THE ULTRASTRUCTURE OF HYDROGENOSOMES IN THIN SECTIONS AND IN FREEZE FRACTURE REPLICAS FROM THE ANAEROBIC CHYTRID FUNGUS *CAECOMYCES* SP.

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Abstract

Hydrogenosomes from the obligately anaerobic rumen fungus *Caecomyces equi* were examined in thin sections and freeze fracture replicates with the transmission electron microscope. These organelles were bounded by a single unit membrane and appeared to contain granular material inside. Sometimes a single unit internal membrane was also present. The dumbbell shape of hydrogenosomes seen in some thin sections and freeze fracture replicas suggests the possibility that these organelles divide by binary fission. There is no evidence that there is an associated organelle genome.

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Introduction

The presence of the hydrogenosomes together with the absence of mitochondria have been observed in all isolates of obligately anaerobic rumen fungi which have been studied with the electron microscope (Munn 1994, Munn *et al.* 1988, Trinci *et al.* 1994). Hydrogenosomes were first discovered in obligately anaerobic protozoa which lack mitochondria (Muller 1988). They are membrane-bound organelles that produce ATP and molecular hydrogen (Muller 1993).

The five known genera of rumen fungi have been placed by Li *et al.* (1993) in the order Neocallimasticales (Chytridiomycetes). The fungi in this order are obligately anaerobic, lack the oxidative type of metabolism found in most fungi and rely on fermentation of carbohydrates for the production of ATP (Trinci *et al.* 1994). Yarlett *et al.* (1986) and Marvin-Sikkema *et al.* (1993a, b) have studied some of the enzyme systems within hydrogenosomes isolated from only one of the five genera, *Neocallimastix*. In fact, with the exception of morphological and nutritional studies, most of the research on rumen fungi has been done with this one genus. The other four genera await further characterization at the molecular level.

The role of the hydrogenosome in the energy metabolism and production of ATP in obligately anaerobic fungi and the possible relationship of hydrogenosomes to mitochondria have been reviewed by Marvin-Sikkema *et al.* (1994), Trinci *et al.* (1994), Embley *et al.* (2002) and Voncken *et al.* (2002).

Prior to 1994 all observations of the ultrastructure of the five genera of rumen fungi showed a single unit membrane around the hydrogenosome, although the quality of fixation and staining varied considerably (Gaillard & Citron 1989, Gold *et al.* 1988, Heath *et al.* 1983, Li *et al.* 1990, Li *et al.* 1991, Marvin-Sikkema *et al.* 1992, Marvin-Sikkema *et al.* 1993a, b, Munn 1994, Munn *et al.* 1981, Munn *et al.* 1988, Webb & Theodorou 1988, Yarlett *et al.* 1986). Since then Benchimol (1997) and van der Giezen (1997) have described a double membrane surrounding hydrogenosomes in *Neocallimastix*. Most of the work on hydrogenosomes involved isolates from the genus *Neocallimastix*, and very little attention has been paid to the internal structure and biochemical properties of the hydrogenosomes in any other genus.

We examined both thin sections and freeze fracture replicas of surfaces of sections through cells of one species from the rumen fungi, *Caecomyces equi* Gold, Heath & Bauchop, with the transmission electron microscope in order to study the structure of the membranes surrounding hydrogenosomes, the internal structure of

hydrogenosomes and other ultrastructural features. The freeze fracture technique had been used only once previously to study ultrastructure of the protoplasm in general or hydrogenosomes in specific in the rumen fungi (Benchimol *et al.* 1997). However, Benchimol *et al.* (1997) only published one photograph and that was of a hydrogenosome from *Neocallimastix*. Data from freeze fracture replicas should give us more insight into the structure of membranes surrounding and within the hydrogenosome in the rumen fungi. The number of membranes surrounding the hydrogenosome is important for understanding the evolutionary origin of this organelle and its possible relationship to the mitochondrion (Benchimol *et al.* 1997, van der Giezen *et al.* 1997).

