

Cytochemical Localization of Hydrogenase Activity in the Anaerobic Protozoa *Trichomonas vaginalis*, *Plagiopyla nasuta* and *Trimyema compressum*

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A cytochemical staining method was developed for the demonstration of hydrogenase activity in hydrogenosomes of *Trichomonas vaginalis*. After fixation in low concentrations of glutaraldehyde under strictly anaerobic conditions and incubation of these cells in the presence of H₂ and the tetrazolium compound 2-(2'-benzothiazolyl)-5-styryl-3-(4'-phthalhydrazidyl)-tetrazolium chloride, an electron-dense deposit was produced in the hydrogenosomes. The method was used to demonstrate the hydrogenosomal nature of similar microbodies of the sapropelic ciliates *Plagiopyla nasuta* and *Trimyema compressum*.

INTRODUCTION

Many anaerobic sapropelic ciliates contain microbodies together with methanogenic endosymbionts (Van Bruggen *et al.*, 1984, 1986; Stumm & Zwart, 1986). Ultrastructurally the microbodies resemble the hydrogenosomes of trichomonads (Lindmark *et al.*, 1975; Müller, 1980) and rumen ciliates (Yarlett *et al.*, 1984). The microbodies are thought to be the source of H₂ for the methanogenic bacteria. Cultivation of sapropelic ciliates in numbers high enough for the cell fractionation studies necessary to characterize these organelles biochemically is not yet possible. Furthermore, preliminary experiments aimed at the localization of hydrogenase activity in the ciliate *Metopus striatus* by cytochemical staining were unsuccessful (K. B. Zwart & C. A. M. Broers, unpublished results). Thus there is a need for a specific staining procedure to demonstrate hydrogenase activity in anaerobic protozoa. In this paper we describe a method which was developed for the cytochemical staining of hydrogenase in the hydrogenosomes of *Trichomonas vaginalis*, an organism in which hydrogenosomes have been characterized thoroughly (Lindmark *et al.*, 1975; Müller, 1980). Furthermore we report the application of this method in two sapropelic ciliates, *Plagiopyla nasuta* and *Trimyema compressum*. (Preliminary results of this study were presented at the Fourth International Symposium on Microbial Ecology, August 24–29, 1986, Ljubljana, Yugoslavia.)

METHODS

Organisms and cultivation. *Trichomonas vaginalis*, isolated from a patient at the local Canisius-Wilhelmina Hospital, was grown anaerobically in serum flasks at 37 °C under a N₂ atmosphere (30 kPa) in the medium of Feinberg & Whittington (1957). Stock cultures were stored at –70 °C in 20% (v/v) glycerol.

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Abbreviations: BSPT, 2-(2'-benzothiazolyl)-5-styryl-3-(4'-phthalhydrazidyl)-tetrazolium chloride; MNA, methylnadnic anhydride.

Plagiopyla nasuta, isolated from the sapropel of a 200 l aquarium, was grown anaerobically in serum flasks at 20 °C under N₂/CO₂ (19:1, v/v) at atmospheric pressure in a dilute salt medium supplemented with 0.02% (w/v) dried grass (Goosen *et al.*, 1988), and a mixture of bacteria enriched anaerobically from the sapropel on nutrient broth (Oxoid). Growing protozoal cells were harvested under anaerobic conditions from culture samples (3 ml) with a cell density of approximately 200 cells ml⁻¹.

Trimyema compressum, isolated from a sludge backing pond of a wastewater treatment plant near Nijmegen, was grown anaerobically in serum flasks at 20 °C under a N₂ atmosphere (30 kPa) in a medium which contained KH₂PO₄/Na₂HPO₄ (4 mM), CaCl₂ (0.1 mM), MgSO₄ (1 mM) and Na₂S (0.4 mM) adjusted to pH 7.3, and supplemented with *Pseudomonas fluorescens* (10⁵–10⁶ cells ml⁻¹). Growing protozoal cells were harvested under anaerobic conditions from culture samples (3 ml) with a cell density of approximately 2000 cells ml⁻¹.

