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Oxygen Consumption by Ruminal Microorganisms: Protozoal and Bacterial Contributions

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The relative contributions to O_2 consumption made by the protozoal and bacterial populations present within the rumen were determined by using an open-type oxygen electrode system. Measurements indicated that two separate microbial populations contributed approximately equally to ruminal O_2 consumption over the O_2 concentration range experienced in situ (0.25 to 1.0 μM). The populations were observed to consume O_2 under liquid-phase O_2 concentrations of up to 7 μM, above which point rapid inactivation of O_2 utilization was observed. K_m values for the mixed population of bacteria and protozoa were 0.36 ± 0.17 and 3.2 ± 0.4 μM at concentrations of <1.6 and >1.6 μM, respectively. O_2 affinity values obtained for both the protozoal and bacterial populations were similar. O_2 affinities of the isolated entodiniomorphid ciliates *Polyplastron multivesiculatum* and *Eudiplodinium maggii* showed O_2 inhibition thresholds of 10 and 5, respectively, and apparent half-saturation constants (K_m values) of 1.7 and 5.2 μM O_2, respectively. Corresponding V_{max} values were 7.8 μM O_2 per min per 10^5 organisms for *P. multivesiculatum* and 3.6 μM O_2 per min per 10^5 organisms for *E. maggii*. Mass spectroscopic analysis detected average rates of H_2 production of 12.0 and 3.7 μM H_2 per min per 10^5 organisms for *P. multivesiculatum* and *E. maggii*, respectively. Trace levels of dissolved O_2 (<0.25 μM) stimulated the H_2 production rate of *E. maggii* eightfold but inhibited that of *P. multivesiculatum* by 18%.

Although the rumen has been commonly regarded as an anaerobic environment (14, 15), direct in situ measurement of dissolved ruminal gases in fistulated sheep has shown that dissolved O_2 is present in ruminal fluid at concentrations as high as 3 μM. These O_2 levels were undetectable (<0.25 μM) 25 min after feeding (11, 23). Various routes for O_2 influx into the rumen have been proposed by Czerkawski (6), who calculated that 10 to 20 liters/day could enter from capillaries through the mucosal lining; lesser amounts originate from saliva and drinking water and during rumination.

Strictly anaerobic species, e.g., methanogens, survive in the rumen under O_2 tensions previously found to be inhibitory to these organisms (23). Therefore, the ruminal microbial population must be able to rapidly utilize O_2 and remove it from the environment of highly O_2-sensitive organisms (20). The ciliate protozoa account for approximately half of the total ruminal microbial biomass and occupy 10% of the total rumen volume (1). In common with other anaerobic protozoa such as the trichomonads (16), certain ciliate species (27–29) have been shown to possess hydrogenosomes, which have been implicated in O_2 utilization (2, 21). The holotrich population alone has been estimated to utilize 7% of ruminal O_2 (25). Mass spectroscopic analyses of the in situ dissolved gases in ruminal fluids of faunated and defaunated (ciliate-free) sheep suggest that the protozoa may contribute to the control of ruminal O_2 fluxes (11).

This study used an oxygen electrode in a stirred reaction vessel open for gas exchange to investigate the ability of the whole microbial population and the separated protozoal and bacterial populations to utilize O_2. The contributions of the subpopulations of this complex microbial community were assessed.

Previous work (11, 30) reports that high affinities for O_2 are characteristic of the ruminal holotrich protozoa *Dasytricha ruminantium* and *Isotricha* spp.; however, no equivalent studies have been undertaken with entodiniomorphid species. However, *Eudiplodinium maggii* (27) possesses pyruvate synthase and hydrogenase enzyme activities associated with a fraction sedimentable at $10^5 \times g$ per min, which is characteristic of hydrogenosome-containing species. Mass spectrometry has proved to be a valuable tool in the determination of in vitro dissolved-H_2 production rates in holotrich ciliates (11) and the hydrogenosome-containing ruminal fungus *Neocallimastix patriciarum* (32). In this study, we used mass spectrometry to demonstrate conclusively that H_2 is produced by *E. maggii* and *Polyplastron multivesiculatum*, both of which are ruminal entodiniomorphid ciliates, and we report the effects of O_2 on H_2 formation.

