OCCURRENCE OF HYDROGENOSOMES IN THE RUMEN CILIATES OPHRYOSCOLECIDAE

Luc SNYERS, Philippe HELLINGS*, Claire BOVY-KESLER and Denyse THINES-SEMPOUX

Laboratoire de Biologie Cellulaire and **Laboratoire de Biochimie de la Nutrition, Université de Louvain, 1348 Louvain-la-Neuve, Belgium*

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1. Introduction

The hydrogenosomes are the microbodies of some protozoa living in anaerobiosis [1]. Their most complete description comes from studies on *Tritrichomonas foetus* [2,3]. The hydrogenosomes play the role of energetic organelles in this organism that lacks mitochondria. They are chromatic granules, the membrane of which seems to be formed by 2 unit membranes joined side by side. Their equilibration density in a sucrose gradient is high (1.24) which allows a good separation from the other organelles. The enzymes studied belong to the metabolism of the pyruvate arisen from the glycolysis. Their most important properties are the malate and the pyruvate oxidation as the acetate and molecular H_2 production. In trichomonads, the hydrogenosomes are able to utilize O_2 as terminal electron acceptor. This 'respiration' occurs in the presence of NADH, α-glycerophosphate, pyruvate and malate supported by NAD(P). ADP or GDP [3,4] and CoA [4] stimulate the pyruvate oxidation giving rise to a phenomenon resembling the mitochondrial respiratory control. Classical respiration inhibitors are ineffective. As important differences with the peroxisomes, one should mention the absence of catalase and the presence of superoxide dismutase and of circular DNA. Peroxisomes are also unable to phosphorylate ADP into ATP. In reality, the hydrogenosomes are near to being more mitochondria than peroxisomes although there exist striking differences at the level of the metabolic processes.

Like *Tritrichomonas foetus*, other anaerobic protozoa do not possess mitochondria. Many of these organisms are susceptible to have hydrogenosomes. Those which are parasitic or symbiotic live in the digestive tract of their host. So are the rumen Ophryoscolecidae which are the subject of this study. Electron microscopical observations of the Ophryoscolecidae have shown some small structures surrounded by a rather thick membrane, located around the oesophage and which could be hydrogenosomes [5]. Our biochemical study has confirmed their occurrence in at least 1 of the 5 organisms studied. Hydrogenosomes have been described in the rumen protozoon *Dasytricha ruminantium* [6].

2. Materials and methods

Samples of rumen juice were collected from a fistulated sheep maintained on a constant hay diet, 90 min after the morning meal. All the manipulations, until the last dosages, were performed in N_2 atmosphere. After filtration on a nylon sieve (106 μm), the juice was allowed to ferment for 3.5 h in order to eliminate the bulk of the vegetal debris. The ciliates were further purified on a 60% glycerol layer after [7] in order to eliminate the bacteria and the vegetal remnants. The resulting pellet of ciliates was washed 4 times with large volumes of unbuffered 0.25 M sucrose. At this step, the organisms were counted and their genus determinated. The cells were suspended in sucrose and disrupted in a Potter-Elvehjem tissue grinder by 40 strokes of the pestle rotating at 1300 rev./min. The suspension was centrifuged for 5 min at 400 × g. This step was repeated twice more giving a pellet called P_0. This pellet made mostly from unbroken cells, fibres, cuticles and cilia has been disregarded. It contains from 30–40% of the total proteins. The cell extracts were further centrifuged at 31 000 × g for 15 min. After being washed in similar conditions, the pellet (P_1) is resuspended in a known

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volume of sucrose. The pooled supernates were submitted to 112 500 × *g* for 120 min giving P_2 and the supernate S.

P_1 has been subfractionated by isopycnic centrifugation in continuous sucrose gradient. A 5 ml sample was layered on top of a 23 ml gradient (ρ = 1.12–1.28 g/cm^3) resting itself on a 10 ml cushion of 1.32 g/cm^3. The gradients were centrifuged at 30 000 × *g* for 15 h in a SW 27.2 rotor. From 10–12 fractions were collected. Results are presented according to [8]. O_2 consumption was measured by polarography with a Clark electrode. Isotonic A medium (3 ml) of [3] maintained at 25°C were supplied by 0.4–3.0 mg protein and by 10 μl samples of the various substrates dissolved in A at a 10 mM final conc. in the test. NAD(P) and NAD(P)H concentrations were 100 and 350 μM, respectively. O_2 concentration of the medium was regularly evaluated (280 μM).