Hydrogenase activity of *Trich. vaginalis*. Exponentially growing cells were harvested under anaerobic conditions by centrifugation at 1000 g for 10 min, when cultures had reached a cell density of approximately 10⁶ cells ml⁻¹ (24–28 h after inoculation). Subsequently, the cells were washed twice in solution A, which contained sucrose (225 mM), KCl (20 mM), KH₂PO₄ (10 mM), MgCl₂ (5 mM), EDTA (1 mM) and Tris (20 mM) adjusted to pH 7.2 with HCl.

Hydrogenase activity of intact cells or of cells disrupted by sonication was measured at 22 °C according to the method of Lindmark & Müller (1973), which was modified as follows. β-Mercaptoethanol was omitted since either the reaction was measured under strictly anaerobic conditions, or the effect of oxygen on enzyme activity was studied. In addition, benzyl viologen instead of methyl viologen was used as an electron acceptor. The effect of fixation on hydrogenase activity was studied by treating cells with formaldehyde or glutaraldehyde (both 0.5 or 1.0%, v/v) for 10 min at 0 °C under aerobic or anaerobic conditions. Protein was measured according to Bradford (1976).

Cytochemistry of *Trich. vaginalis*. Cells were harvested, rinsed twice in solution A, prefixed for 10 minutes at 0 °C in 0.5% (v/v) glutaraldehyde in 0.1 M-KH₂PO₄/K₂HPO₄ buffer (PPB), pH 7.2, containing 0.25 M-sucrose and subsequently rinsed again twice in PPB plus sucrose to remove glutaraldehyde. Prefixed cells were incubated in serum flasks (10 ml) for 30–90 min at 22 °C in PPB containing sucrose and 2-(2'-benzothiazolyl)-5-styryl-3-(4'-phthalhydrazidyl)-tetrazolium chloride (BSPT, 0.25 mg ml⁻¹) as electron acceptor (Doddema *et al.*, 1979;

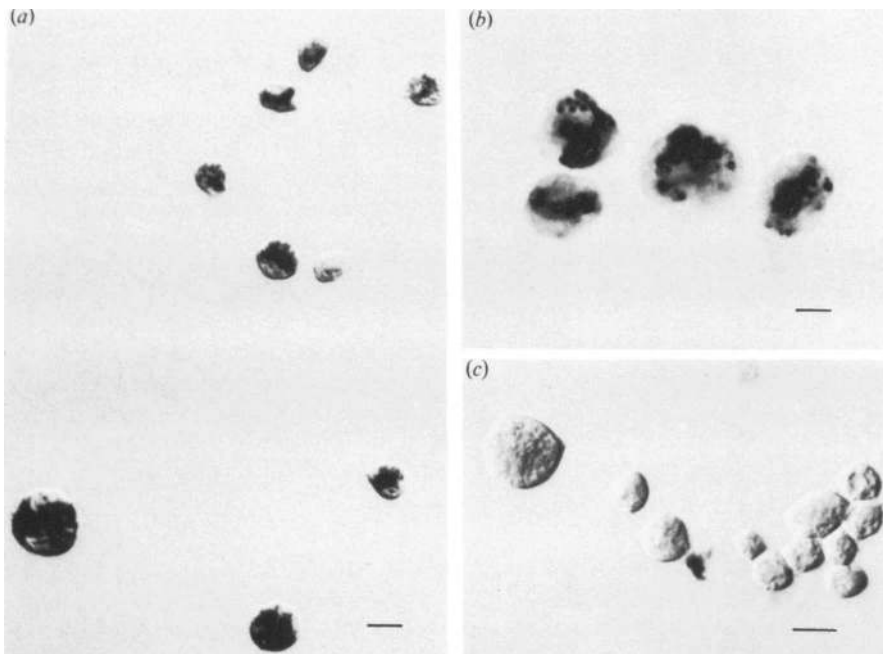


Fig. 1. Light micrographs of *Trich. vaginalis* fixed anaerobically in 0.5% glutaraldehyde and stained for hydrogenase activity with BSPT as electron acceptor. (a) Cells incubated in the presence of H₂ showing a positive reaction in the majority of cells (differential interference contrast, DIC). (b) Detail of cells incubated in the presence of H₂ showing positively stained granules (bright field). (c) Control cells incubated in the presence of N₂ showing no staining (DIC). Bars, 10 μm.