MATERIALS AND METHODS

Preparation of rumen samples for the mixed-population study. Rumen contents were obtained from fistulated cattle fed a daily ration of ryegrass hay (4.1 kg) and concentrates (2.8 kg) (Dalgety Agriculture Ltd., Bristol, England) in two equal portions. Water was given ad lib. Each sample was withdrawn before the morning ration was offered and was transported to the laboratory in a sealed, insulated container maintained at 39°C in a portable incubator.

The sample was strained through a double layer of surgical gauze to remove the large particulate material and was diluted twofold with deoxygenated Simplex-type buffer (buffer S) (5), which was prepared by using distilled H_2O that had been boiled and cooled by N_2 gassing. Anaerobic conditions were maintained throughout. Glucose was then added to give a concentration of 5 mg of glucose per ml, and the sample was transferred to a separating funnel at 39°C and allowed to stand for 30 to 60 min, during which time a scum layer formed and was removed. The protozoal fraction was obtained by centrifuging 10-ml samples of diluted ruminal contents at $68 \times g$ for 10 min. The pellet was washed and recentrifuged several times at $68 \times g$ for 30 s to remove

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bacterial contamination. The washed pellet was suspended in 10 ml of buffer S at 39°C under N_2. The bacterial fraction was obtained by centrifuging the supernatant from the 68 × *g* centrifugation at 17,000 × *g* for 30 min. The resultant pellet was suspended under N_2 in 10 ml of buffer S at 39°C.

Preparation of defined protozoal suspensions. Two fistulated sheep were used as sources for the isolation of defined protozoal suspensions. One contained a coculture of *E. maggii* and *Entodinium* spp., and the other contained a coculture of *P. multivesiculatum* and *Entodinium simplex*. These defined populations had been established in the ruminal contents of defaunated animals. Samples (500 ml) were withdrawn 1 h after feeding and transported to the laboratory in a portable incubator maintained at 39°C in sealed N_2-gassed, insulated containers. Prior to transportation, each rumen sample was strained and diluted twofold in buffer D (5) by using the method previously described. The sample never fell below 37°C, and the pH remained between 6.7 and 7.0. Rumen contents were strained and diluted twofold and placed into N_2-gassed conical flasks. Whole-meal flour (5 mg/ml) was added, and the sample was incubated in a water bath at 39°C until contaminating plant particles formed a scum layer and could be removed. The protozoa were collected on defined-aperture textiles (45-μm pore size) following prefiltration through a filter cloth (100-μm pore size). The protozoa were thoroughly washed with buffer D on the 45-μm-pore-size filter to remove bacteria. Anaerobiosis was maintained during the isolation procedures by thorough gassing with N_2. Buffer D was rendered O_2 free as described for buffer S. The washed protozoal suspensions were sedimented by centrifugation (500 × *g*) and suspended in buffer D. Contamination of the preparation by other species of protozoa was negligible (<1%) (26). *E. maggii* cells were suspended in 200 ml of buffer S at 39°C under a CO_2 atmosphere. *P. multivesiculatum* cells were maintained similarly except that Hungate-type buffer was used under an inert N_2 atmosphere as recommended by Coleman (5). When required, the cells were collected by centrifugation at 500 × *g* and were suspended in 10 ml of buffer D at 39°C.

Oxygen consumption measurements. The O_2 utilization rate of ruminal organisms at 39°C was measured with a 25-μm-thick Teflon membrane-covered oxygen electrode (Radiometer A/S, Copenhagen, Denmark) by using the open electrode system described by Degn et al. (10). Each sample was stirred with a cross-shaped stirrer fixed to a stainless steel shaft entering through a hole in the lid and driven by a synchronous motor at 790 rpm. The incubation vessel volume was 5.5 ml. Oxygen uptake rates in the open electrode system were calculated as described by Lloyd and Scott (18). The solubility of O_2 in air at 39°C was assumed to be 215 μM (24). The composition of the gas mixture (O_2 in N_2) passing over the surface of the cell suspension was controlled by using a digital gas mixer (9). K_m and V_{max} values were determined from double-reciprocal plots of O_2 uptake versus O_2 concentration in the liquid phase. Protozoa were counted by using a standard counting chamber (Weber Scientific International Ltd., Sussex, England).