Hydrogenase was assayed spectrophotometrically after [9] and pyruvate synthase was measured by the pyruvate–CO_2 exchange reaction [10]. The catalase [11] has been assayed at 0, 25 and 37°C for as long as 2 h. Acid phosphatase was determined at 37°C and pH 5.5 on pre-frozen samples with 45 mM *p*-nitrophenylphosphate. Proteins were measured according to [12] with BSA as standard.

Samples of ciliates and of the various subcellular fractions were fixed in 2.5% glutaraldehyde, postfixed in 2% osmium tetroxide, both in 0.1 M cacodylate buffer at pH 7.4 and embedded in Epon. The sections were counterstained with lead citrate before examination with a Philips EM 301.

3. Results

Table 1 gives the average composition of the samples. The genus *Epidinium* is the most important one while the *Holotriches* were nearly absent. Microscopical observation confirmed the relative scarcity of exo- or endobacteria in the ciliate samples, suggesting that they could not invalidate the results.

Table 1
Average composition of the ciliate samples

Genus	% (mean ± σ)
Entodinium	17.47 ± 15.71
Epidinium	30.96 ± 14.59
Diplodinium	27.93 ± 15.52
Eudiplodinium	21.72 ± 21.39
Holotriches	1.93 ± 2.7

Means of 12 samples containing 620.9 ± 405.22 ml ciliate/l

Table 2
Respiratory activity of P_1

Substrates	*n*	nmol O_2 . min^{-1} . mg protein^{-1}
Malate–NAD	9	8.43 ± 3.62
Pyruvate	2	2.97 ± 0.50
NADH	2	2.42 ± 0.35

The activities have been measured in isotonic A medium [3]; means ± σ; *n*, no. samples

3.1. *Detection of the respiratory activity*

Rumen Ophryoscolecidae are able to utilize molecular O_2. Their respiratory activities measured on P_1 in isotonic conditions are given in table 2. Basal O_2 consumption is not significantly increased by malate except when NAD(P) is added.

The malate-NAD respiration exhibits some latency: 0.05% Triton X-100 or freezing/thawing increases it 1.5 times. Pyruvate and NADH respirations are very low in isotonic conditions. Pyruvate oxidation is not increased by Triton X-100 but is stimulated 3–6 times by 100 μM CoA, in the presence of Triton X-100 only (fig.1). Addition of 0.05% Triton X-100 produced a stimulation of NADH oxidation 3–6 times (fig.2).

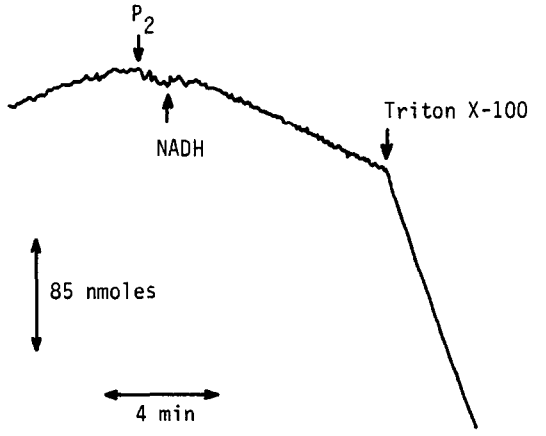


Fig.1. Effect of the addition of 0.05% Triton X-100 on the NADH-supported O_2 uptake by a P_1 fraction maintained at 25°C in the isotonic A medium of [3]; 4 mg protein in the test.

