Respiration of the rumen ciliate *Dasytricha ruminantium* Schuberg

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The endogenous respiration of the rumen ciliate *Dasytricha ruminantium* maintained under an O₂ tension of 2 kPa (~0.02 atm) was partially inhibited by KCN (40% inhibition) and NaN₃ (58% inhibition). The organisms lack cytochromes, and sensitivity of respiration to KCN, NaN₃, chloroquine and quercetin suggest that the operation of flavoprotein–iron–sulphur-mediated electron transport. As in *Tritrichomonas foetus*, hydrogenosomal respiration can be stimulated by the addition of CoA in the presence of 0.025% Triton X-100; stimulation by ADP was not detected. Stimulation of pyruvate-supported O₂ uptake by P_i suggests that acetate is produced via acetyl phosphate.

In the holotrich ciliate *Dasytricha ruminantium* the enzymes transforming pyruvate to acetate and hydrogen are located within a special organelle (Yarlett *et al.*, 1981). This organelle, the 'hydrogenosome', was first described in the aerotolerant anaerobe *Tritrichomonas foetus* (Lindmark & Müller, 1973) and has subsequently been implicated in O₂ utilization (Čerkasov *et al.*, 1978; Müller & Lindmark, 1978). Recent investigations have shown that the rumen protozoon *D. ruminantium* is able to respire under low O₂ tensions, where it exhibits a relatively high apparent K_m for O₂ (Lloyd *et al.*, 1982a).

The present paper examines the survival of the organism under different O₂ tensions and the effects of various inhibitors on endogenous respiration. In addition, the respiratory involvement of the hydrogenosomes suggests a mechanism for the formation of acetate from acetyl-CoA.

Methods and materials

Isolation of the organism

D. ruminantium was isolated from rumen contents by filtration as described by Williams & Yarlett (1982). Na₂S was omitted from the isolation buffer because of potential interference in the oxygen-electrode system.

Disruption technique

The washed cell pellet was disrupted by a motor-driven homogenizer and the large-granule fraction obtained by differential centrifugation as described previously (Yarlett *et al.*, 1981).

Electrode techniques

The respiration of intact cells of *D. ruminantium* was measured at 39°C by using the open oxygen-electrode system described by Degn *et al.* (1980) and Lloyd *et al.* (1981). A digital gas mixer was used to obtain various mixtures of O₂ in N₂. Effects of respiratory inhibitors were determined on whole cells maintained under a pO₂ of 2 kPa (0.02 atm) at 39°C. The inhibitors used, KCN, NaN₃, quercetin [2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one], quinacrine dihydrochloride, chloroquine hydrochloride, metronidazole and antimycin A were obtained from Sigma, Poole, Dorset, U.K.; 4-chloro-7-nitrobenzofurazan was from Aldrich Chemical Co., Wembley, Middx., U.K.; and 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone was from Dr. W. Draber, Bayer AG, BRD 56 Wuppertal, Germany. Additions were made through the gas exit port by using a micropipette. Incubations with metronidazole {10–100 µg/ml, in Hepes [4-(2-hydroxymethyl)-1-piperazine-ethanesulphonic acid] buffer (100 mM, pH 7.2)}, were conducted under an atmosphere of N₂ in 5 ml bottles fitted with Suba-Seals. Cells were introduced with a syringe and incubated at 39°C for various periods of time (1–6.75 h). The respiration of the treated cells was compared polarographically with that in cells that had been subjected to identical preincubation conditions without metronidazole. Cells were counted in a standard counting chamber (Hawkesley, Lancing, Sussex, U.K.).

Respiration of large granule fractions was measured under a gas phase of O₂ (1 kPa, ~0.01 atm);

again all additions were made through the gas outlet port. The concentration of dissolved O_2 at $39^\circ C$ was taken to be $250 \mu M$.