Materials and Methods

NM1, NJ11 and NJ12 were isolated from the rumen of sheep in Australia using the procedure described by Phillips & Gordon (1988, 1989) and were maintained at 39°C since isolation by transfer to fresh anaerobic growth medium every 4 to 5 days. These isolates of obligately anaerobic fungi resembled *Sphaeromonas communis* Orpin in morphology (Orpin 1976), but are now classified as *Caecomyces communis* Gold, Heath & Bauchop (Gold *et al.* 1988).

The fungi were grown anaerobically in serum bottles with butyl rubber stoppers and aluminium crimp seals at 39°C in liquid, pre-reduced basal medium 10 plus either glucose at a concentration of 5 g per litre or finely milled wheat straw (passing a 1 mm screen) at a concentration of 10 g per litre. No agar was added to the medium. The complete composition of pre-reduced basal medium 10 and the method for medium preparation are described by Phillips & Gordon (1988, 1989).

The fungal cells from NM1, NJ11 and NJ12 were fixed in anaerobic cultures by slowly adding 50% glutaraldehyde solution from a syringe through the stopper into the culture bottle to give a final concentration of 3% (v/v) in the growth medium. For cells used to make thin sections the fixation time was 3 hours at room temperature. After washing three times with 0.1 M sodium cacodylate buffer at pH 7.4, the cells were post-fixed for two hours with 1% (w/v) osmium tetroxide in 0.1 M sodium cacodylate buffer at pH 7.4. After post-fixation the cells were washed again three times with cacodylate buffer, dehydrated through a series of graded ethanol solutions to 100% ethanol either with or without 100% propylene oxide in the last step prior to infiltration and then were infiltrated and embedded in low viscosity epoxy resin or araldite resin. Ultra thin sections were cut using a diamond knife fitted to a Reichert-Jung Ultracut C ultramicrotome. The sections were then stained using stabilised solutions of uranyl acetate and lead citrate and placed on a grid prior to examination with a Philips EM 300 transmission electron microscope.

For cells of NM1 used for freeze fracture, the fixation time with 3% glutaraldehyde was 1 hour at room temperature. After fixation the cells were infiltrated with 30% glycerol (v/v) for 1 hour and then concentrated by low speed centrifugation. The cells were frozen as quickly as possible by plunging them into liquid nitrogen cooled freon F22 and then were transferred into liquid nitrogen. The cells were fractured using a Balzers high vacuum freeze fracture unit (Model BAE 300) while the specimen was maintained at -100°C and the cutting knife at -196°C. The surface of the fractured cells was shadowed with platinum-carbon to give a thickness of 20 nm and then coated with a carbon layer for backing with a thickness also of 20 nm. The remains of the living tissue were removed from the replica by digestion overnight in 50% chromic acid at room temperature. The replica was then washed twice with distilled water, placed on a grid and examined with the electron microscope.

Results

Examination of thin sections of sporangia and rhizoids of *Caecomyces equi* NM1, NJ11 and NJ12 revealed that the most conspicuous organelles within the cell were nuclei, hydrogenosomes, crystal bodies, vacuoles, and flagella (formed within vacuoles in the sporangia). Other structures seen within the cells include ribosomes (in packets in zoospores developing within the sporangium and free within the cytoplasm of other cells), microtubules (in flagella and forming intra-nuclear spindles within dividing nuclei), rarely endoplasmic reticulum, cell membrane, vacuolar membrane and cell wall. The cell wall often had a fibrillar coating on the external surface, which probably is involved in attachment to substrates. We used nuclear structure as an indication of quality of fixation and staining. The nucleus was bounded by a double membrane with nuclear pores as in all eukaryotic cells (Fig. 1). Therefore the procedure for fixation and staining appeared to give good results.

Examination of thin sections of hydrogenosomes indicated that they were bounded by a single unit membrane and appeared to contain granular material (Figs 2 and 3). In occasional hydrogenosomes, a single unit internal membrane was present (Fig. 3). One of these was formed at right angles to the outer membrane. They were not invaginations of the outer membrane and do not completely cross the centre of the hydrogenosome. We did not see any hydrogenosomes in which cytoplasmic regions of the cell extended into the centre of the hydrogenosome. We found one example of several internal membranes within a single hydrogenosome, probably a megahydrogenosome (not shown in the figures). The size and shape of hydrogenosomes were variable; some were spherical, some sausage shaped (elongated) and some dumbbell in shape. No DNA strands were seen inside the hydrogenosomes.