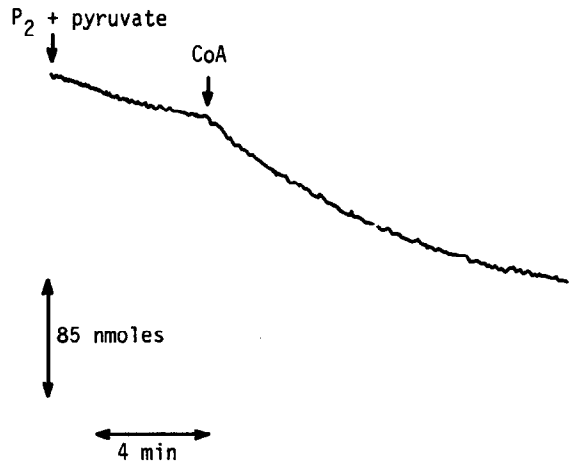


Fig.2. Effect of the addition of 100 μM on the pyruvate-supported O_2 consumption by a subfraction of $\rho = 1.218$ (E) obtained after isopycnic centrifugation of a P_1 fraction. The assay was performed with ~2 mg protein at 25°C in the isotonic A medium of [3], added with 0.05% Triton X-100.

Table 3
Subcellular distribution after differential centrifugation

Measure	Spec. act.[a]	Distribution in fractions (%)		
		P_1	P_2	S
Protein[d]	435.40[b]	43.8	7.1	49.1
O_2 consumption with				
Malate–NAD[c]	5.47	49.8	2.1	48.1
Pyruvate	2.62	100.0	0.0	0.0
NADH	3.68	20.7	5.6	73.7
NADH (Triton X-100)	5.78	45.2	3.8	51.0
Pyruvate synthase	9.50	96.0	0.0	4.0
Hydrogenase	960.00	66.0	0.6	33.4
Acid phosphatase[d]	51.16	89.2	6.7	4.4
Catalase	n.d.			

[a] Specific activity expressed in mU/mg proteins
[b] Total protein in mg; n.d., not detected
[c] Mean of 2 expt; [d] mean of 5 expt

Conditions as in section 2

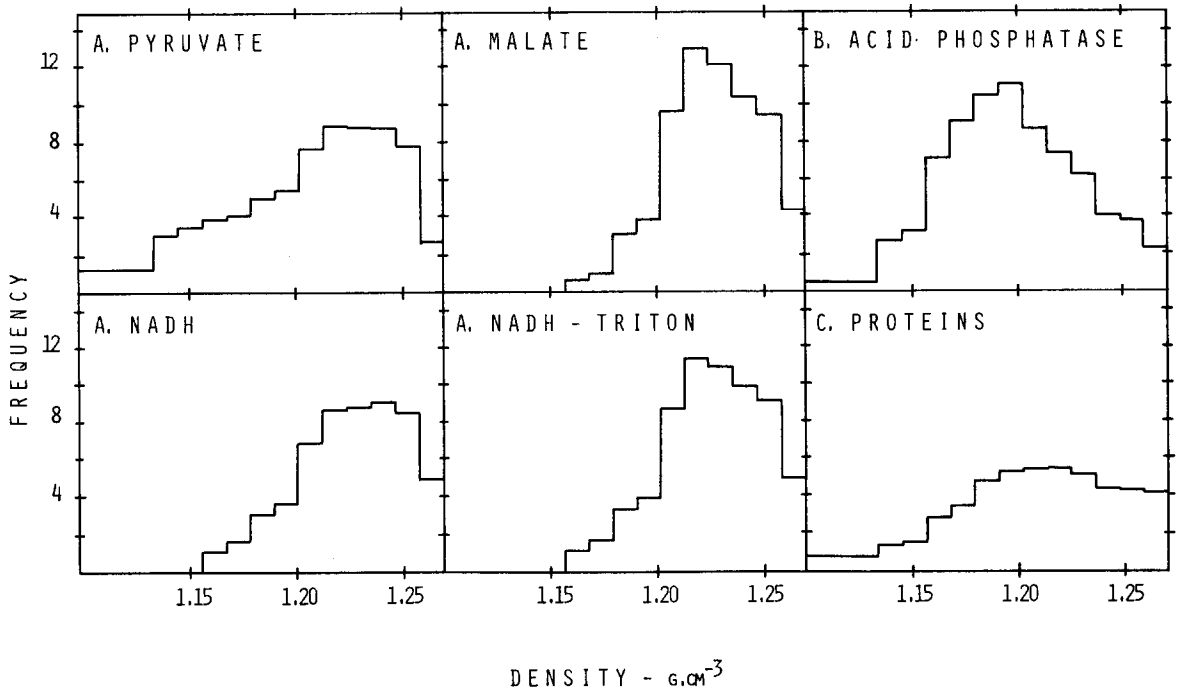


Fig.3. Distribution profiles of some activities of a P_1 fraction after isopycnic centrifugation. Results of a single, representative experiment, presented as normalized density frequency plots after [8]. The densities of the fractions were calculated from their refractive index. (A) O_2 consumption in the presence of 3 various substrates. Conditions as in section 2. Triton X-100 was at 0.05%.