Enzyme assays

Acetate kinase (EC 2.7.2.1), butyrate kinase (EC 2.7.2.7) and phosphate acetyltransferase (EC

Fig. 1. Respiration of *D. ruminantium* in an open oxygen-electrode system

Gas mixtures were produced with a digital gas mixer. Cells (14×10^5) were suspended in a 100 mM-Hepes/50 mM-phosphate buffer, pH 7.4, at $40^\circ C$ in a total volume of 5 ml. pO_2 : (a) 21 kPa; (b) 10 kPa; (c) 6 kPa; (d) 2 kPa T_L is the O_2 tension in the liquid.

2.3.1.8) were assayed as described by Miller & Jenesel (1979). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Difference spectra

Difference spectra of whole cells stored anaerobically at 77 K were measured at liquid-N₂ temperatures in a cell of path length 2 mm, with a Pye-Unicam SP. 1800 spectrophotometer.

Results

Respiration and survival of *D. ruminantium* at different O₂ tensions

The effects of O₂ on *D. ruminantium* were investigated by determining respiration during incubation under various O₂ tensions (1–21 kPa of O₂) for periods up to 2 h. Figs. 1(a)–1(d) show that at O₂

tensions above 2 kPa the respiration decreased rapidly to an undetectable rate; when maintained at lower O₂ concentrations the respiration was stable for several hours, and at 1 kPa for periods in excess of 12 h (results not shown).

Effects of respiratory inhibitors

The effects of various respiratory inhibitors on the endogenous respiration of whole cells maintained under a gas phase of 2 kPa of O₂ are shown in Table 1. Organisms maintained at this O₂ tension in the absence of inhibitors show rapid motility and a non-vacuolate appearance. After incubation with KCN or NaN₃ the organisms became slowly motile and vacuolate; antimycin A had no cytologically detectable effect. Incubation with chloroquine, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone, quercetin, 4-chloro-7-nitrobenzofurazan or metronidazole gave cells that were non-motile and non-

Table 1. *Effects of various respiratory inhibitors on whole cells of D. ruminantium*
I_{max.} is the maximum inhibition achieved on addition of excess inhibitor. Abbreviation used: ND, not determined.

Inhibitor	Concentration [(mm) or μmol/mg of protein]	Activity (%)	I _{max.} (%)
KCN	(3.6)	70	40
NaN ₃	(1.0)	67	58
Antimycin A	0.002	100	0
Chloroquine	0.15	77	36.5
2,5-Dibromo-3-methyl-6- isopropyl- <i>p</i> -benzoquinone	0.023	34.4	100
Quercetin	0.13	90	ND
4-Chloro-7-nitrobenzofurazan	0.009	48	100
Metronidazole	0.04	75	ND
Quinacrine	0.56	29.7	ND

Fig. 2. *Effects of various concentrations of CoA on O₂ uptake by the large-granule fraction (LGF) of D. ruminantium*
The vessel contained 1.3 mg of protein suspended in buffered sucrose (total vol. 5.0 ml) at a temperature of 39°C. The gas atmosphere consisted of 1 kPa of O₂ in N₂.

Table 2. *Respiratory activity of the large-granule fraction from D. ruminantium*

Values given are as nmol of O_2 /min per mg of protein, calculated by using the formula $K(T_G - T_L)$, where $K = 0.693/t_i$ and T_G and T_L are the O_2 tensions in the gas and liquid phases respectively. All measurements were made at $39^\circ C$ under an atmosphere of 1 kPa of O_2 in N_2 . The average activity for endogenous respiration of the large-granule fraction was 1.18 ± 0.67 (s.d., five expts.) nmol of O_2 /min per mg of protein. Abbreviation used: NT, not tested.