Mass spectrometry. The quadrupole mass spectrometer (Dataquad; Spectrum Scientific, Congleton, Cheshire, United Kingdom) was fitted with a silicone membrane-covered inlet probe (17). This system was used to measure H_2 and O_2 concentrations simultaneously and continuously. A turbomolecular pump (Pfeiffer, Asstar, Federal Republic of Germany) and a rotary backing-pump were used to establish a strong vacuum. The incubation vessel, which was stainless steel with a 5-ml working volume, was stirred at 890 rpm and maintained at 39°C. Calibration was carried out by using gas mixtures of known H_2 and O_2 concentrations in buffer D at 39°C and pH 7.0. Dissolved O_2 was detected at a mass/charge ratio (*m/z*) of 32, and dissolved H_2 was detected at an *m/z* of 2. V_r or V_{H_2} (velocity of O_2 uptake or H_2 production) was calculated for each addition from $k(T_L - T_G)$, where k is a constant $0.693/t_{1/2}$ ($t_{1/2}$ is the half time for O_2 or H_2 equilibration in the buffer in the absence of organisms at 39°C), T_L is the O_2 (H_2) tension in the liquid phase, and T_G is the O_2 (H_2) tension in the gas phase (17). The solubilities of H_2 and O_2 in saturated buffer at 39°C were 740 and 215 μM, respectively (24).

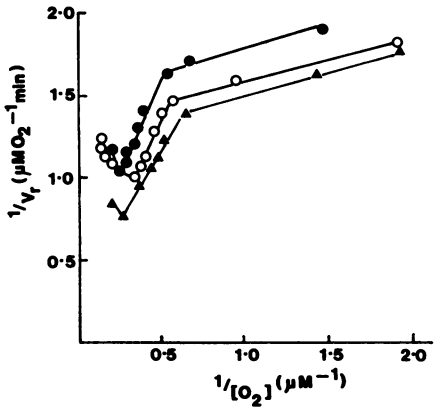

FIG. 1. Effects of increasing O_2 concentration on the O_2 consumption rates of mixed ruminal microorganisms (3.4×10^3 protozoa per ml) (●), protozoal population (4.3×10^2 protozoa per ml) (○), and bacterial population (▲). Two linear portions were obtained for each population type, and extrapolations show two apparent half-saturation constants (K_m values) for each respective component up to the O_2 inhibition thresholds. V_r, Velocity of O_2 uptake.

RESULTS

Oxygen utilization by microbial populations in the rumen. The effects of O_2 on the respiratory activities of mixed ruminal organisms, protozoal and bacterial, were investigated by increasing the concentration of dissolved O_2 from 0 to 51 μM in 5% steps, each lasting 15 min. Inhibition of O_2 utilization occurred with all three population types when the levels of dissolved O_2 were greater than physiological levels in the rumen (<0.25 to 3 μM). Although there was some intersample variation in threshold levels, the results from six experiments confirmed that O_2 concentrations of >7 μM led to a total inhibition of O_2 consumption for each population type.

Double-reciprocal plots of O_2 utilization rates versus dissolved-O_2 tensions (Fig. 1) showed that the three popu-

TABLE 1. Apparent K_m values for O_2 consumption by ruminal microorganisms

Ruminal population	K_m values at[a]:	
	<1.6 μM O_2	>1.6 μM O_2
Mixed organisms	0.36 ± 0.17	3.2 ± 0.4
Protozoa	0.31 ± 0.2	5.0 ± 2.1
Bacteria	0.45 ± 0.17	3.1 ± 2.1

[a] Means ± standard deviations obtained from six values.

TABLE 2. Contributions to ruminal O_2 consumption made by protozoa and bacteria[a]

Ruminal population	Total respiratory activity (μM O_2/min per ml)[b]	O_2 recovered (%)
Mixed organisms	4.4	100
Protozoa	3.24	73.6
Bacteria	3.43	77.9

[a] Measurements taken at 1 μM O_2
[b] Values were recalculated from the diluted samples in order to represent an undiluted ruminal population.

lations exhibited similar features. O_2 consumption rates measured at O_2 tensions of <1.6 μM had apparent *Km* values 1 order of magnitude lower than those attained at O_2 tensions of >1.6 μM (Table 1).

