possible involvement of asparaginase has also been invoked to explain the asparagine uptake and aspartic acid production observed in species of the anaerobic parasite *Entamoeba* (47).

Hexamita has been found in the water column of marine basins at depths where the O₂ tension ranged from 0 to 30 μ M O₂ (14). We have shown that it is in this microaerobic realm that *Hexamita* optimally produces CO₂ and organic acids at the highest rates. Significantly, the rapid consumption of arginine suggests that carbohydrates are not its sole source of energy, a function which may be a result of its free-living mode.

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