

## OCCURRENCE OF HYDROGENOSOMES IN THE RUMEN CILIATES OPHRYOSCOLECIDAE

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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<sub>2</sub> production. In trichomonads, the hydrogenosomes are able to utilize O<sub>2</sub> as terminal electron acceptor. This 'respiration' occurs in the presence of NADH,  $\alpha$ -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<sub>2</sub> atmosphere. After filtration on a nylon sieve (106  $\mu$ m), 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 determined. 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  $\times$  g. This step was repeated twice more giving a pellet called P<sub>0</sub>. 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  $\times$  g for 15 min. After being washed in similar conditions, the pellet (P<sub>1</sub>) is resuspended in a known

volume of sucrose. The pooled supernates were submitted to  $112\,500 \times g$  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 ( $\rho = 1.12\text{--}1.28\text{ g/cm}^3$ ) resting itself on a 10 ml cushion of  $1.32\text{ g/cm}^3$ . The gradients were centrifuged at  $30\,000 \times g$  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  $25^\circ\text{C}$  were supplied by 0.4–3.0 mg protein and by 10  $\mu\text{l}$  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  $\mu\text{M}$ , respectively.  $O_2$  concentration of the medium was regularly evaluated (280  $\mu\text{M}$ ).

Hydrogenase was assayed spectrophotometrically after [9] and pyruvate synthase was measured by the pyruvate– $\text{CO}_2$  exchange reaction [10]. The catalase [11] has been assayed at 0, 25 and  $37^\circ\text{C}$  for as long as 2 h. Acid phosphatase was determined at  $37^\circ\text{C}$  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. Microscopi-

Table 1  
Average composition of the ciliate samples

Genus	% (mean $\pm$ $\sigma$ )
<i>Entodinium</i>	17.47 $\pm$ 15.71
<i>Epidinium</i>	30.96 $\pm$ 14.59
<i>Diplodinium</i>	27.93 $\pm$ 15.52
<i>Eudiplodinium</i>	21.72 $\pm$ 21.39
<i>Holotriches</i>	1.93 $\pm$ 2.7

Means of 12 samples containing  $620.9 \pm 405.22$  ml ciliate/l

Table 2  
Respiratory activity of  $P_1$

Substrates	<i>n</i>	nmol $O_2 \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$
Malate–NAD	9	8.43 $\pm$ 3.62
Pyruvate	2	2.97 $\pm$ 0.50
NADH	2	2.42 $\pm$ 0.35

The activities have been measured in isotonic A medium [3]; means  $\pm$   $\sigma$ ; *n*, no. samples

cal observation confirmed the relative scarcity of exo- or endobacteria in the ciliate samples, suggesting that they could not invalidate the results.

#### 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  $\mu\text{M}$  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).

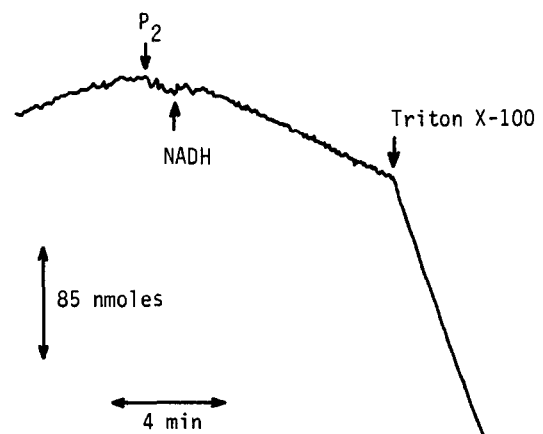


Fig.1. Effect of the addition of 0.05% Triton X-100 on the NADH-supported  $O_2$  uptake by a  $P_1$  fraction maintained at  $25^\circ\text{C}$  in the isotonic A medium of [3]; 4 mg protein in the test.

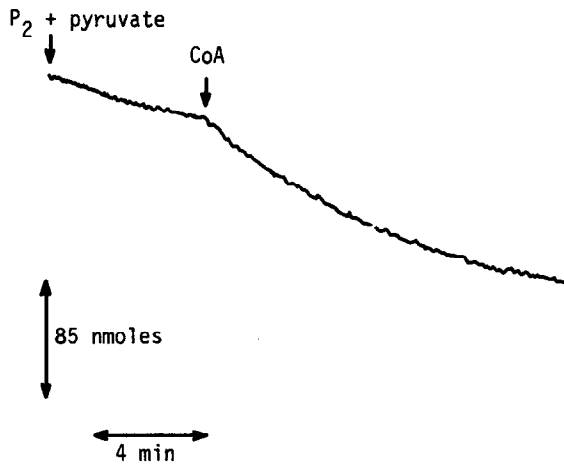


Fig. 2. Effect of the addition of 100  $\mu$ M on the pyruvate-supported O<sub>2</sub> consumption by a subfraction of  $\rho = 1.218$  (E) obtained after isopycnic centrifugation of a P<sub>1</sub> fraction. The assay was performed with  $\sim 2$  mg protein at 25°C in the isotonic A medium of [3], added with 0.05% Triton X-100.

Table 3  
Subcellular distribution after differential centrifugation

Measure	Spec. act. <sup>a</sup>	Distribution in fractions (%)		
		P <sub>1</sub>	P <sub>2</sub>	S
Protein <sup>d</sup>	435.40 <sup>b</sup>	43.8	7.1	49.1
O <sub>2</sub> consumption with				
Malate-NAD <sup>c</sup>	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 <sup>d</sup>	51.16	89.2	6.7	4.4
Catalase	n.d.			