Nuclei, hydrogenosomes and the cell walls were easily observed in replicas of sections made through cells of NM1 by the freeze fracture technique. Flagella were observed in sporangia. A surface coating was visible on the outside of the cell wall in replicas as well. Examination of surfaces of the nuclei revealed a double membrane and nuclear pores (Figs 4 and 5). The single unit membrane surrounding the hydrogenosome appeared like an egg shell when the internal contents were absent (Figs 6 and 7). When the fracture plane passed through a hydrogenosome and when the internal contents were present, they appeared granular in texture (Figs 8 and 9). A three dimensional view of the dumbbell shaped hydrogenosome was seen in freeze fracture replicas (Fig. 9). No internal membranes inside the hydrogenosome were seen by this technique, but a surface view of one hydrogenosome showed a groove that may be a slight invagination of the outer membrane (Fig. 10).

Discussion

In general the ultrastructure of *Caecomyces equi* isolates NM1, NJ11 and NJ12 appeared similar to that of the isolates of this genus which have been previously studied by Gaillard & Citron (1989), Gold *et al.* (1988) and Munn *et al.* (1988) and the isolates of other genera of obligately anaerobic rumen fungi (Gaillard & Citron 1989, Gaillard-Martinie *et al.* 1992, Heath & Bauchop 1985, Li *et al.* 1990, Li *et al.* 1991, Marvin-Sikkema *et al.* 1992, Marvin-Sikkema *et al.* 1993a, b, Munn 1994, Munn *et al.* 1981, Munn *et al.* 1987, Munn *et al.* 1988, Webb & Theodorou 1988, Yarlett *et al.* 1986). The observations of the structure of hydrogenosomes in *Caecomyces* NM1 using the freeze fracture technique confirm that the hydrogenosome in the fungi in the Order Neocallimasticales is bounded by a single unit membrane as seen in thin sections in this study as well as in other studies by various authors (Gaillard & Citron 1989, Gold *et al.* 1988, Heath *et al.* 1983, 1985, Li *et al.* 1990, Li *et al.* 1991, Marvin-Sikkema *et al.* 1992, Marvin-Sikkema *et al.* 1993, Munn 1994, Munn *et al.* 1981, Munn *et al.* 1988, Webb & Theodorou 1988, Yarlett *et al.* 1986). The internal membrane can be seen in several electron micrographs of thin sections of hydrogenosomes from *Neocallimastix* (Marvin-Sikkema *et al.* 1992, Marvin-Sikkema *et al.* 1993a, Munn 1994). In our study, one inner membrane appears to touch the outer membrane at a right angle. The resolution in our sections was not good enough to determine precisely the continuity of the lipid bi-layers at this point, but all membranes consist of one unit membrane, approximately the same thickness as the cell and vacuolar membranes. Furthermore, the exact origin of the inner membrane cannot be determined by the methods used here. Because it does not completely cross the internal volume of the hydrogenosome, it may be formed independently of the outer membrane. The inner membranes within the hydrogenosomes were always a single unit membrane. The internal matrix of the hydrosome appears to be granular which is consistent with the fact that it is filled with enzymes (Benchimol *et al.* 1997, Marvin-Sikkema *et al.* 1994, Yarlett *et al.* 1986).

The dumbbell shape of some hydrogenosomes in thin sections and freeze fracture replicas suggests the possibility that these organelles divide by binary fission or that this organelle has plasticity in shape. This has been proposed previously by Munn (1994) and by Benchimol *et al.* (1997).