Shannon, 1982) with H_2 (30 kPa) in the headspace. In control experiments N_2 (30 kPa) was used instead of H_2 . The reaction was followed by light microscopy and stopped by rinsing the cells in PPB plus sucrose. Up to this point strictly anaerobic conditions were maintained. Reduced BSPT formed during the incubation was modified into an electron-dense product in a reaction with 2% (w/v) OsO_4 in distilled water (Shannon, 1982). After rinsing in PPB containing sucrose, the cells were postfixed in 3% (v/v) glutaraldehyde. Specimens were dehydrated in an alcohol series and embedded in Araldite or Epon 812 resin with or without methylnadac anhydride (MNA). Ultrathin sections were cut with a diamond knife and examined in a Philips EM 201 or EM 300.

Cytochemistry of sapropelic ciliates. Cytochemical procedures for sapropelic ciliates were applied as described for *Trich. vaginalis* with the following modifications. Cells were harvested, immediately prefixed in 0.35% (v/v, final concentration) glutaraldehyde in culture medium for 10 minutes at 0 °C and rinsed three times in culture medium without sulphide. The conditions for incubation with BSPT were as described for *Trich. vaginalis*, except that the incubation time was 3 h instead of 30–90 min. The reaction was stopped by rinsing the cells twice in PPB plus sucrose and subsequently in 0.1 M cacodylate buffer, pH 7.2. After formation of an electron-dense product with 2% OsO_4 in cacodylate buffer, cells of *Trim. compressum* were rinsed and postfixed in 6% (v/v) glutaraldehyde in cacodylate buffer. Cells of *P. nasuta* were postfixed in 3% (v/v) glutaraldehyde in cacodylate buffer prior to the reaction with 1% OsO_4 . This method resulted in a better preservation of the ultrastructure of ciliates than the method used for *Trich. vaginalis*. After embedding in Araldite, ultrathin sections were cut with glass knives.

RESULTS AND DISCUSSION

Preliminary experiments with *Metopus striatus* cells had indicated that fixation in 2.5% glutaraldehyde severely inhibited hydrogenase activity (unpublished results). Therefore the