	Respiratory activity (nmol/min per mg of protein)		
	Endogenous	+Substrate	+Substrate + Triton X-100
CoA (30 μM)	1.15	1.61	7.12
Succinyl-CoA (30 μM)	0.96	1.40	3.58
Acetyl-CoA (30 μM)	0.94	1.17	1.87
NADH (50 μM)	0.96	0.96	4.78
Pyruvate (6 mM)	2.36	3.87	NT
+ P_i (4 μM)	2.36	10.10	NT
+ P_i + ADP (30 μM)	2.36	11.17	11.17
+ CoA (15 μM)	0.51	0.51	13.17
+ Succinate + CoA (30 μM)	0.94	2.81	NT
NADPH (30 μM)	0.94	0.94	2.11
NADH (50 μM)	0.96	0.96	4.40

Fig. 3. *Effects of CoA and CoA derivatives on O_2 uptake by large-granule fraction (LGF) of D. ruminantium*. The vessel contained 1.57 mg of protein suspended in buffered sucrose of total volume 5.0 ml. The gas phase consisted of 1 kPa of O_2 . (a) CoA, 30 μM ; Triton X-100, 0.025%; pyruvate, 6 mM. (b) succinyl-CoA, 30 μM ; Triton X-100, 0.025%; acetyl-CoA, 30 μM . (c) acetyl-CoA, 30 μM , Triton X-100, 0.025%, succinate, 1 mM.

vacuolate, and with quinacrine the cells appeared non-motile even at concentrations at which the respiration was apparently unaffected.

Respiration of the large-granule fraction

O_2 uptake by the large-granule fraction was investigated under a gas phase of 1 kPa of O_2 in N_2 in the open-electrode system. In the absence of exogenous substrate the organelles in this fraction showed only a small endogenous respiration, even after treatment with 0.025% Triton X-100 (Fig. 2a and Table 2) or three freeze-thawing cycles. Some variation in activities in different preparations are likely to be due to inadvertent exposure to O_2 during processing. Addition of pyruvate alone to intact or Triton-disrupted particles gave little stimulation of respiration, but CoA produced a marked effect (Fig. 2a), even in the absence of substrate (Fig. 3a). A transient decrease in the steady-state O_2 level indicated a 24-fold stimulation with 15 μM -CoA. Successive equal additions of CoA showed steadily decreasing effects both in terms of the maximal stimulation of respiration rates and total O_2 consumption (integrated areas) (Figs. 2b). This progressive loss of stimulation by CoA was not prevented by catalase [60 EC ($\mu mol/min$) units] or superoxide dismutase (60 units). After disruption with Triton X-100, succinyl-CoA stimulated endogenous respiration to a lesser extent than CoA (Fig. 3b); acetyl-CoA gave a smaller effect (Fig. 3c) and glutathione (result not shown) had no effect.

The respiration of pyruvate by the large-granule fraction was further stimulated by potassium phosphate; addition of ADP in the presence or absence of phosphate caused only slight stimulation (Fig. 4).

Fig. 4. O_2 uptake by large-granule fraction (LGF) of *D. ruminantium*

The vessel contained 2.10 mg of protein suspended in buffered sucrose (total vol. 5.0 ml). The gas phase consisted of 1 kPa of O_2 in N_2 . Compounds were added to give the following final concentrations: pyruvate, 6 mM; phosphate, 4 mM; ADP, 60 μ M; isocitrate, 1 mM; ADP, 30 μ M; AMP, 30 μ M; succinate 1 mM; Triton X-100, 0.025%.

Table 3. Activities of enzymes in cell-free extracts of *D. ruminantium*

Specific activities are expressed as enzyme units (e.u.) (μ g of acylhydroxamate formed/min per mg of protein) $\pm$ s.d. for three experiments. Fraction P_2 sediments at 10^5 g-min, S is the non-sedimentable fraction.