<sup>a</sup> Specific activity expressed in mU/mg proteins

<sup>b</sup> Total protein in mg; n.d., not detected

<sup>c</sup> Mean of 2 expt; <sup>d</sup> mean of 5 expt

Conditions as in section 2

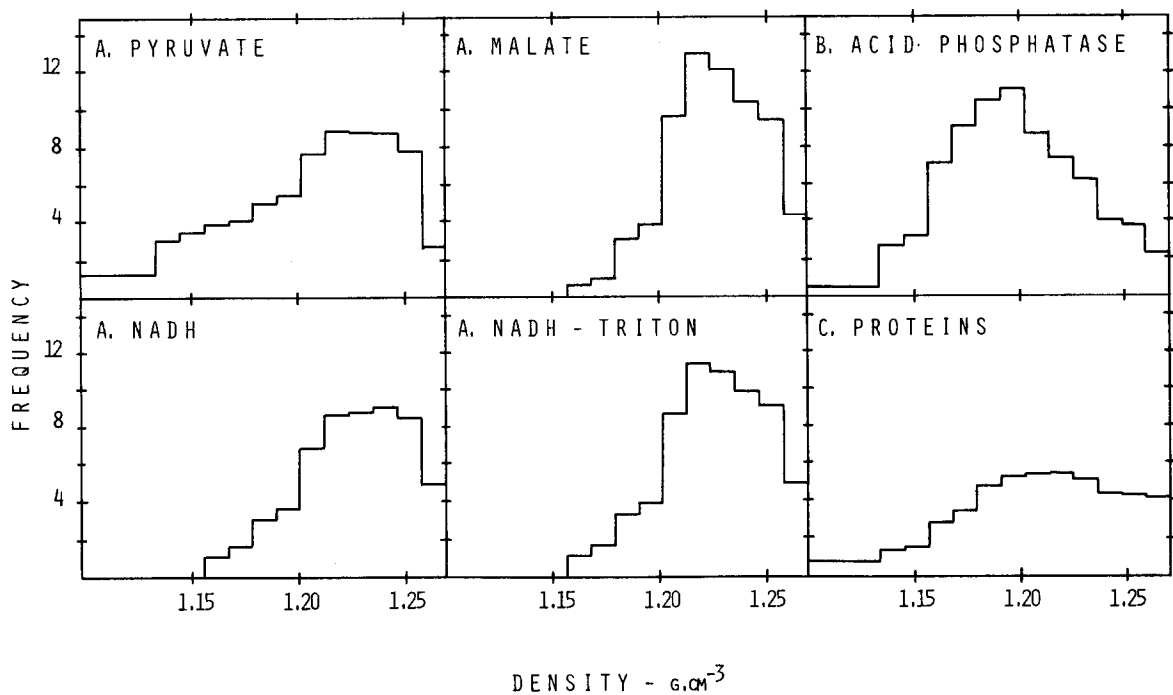


Fig. 3. Distribution profiles of some activities of a P<sub>1</sub> fraction after isopycnic centrifugation. Results of a single, representative experiment, presented as normalized density frequency plots after [8]. The densities of the fractions were calculated from their refractive index. (A) O<sub>2</sub> 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  $10 \mu\text{M CN}^-$ . Succinate, fumarate, isocitrate,  $\alpha$ -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  $\text{O}_2$  consumption.

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

Pyruvate oxidation is entirely sedimentable at  $450\,000 \times g_{\text{min}}$  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  $\sim 7.5$ – $8.5$ . 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  $37^\circ\text{C}$ .

### 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  $0.3 \mu\text{m}$  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.

## 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  $\text{O}_2$ . This respiration is particulate and linked to microbody-like particles,  $0.3 \mu\text{m}$  diam., equilibrating at densities 1.22–1.24 in sucrose gradients. These gran-

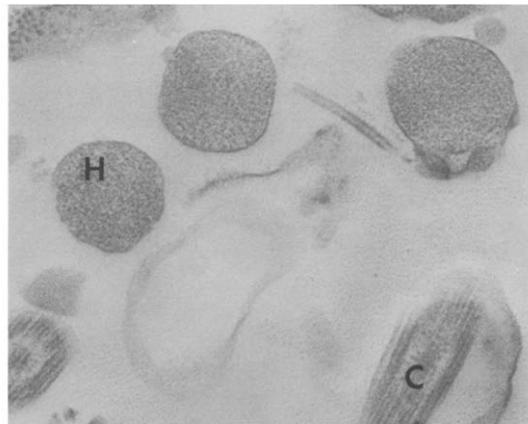
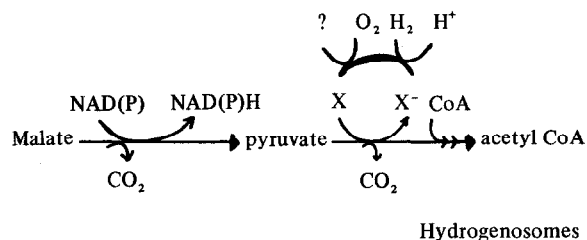


Fig.4. Electron micrograph of a subfraction of  $\rho = 1.210$  obtained after isopycnic centrifugation of a  $P_1$  fraction: hydrogenosomes (H); cilia (C);  $\times 42\,500$ .

ules differ from the mitochondria because the oxidations are  $\text{CN}^-$  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 a hydrogenase and that the pyruvate is decarboxylated in anaerobiosis, reaction stimulated by CoA. Our observations may be summarized thus:



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