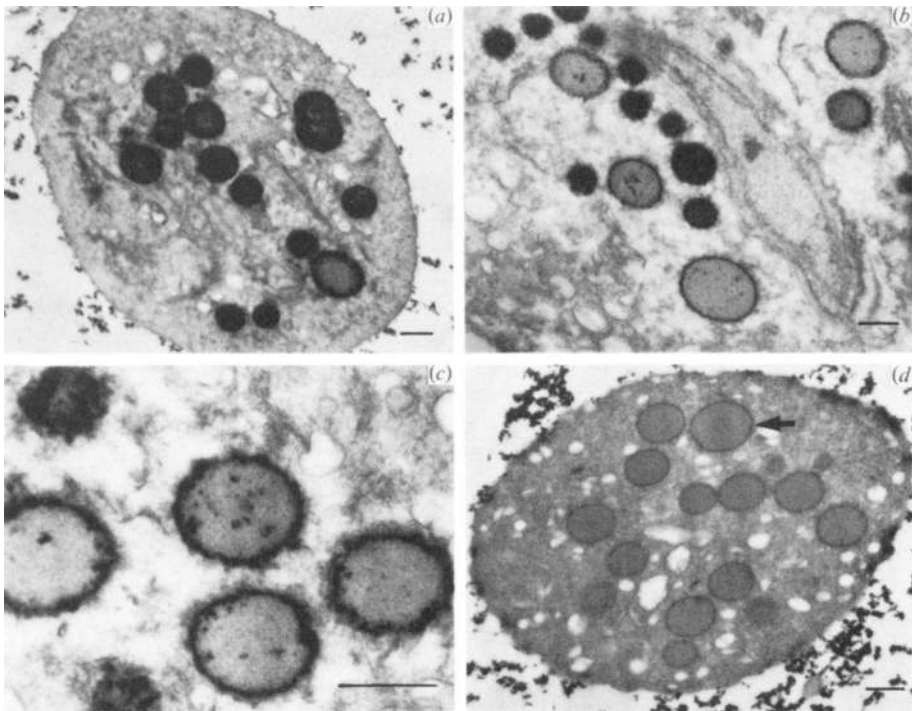


Fig. 2. Electron micrographs of *Trich. vaginalis* fixed and stained as described for Fig. 1. (a) Cell incubated in the presence of H_2 . A positive reaction is shown in the hydrogenosomes. The deposit outside the cells is a result of a non-specific reaction also occurring in control cells (see d). (b) Detail of a cell incubated in the presence of H_2 . In some of the hydrogenosomes the centre of the matrix remained unstained. (c) Detail of a cell incubated in the presence of H_2 showing partly stained hydrogenosomes. (d) Control cell incubated in the presence of N_2 . Note the absence of staining products in the hydrogenosomal matrix, the light deposit on the hydrogenosomal membrane (arrow) and the deposit outside the cell. Bars, 0.5 μm .

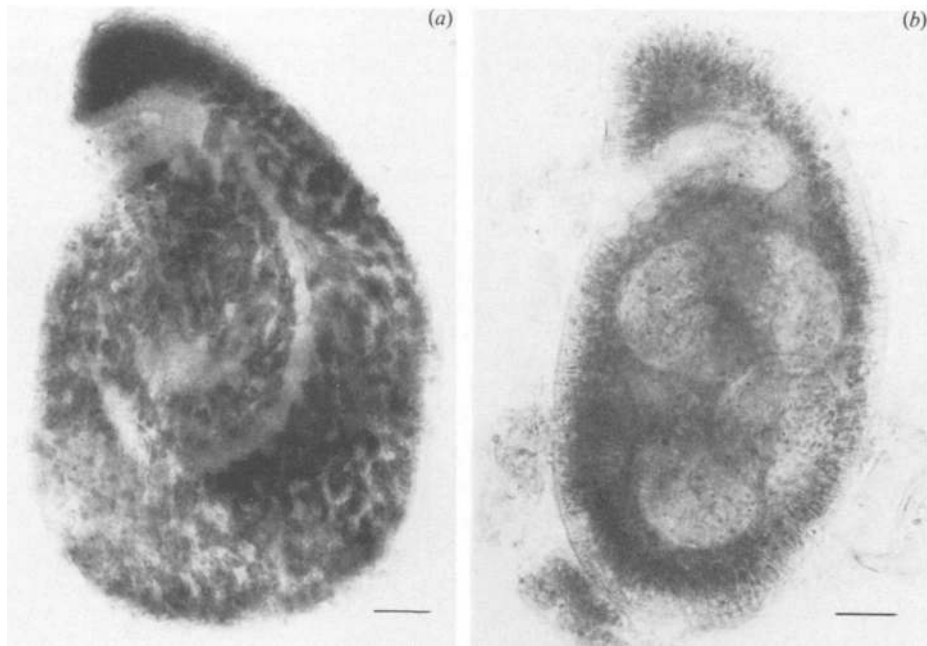


Fig. 3. Light micrographs (bright field) of *P. nasuta* fixed anaerobically in 0.35% glutaraldehyde and stained for hydrogenase activity with BSPT as electron acceptor. (a) Cell incubated in the presence of H_2 showing positive staining located in clusters. (b) Control cell incubated in the presence of N_2 showing no staining. Bars, 10 μm .