Enzyme	pH	Specific activity (e.u.):		
		In whole homogenate	In fractions	
			P_2	S
Acetate kinase	7.4	1.1 ± 0.05	1.7 ± 0.11	0.63 ± 0.02
Butyrate kinase	7.4	1.1 ± 0.07	1.8 ± 0.12	0.90 ± 0.05
Phosphate acetyltransferase	8.0	4.7 ± 0.50	6.8 ± 0.51	7.50 ± 0.67

O₂ uptake by the non-sedimentable fraction

In the absence of added substrate the non-sedimentable fraction respired slowly (0.04 nmol of O_2 /min per mg of protein); however, addition of NADH (30 μ M) or NADPH (30 μ M) increased O_2 consumption to 1.61 and 2.47 nmol of O_2 /min per mg of protein respectively.

Cytochrome spectra

Difference spectra (dithionite-reduced minus aerated-oxidized) of whole cells of *D. ruminantium* are featureless over the range 430–610 nm, indicating the absence of any detectable cytochromes (limit of detectability <2 pmol of cytochrome c/mg of protein). Oxidation of the reference cuvette contents with $K_3[Fe(CN)_6]$ also failed to reveal cytochromes.

Enzyme distribution

The enzymes converting acetyl-CoA to acetate in

D. ruminantium were shown to be acetate kinase and phosphate acetyltransferase. Specific activities of these enzymes, together with that of butyrate kinase, are shown in Table 3. Differential centrifugation of cell-free extracts revealed that significant activities of the enzymes were associated with the fraction sedimentable at 10^5 g-min and termed the 'large-granule fraction' (Fig. 5); this fraction has previously been shown to contain the organelles converting pyruvate to acetyl-CoA (Yarlett *et al.*, 1981). The activities of phosphate acetyltransferase and pyruvate synthase (a hydrogenosomal marker enzyme) have a mean equilibrium density of 1.19 g/ml in sucrose solutions (Fig. 6). Acetate kinase associated with the large-granule fraction, when centrifuged through a sucrose gradient, showed a heterogeneous distribution, with 45% remaining in the sample zone. This behaviour is reminiscent of that of lactate dehydrogenase, which has previously been shown to be only loosely associated with the hydrogenosome-enriched fraction.

Fig. 5. Distribution of enzymes in fractions obtained by differential centrifugation of a cell-free extract of *D. ruminantium*.

Relative specific activities (the ratios of specific activities in fractions of those in the cell-free extract) were plotted against cumulative percentage of protein recovered in each fraction. The centrifugal field increases from left to right. The far-right-hand bar represents the fraction containing particles non-sedimentable at 6×10^6 g-min. Percentage recoveries (based on enzyme units in the cell-free extract) were: acetate kinase, 49.4%; butyrate kinase, 53.7%; phosphate acetyltransferase, 102.7%, protein, 102%. Material from the shaded area was subjected to equilibrium density centrifugation (Fig. 6).

Fig. 6. Distribution of pyruvate synthase, acetate kinase, phosphate acetyl transferase and protein after equilibrium density-gradient centrifugation in a sucrose gradient of the 10^5 g-min fraction.

The direction of sedimentability was from left to right. The specific activities of acetate kinase and phosphate acetyltransferase (μ g of acylhydroxamate formed/min per mg of protein) and percentage recovery (in parentheses) were 0.76 (59%) and 38 (82%) respectively. Pyruvate synthase specific activity was 2μ mol/min per mg of protein (77%). Recovery of protein was 104%. Abbreviation used: e.u., enzyme units (see the text).