The protozoal and bacterial populations had equivalent rates of O_2 utilization both at the lower detectable level of O_2 within the rumen (0.25 μM) and at that concentration range attained immediately prior to feeding (1 to 1.5 μM) (Table 2). Separated suspended bacterial and protozoal components had slightly increased O_2 consumption rates at every O_2 tension tested when compared with the original mixed population.

Oxygen uptake by *P. multivesiculatum* and *E. maggii*. *P. multivesiculatum* and *E. maggii* consumed O_2 at linear rates up to the inhibition threshold for each respective species (Fig. 2). Exposure to O_2 concentrations in excess of those tensions resulted in rapid inactivation of the observed O_2 consumption (Table 3).

Oxygen consumption and protozoal hydrogen production. Endogenous H_2 production by both protozoal species was detected by using in vitro mass spectroscopic analysis of dissolved gases. An average value obtained from measurements of three separate preparations is presented (Table 3). The addition of glucose stimulated H_2 production in *P. multivesiculatum* at concentrations of 5 and 10 mM by 6 and 10%, respectively. No effect on *E. maggii* H_2 production was observed when glucose was added. However, exposure to 5.2 kPa of O_2 (Fig. 3) resulted in a rate of H_2 production by *E. maggii* eight times higher than that previously detected. Similar exposure of *P. multivesiculatum* to O_2 decreased the evolution of H_2 by 18%. In both cases, the levels of dissolved O_2 never increased above the limit of detectability for the mass spectrometer (0.25 μM). Switching back

TABLE 3. Effects of O_2 concentration on ruminal O_2 consumption and H_2 production rates at 39°C by the entodiniomorphid ciliates *P. multivesiculatum* and *E. maggii*[a]

Ruminal organism	O_2 inhibition threshold (μM O_2)	Apparent K_m for O_2 (μM O_2)	μM/min per 10^5 organisms	
			V_{max} for O_2	H_2 production rate
P. multivesiculatum	10	1.7 ± 0.8	7.8	12.0
E. maggii	5	5.2 ± 0.7	3.6	3.7

[a] Values are from three measurements.

to an N_2 atmosphere resulted in endogenous H_2 production returning to its former rate in *E. maggii* but increasing by 18% in *P. multivesiculatum*.

DISCUSSION

The population of ruminal microorganisms clearly comprises a number of species which are somewhat tolerant to low O_2 concentrations and are able to utilize O_2 at low tensions. They show diminished O_2 consumption at levels above normal physiological levels in the rumen. Thus, aerotolerant anaerobes exist within both the bacterial and protozoal populations. Previous work (8, 23) has shown that methane production rates are dependent on dissolved O_2 tensions in ruminal fluid; very low concentrations of O_2 (<30 nM) are inhibitory (22), and this reflects the high sensitivity of the methanogenic bacteria to O_2. In the presence of protozoa, these organisms tolerate higher O_2 concentrations (13). The aerotolerant species remove O_2 from the environment of more sensitive microorganisms and function in a protective capacity (19). The question of the extent of O_2 utilization by facultatively anaerobic bacteria in the rumen has not been specifically addressed. The hydrogenosome-containing ruminal ciliates possess a high affinity for O_2. K_m values for *D. ruminantium* and *Isotricha* spp. at 39°C were determined to be 0.34 ± 0.2 and 2.5 ± 0.5 μM, respectively (11). The values obtained for the mixed protozoal population and the isolates of *P. multivesiculatum* and *E. maggii* are of the same order as those for *D. ruminantium* and *Isotricha*

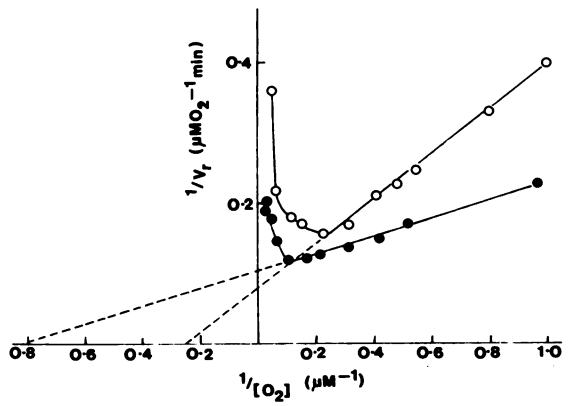

FIG. 2. Double-reciprocal plot of the O_2 dependence on O_2 consumption of *P. multivesiculatum* (1.3×10^4 cells per ml) (●) and *E. maggii* (2.1×10^4 cells per ml) (○).