effect of fixation on hydrogenase activity was studied in more detail in *Trich. vaginalis*. Unfixed cells showed a specific hydrogenase activity of $7.1 \mu mol \text{ min}^{-1} (\text{mg protein})^{-1}$ both in the absence and the presence of oxygen. Fixation under aerobic conditions inhibited the reaction completely. *Trich. vaginalis* hydrogenase is oxygen labile (Lindmark & Müller, 1973; Lindmark *et al.*, 1975), and possibly fixation facilitated passage of oxygen across the cell membrane, which resulted in enzyme inactivation. Anaerobic fixation in 1% glutaraldehyde or 1% formaldehyde strongly inhibited enzyme activity to 15% of the original value, but after anaerobic fixation in 0.5% glutaraldehyde 66% of the original activity was retained.

When cells were fixed anaerobically in 0.5% glutaraldehyde and subsequently incubated in the reaction mixture for hydrogenase staining in the presence of H_2 , they showed blue/violet-stained granules inside 40–90% of the cells (Fig. 1*a, b*), whereas in control incubations staining was absent (Fig. 1*c*). In ultrathin sections of these cells embedded in Araldite, an electron-dense deposit was observed exclusively in the hydrogenosomes (Fig. 2*a, b*). Similar results were found in cells embedded in Epon 812 with or without MNA (not shown). In some of the organelles the centre of the matrix remained unstained (Fig. 2*b, c*). Whether this was due to a peripheral localization of the enzyme or to poor penetration of staining chemicals is not known. Obviously, in *Trich. vaginalis* strictly anaerobic conditions during the entire staining procedure, i.e. until the reduction of BSPT was completed, were required for a successful result. In control cells no deposit was observed within the matrix (Fig. 2*d*) but in some of the cells the membrane of the microbodies stained slightly (Fig. 2*d*). At present no conclusive explanation of this phenomenon can be given. Possibly it is due to endogenous activity of malate dehydrogenase, but no experiments were done to confirm this hypothesis. To our knowledge, the described result is the first cytochemical demonstration of hydrogenase activity in a eukaryotic organism. The technique was used earlier in bacteria by Doddema *et al.* (1979).

After having established the proper reaction conditions for *Trich. vaginalis*, the technique was applied for the localization of hydrogenase activity in two anaerobic sapropelic ciliates,