Discussion

No organelles recognizable as mitochondria occur in electron micrographs of thin sections of *D. ruminantium* (Yarlett *et al.*, 1981); furthermore, cytochromes are not detectable (limits of detectability $<2\text{ pmol/mg}$ of protein). Maintenance of the organism under $p\text{O}_2$ values in the range 1–2 kPa clearly indicates that this protozoon remains viable for long periods under low O_2 tensions. The O_2 tension of the rumen gas phase as measured by McArthur & Miltimore (1961) by g.l.c. was 0.5 kPa (0.6%); the O_2 concentration of rumen fluid has been measured at $0.37\text{--}0.45\text{ }\mu\text{M}$ ($31.5\text{--}38.3\text{ Pa}$) (N. Yarlett, D. Lloyd & A. G. Williams, unpublished work). Respiration may thus be of great importance to the organism *in situ*. Abe *et al.* (1981) have shown that the holotrich protozoa migrate to the rumen-reticulum wall about 2 h after feeding of the host, suggesting that the organism may encounter large variations in O_2 concentrations. Results with inhibitors on whole cells of *D. ruminantium* showed that respiration was unaffected by the mitochondrial inhibitor antimycin A, and was only sensitive to high concentrations of NaN_3 and KCN. NaN_3 has been shown to inhibit the respiration of *T. foetus*, where it is known to act both on electron transport in hydrogenosomes and cytosolic NADH oxidase (Lloyd *et al.*, 1982b); the latter enzyme system of *D. ruminantium* was 75% inhibited by 0.8 mM-KCN. Of the other compounds tested 4-chloro-7-nitrobenzofurazan and 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone were the most active inhibitors of O_2 consumption, chloroquine gave more than 25% and quercetin up to 20% inhibition. 4-Chloro-7-nitrobenzofurazan and quercetin are potent mitochondrial ATPase inhibitors (Lloyd & Edwards, 1977), and have been shown to be active inhibitors of the ATPase from *T. foetus* (Lloyd *et al.*, 1979), but their sites of action as respiratory inhibitors remain to be determined.

The action of metronidazole on whole cells was greatly enhanced by preincubation in an anaerobic buffer containing the inhibitor. O_2 uptake was not affected after anaerobic incubation for 6.75 h at 39°C , in which the concentration of metronidazole was less than $3\text{ }\mu\text{g/mg}$ of protein, whereas $6.8\text{ }\mu\text{g}$ of metronidazole/mg of protein for 2.5 h resulted in 25% inhibition of respiration. This suggests that a threshold concentration was necessary; similar observations have been reported for *T. foetus* (Müller & Lindmark, 1976). Sensitivity to chloroquine and other quinoline derivatives would indicate the presence of flavoprotein-mediated electron transport.

The data presented confirm that whole cells of *D. ruminantium* are able to utilize O_2 as terminal acceptor. The nature of the terminal oxidase is not known, but, as reported here, cytochromes are not involved. The respiration was not stimulated by intermediates of the tricarboxylic acid cycle, confirming that this cycle is not involved (Yarlett *et al.*, 1981). As in *T. foetus*, respiration by disrupted organelles was highest in the presence of CoA in combination with pyruvate (Müller & Lindmark, 1978), suggesting that O_2 uptake accompanied the conversion of pyruvate into acetyl-CoA. Various substrates were tested as potential mediators in the release of bound CoA, leading to stimulated respiration; oxalate, glyoxylate, formate, succinate and FAD were ineffective. Respiratory control by ADP shown in *T. foetus* by Čerkasov *et al.*, (1978) could not be demonstrated in hydrogenosomes from *D. ruminantium*. Respiration of pyruvate was, however, stimulated by phosphate. The association of phosphate acetyltransferase and acetate kinase with the organelle explains this stimulation (Scheme 1). This Scheme resembles that for O_2 consumption in *T. foetus* (Müller & Lindmark, 1978); however, the mechanism for regeneration of free CoA in the rumen ciliate does not necessitate the succinate thiokinase system observed in the trichomonad.

Scheme 1. Metabolic reactions in *D. ruminantium* hydrogenosomes

The scheme is based on the data reported by Yarlett *et al.* (1981) and that of present work. Key to enzymes: 1, lactate dehydrogenase; 2, malate dehydrogenase (decarboxylating); 3, pyruvate synthase; 4, hydrogenase; 5, phosphate acetyltransferase; 6, acetate kinase. X is the uncharacterized low-oxidation-reduction-potential electron-transporting component.

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