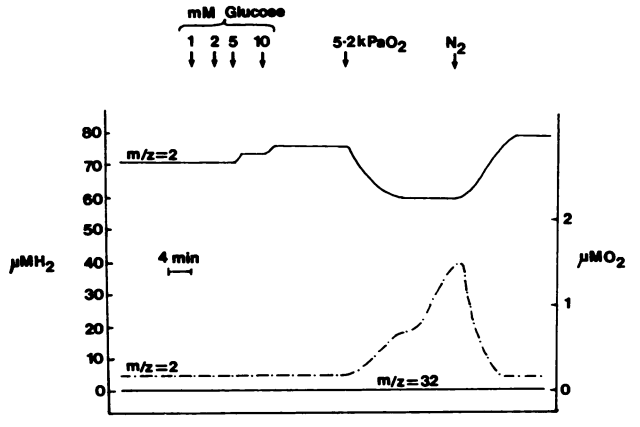

FIG. 3. Effects of glucose addition and an increase in gaseous O_2 concentration on H_2 production by *P. multivesiculatum* (3.8×10^4 cells per ml) (——) and *E. maggii* (2.6×10^5 cells per ml) (– · – · –) over time. Dissolved O_2, *m/z* = 32; dissolved H_2, *m/z* = 2.

spp. and can be compared directly, indicating the potential of the ruminal ciliates for scavenging O_2 over the entire range of O_2 levels encountered in situ. The contributions that bacterial and protozoal populations make to the overall O_2 utilization ability of the ruminal microbiota were found to be approximately equal at physiological concentrations.

Double-reciprocal plots of O_2 uptake versus dissolved O_2 in mixed populations indicate the presence of diverse subpopulations of not only protozoa but also bacteria with different O_2 affinities. Defaunation studies do, however, reveal transient O_2 fluctuations in sheep lacking protozoa which do not exist in the normal faunated rumen (12). It is therefore proposed that ruminal ciliates significantly contribute to the control of ruminal O_2 fluxes in situ and therefore aid in the stabilization of ruminal metabolism. The present study ignores ruminal phycomycetes, which contribute less than 8% of the total ruminal biomass (3, 7).

P. multivesiculatum and *E. maggii* form hydrogen. This characteristic is shared by the other hydrogenosome-containing ruminal organisms *D. ruminantium* and *Isotricha* spp. (11) and the anaerobic ruminal fungus *N. patriciarum* (32). These eucaryotes have adapted to the anaerobic environment and lack mitochondria but possess H_2-evolving organelles (27–29, 31). The kind of inhibition of H_2 production on exposure to O_2 found in *P. multivesiculatum* has previously been observed in *D. ruminantium*, *Isotricha* spp., and *N. patriciarum* (11, 32, 33). This is presumed to be due to the inactivation of the H_2-evolving system either directly by O_2 or possibly by a product of univalent O_2 reduction, i.e., singlet O_2, hydroxyl radical, superoxide, or peroxide. However, the exposure of *E. maggii* to undetectably low O_2 concentrations (<0.25 μM) resulted in the stimulation of H_2 production. This effect has been previously reported in the cattle parasite *Tritrichomonas foetus* (17) but is the opposite of that observed in other H_2-producing eucaryotes. Stimulation of H_2 production by the addition of glucose, as observed in *P. multivesiculatum*, did not occur in *E. maggii*. This supports the proposal of Coleman (4) that *E. maggii* possesses a mechanism for maintaining a constant intracellular glucose concentration and hence the rate at which it is metabolized.

Further work is necessary to determine the specific contributions of the entodiniomorphid ciliates which, like the holotrichs, play a key role in maintaining the rumen under conditions of near anaerobiosis.

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