Malate oxidation is insensitive to 10 μM CN^-. Succinate, fumarate, isocitrate, α-glycerophosphate, ethanol and acetaldehyde (10 mM) did not improve the basal respiration. Neither succinate, malate or isocitrate (10 mM) added to the pyruvate nor ADP or GDP (50 mM) added to the malate or the pyruvate influenced the O_2 consumption.

3.2. *Distribution of the enzymatic activities in the subcellular fractions: differential centrifugation (table 3)*

Pyruvate oxidation is entirely sedimentable at 450 000 × g_{min} whereas malate-NAD and NADH (Triton) oxidation have 50% of their total activity which is soluble. The NADH oxidation is latent in the P_1 fraction only. The malate oxidation by S requires NAD(P) as the sedimentable activity. Its app. K_m is 5.00 mM whereas it is 1.11 mM for the P_1 activity. Both activities exhibit an optimal pH ~7.5–8.5. The pyruvate synthase is largely recovered in P_1 whereas a part of the hydrogenase is soluble. The acid phosphatase is entirely sedimentable. Catalase was not detected even at 37°C.

3.3. *Isopycnic centrifugation of P_2*

The distribution profiles of the 3 oxidation reactions exhibit a similar pattern with the peak activity at densities 1.22–1.24. The distribution of the proteins is rather flat. Acid nitrophenylphosphatase, a lysosome marker, equilibrates at a density about 1.17–1.19 (fig.3).

3.4. *Morphology of the subcellular fractions*

Microscopical observation of subcellular fractions of density 1.21–1.24 indicates that their most prominent components are cilia fragments and round bodies of 0.3 μm av. diam. with a usually well-preserved membrane and a finely granular matrix (fig.4). This fraction contains also few sheets of membranes and some rough endoplasmic reticulum profiles.

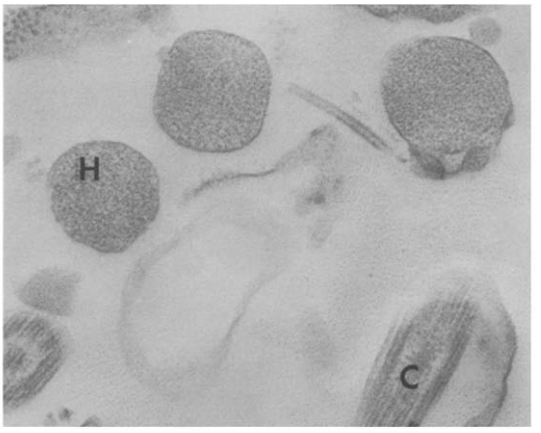
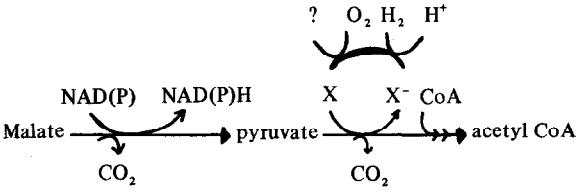


Fig.4. Electron micrograph of a subfraction of ρ = 1.210 obtained after isopycnic centrifugation of a P_1 fraction: hydrogenosomes (H); cilia (C); ×42 500.

4. Discussion

We show that although described as strict anaerobes, the rumen ciliates are able to oxidize malate-NAD(P), pyruvate and NADH in the presence of molecular O_2. This respiration is particulate and linked to microbody-like particles, 0.3 μm diam., equilibrating at densities 1.22–1.24 in sucrose gradients. These granules differ from the mitochondria because the oxidations are CN^- insensitive, and from the peroxisomes by the absence of detectable catalase. Their membrane is particularly impermeable to NADH and CoA.

Preliminary results indicate that they would contain an hydrogenase and that the pyruvate is decarboxylated in anaerobiosis, reaction stimulated by CoA. Our observations may be summarized thus:



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