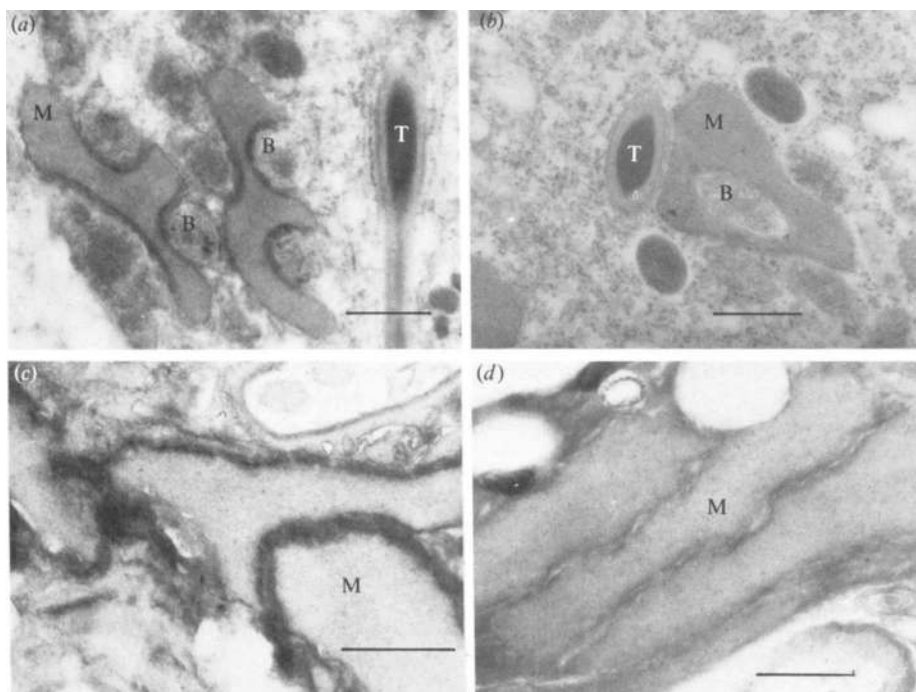


Fig. 4. Electron micrographs of *P. nasuta* (a, b) and *Trim. compressum* (c, d) fixed and stained as described for Fig. 3. (a) Detail of a cell incubated in the presence of H_2 . A positive reaction is shown with the staining product located on the membranes of the microbodies, especially at the sides associated with the methanogens. The electron density of trichocysts is a normal phenomenon and not due to the presence of BSPT. (b) Detail of a control cell incubated in the presence of N_2 . No staining product is visible. (c) Detail of a cell incubated in the presence of H_2 , showing a staining product located on the membranes of the microbodies. Note the absence of endosymbionts. (d) Detail of a control cell incubated in the presence of N_2 . No staining product is formed. M, microbodies; B, methanogenic endosymbionts; T, trichocysts. Bars, 0.5 μm .

Plagiopyla nasuta and *Trim. compressum*. In *P. nasuta* clusters of microbodies in a very close association with methanogenic bacteria are scattered throughout the cytoplasm (Stumm & Zwart, 1986; Goosen *et al.*, 1988). The results are shown in Figs 3 and 4. After incubation for 3 h in the reaction mixture for staining hydrogenase activity in the presence of H_2 , preparations examined by light microscopy showed a blue/violet deposit arranged cluster-wise in each cell of *P. nasuta* (Fig. 3a). In control cells, no reaction occurred (Fig. 3b). In ultrathin sections of cells embedded in Araldite, an electron-dense deposit was formed close to the membrane of the microbodies, especially at the immediate vicinity of the methanogens (Fig. 4a). In control cells no reaction product was formed (Fig. 4b).

Cells of *Trim. compressum* were harvested that had lost their endosymbiotic methanogens, as confirmed by epifluorescence microscopy (Doddema & Vogels, 1978) and by electron microscopy. The phenomenon of loss of endosymbionts was also reported by Wagener & Pfennig (1987). Microbodies, however, were still present and these organelles showed a positive reaction after staining for hydrogenase activity (Fig. 4c). The reaction product was not uniformly distributed over the organelles but was deposited at the periphery. Again in control cells no deposit was found (Fig. 4d).

The incubation time used for ciliates was two to three times longer than that for *Trich. vaginalis*, probably indicating a much lower enzymic activity. This may also explain why in ciliates no reaction product was formed throughout the microbodies and why in control cells no staining of the membrane occurred. On the other hand, hydrogenase may indeed be located on

the microbody membrane, and malate dehydrogenase may be absent in these cells since it could not be demonstrated in hydrogenosomes of some rumen ciliates (Yarlett *et al.*, 1981, 1983, 1984). The results strongly indicate the presence of hydrogenase activity in the microbodies of sapropelic ciliates. This study validates the hypothesis mentioned by Van Bruggen *et al.* (1984) that these organelles might produce H₂, which is utilized subsequently by the methanogenic endosymbionts, and consequently the methanogen-associated organelles may be referred to as hydrogenosomes. Further characterization of these organelles by cytochemical techniques is being undertaken at present in order to elucidate their metabolic activities. The technique for staining hydrogenase activity as described in the present paper may be helpful in characterizing organelles of cells which cannot yet be cultured in numbers high enough for the study of isolated organelles.

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