Benchimol *et al.* (1997) and van der Giezen *et al.* (1997) both found two closely apposed membranes surrounding the hydrogenosomes and, in some preparations, double membranes within the hydrogenosomes from *Neocallimastix*. There was no inter-membrane space between either the two outer or the two internal membranes. Benchimol (1997) observed two membranes surrounding the hydrogenosome in freeze-fracture replicas as well. Benchimol *et al.* (1997) explains that their discovery of double membranes in the hydrogenosome is due to better fixation, staining and sectioning methods and to higher magnification. However, in our study with *Caecomyces* two membranes were not seen in either thin sections or freeze-fracture replicas. This raises the possibility that the outer and inner membranes can be either double or single in the rumen fungi.

Hydrogenosomes have also been found in some obligately anaerobic protozoa from a number of unrelated evolutionary lines: for example, parasitic parabasalian flagellates (Order Trichomonadida), rumen and intestinal ciliates (Orders Entodiniomorphida and Trichostomatida), some free living ciliates (Order Heterotrichida, Order Odontostomatida and several other orders) and in at least one amoeboflagellate (Genus *Psalteriomonas*, *Heterolobosea*) (Muller 1993). A number of other species of obligately anaerobic protozoa lack both hydrogenosomes and mitochondria (Muller 1993). In the trichomonads the hydrogenosome is bounded by a double unit membrane as seen in thin sections and freeze fracture replicates (Honigberg *et al.* 1984). In the ciliates, the hydrogenosome can be bounded by either a single or a double unit membrane (Muller 1993). This and other data have lead Muller (1993) to speculate that the hydrogenosome has evolved independently many times. Currently mitochondria and hydrogenosomes are considered to be two forms of the same organelle (Embley *et al.* 2002). However, it is necessary to make sure that the fungal hydrogenosome does not have a second membrane that is destroyed by conventional processing for observation in the transmission electron microscope. Our freeze fracture data shows that this is not the case and the fungal hydrogenosome is surrounded by one unit membrane. More investigation is needed to resolve this issue.

Electron microscope studies have never provided evidence for an organelle genome in the hydrogenosome. Furthermore, van der Giezen *et al.* (1997) were unable to detect any DNA in hydrogenosomal fractions from *Neocallimastix* in contrast to mitochondria from aerobic fungi.

Both obligately anaerobic rumen fungi (Trinci *et al.* 1994) and obligately anaerobic protozoa (Muller 1988) can synthesize ATP by fermentation of glucose to either lactic acid or ethanol. Enzymes that catalyze the chemical conversion of glucose to lactic acid or ethanol are normally found in the cytosol. The enzymes in the hydrogenosome provide an alternative pathway for fermentation of glucose with the production of hydrogen gas (Marvin-Sikkema *et al.* 1994, Trinci *et al.* 1994). However, several isolates of fungi in the genus *Blastocladia*, which is related to the rumen fungi (Gleason & Gordon 1989), and many genera of protozoa related to the orders mentioned above (Muller 1993) are capable of growth under anaerobic conditions without hydrogenosomes because they have all necessary enzymes in the cytosol. Why then is the hydrogenosome found in evolutionarily unrelated groups of obligately anaerobic eukaryotic micro-organisms? Voncken *et al.* (2002) suggest multiple origins of hydrogenosomes. The hydrogenosome forms a compartment separate from the cytosol where different chemical reactions can take place. Marvin-Sikkema *et al.* (1994) and Yarlett *et al.* (1986) have isolated a number of enzymes from the hydrogenosome that catalyze reactions leading to the production of hydrogen gas. The function of the outer membrane of the hydrogenosome is not understood, although Marvin-Sikkema *et al.* (1994) propose that an ATPase is involved in maintenance of a protomotive force across the membrane. They suggest that the protomotive force may function in transport of metabolites both into and out of the hydrogenosome and possibly transport of proteins into the hydrogenosome. Also, they suggest that proton pumping from the matrix of the hydrogenosome into the cytosol results in the generation of a pH gradient across the hydrogenosomal membrane. This could provide an optimal pH in the matrix to allow efficient enzymatic conversion of the metabolites that have been transported inside. According to Voncken *et al.* (2002) a mitochondrial-type ADP/ATP carrier has been identified in the hydrogenosomes of *Neocallimastix*. This provides more evidence that the hydrogenosomes of rumen fungi evolved from fungal mitochondria (Voncken *et al.* 2002).

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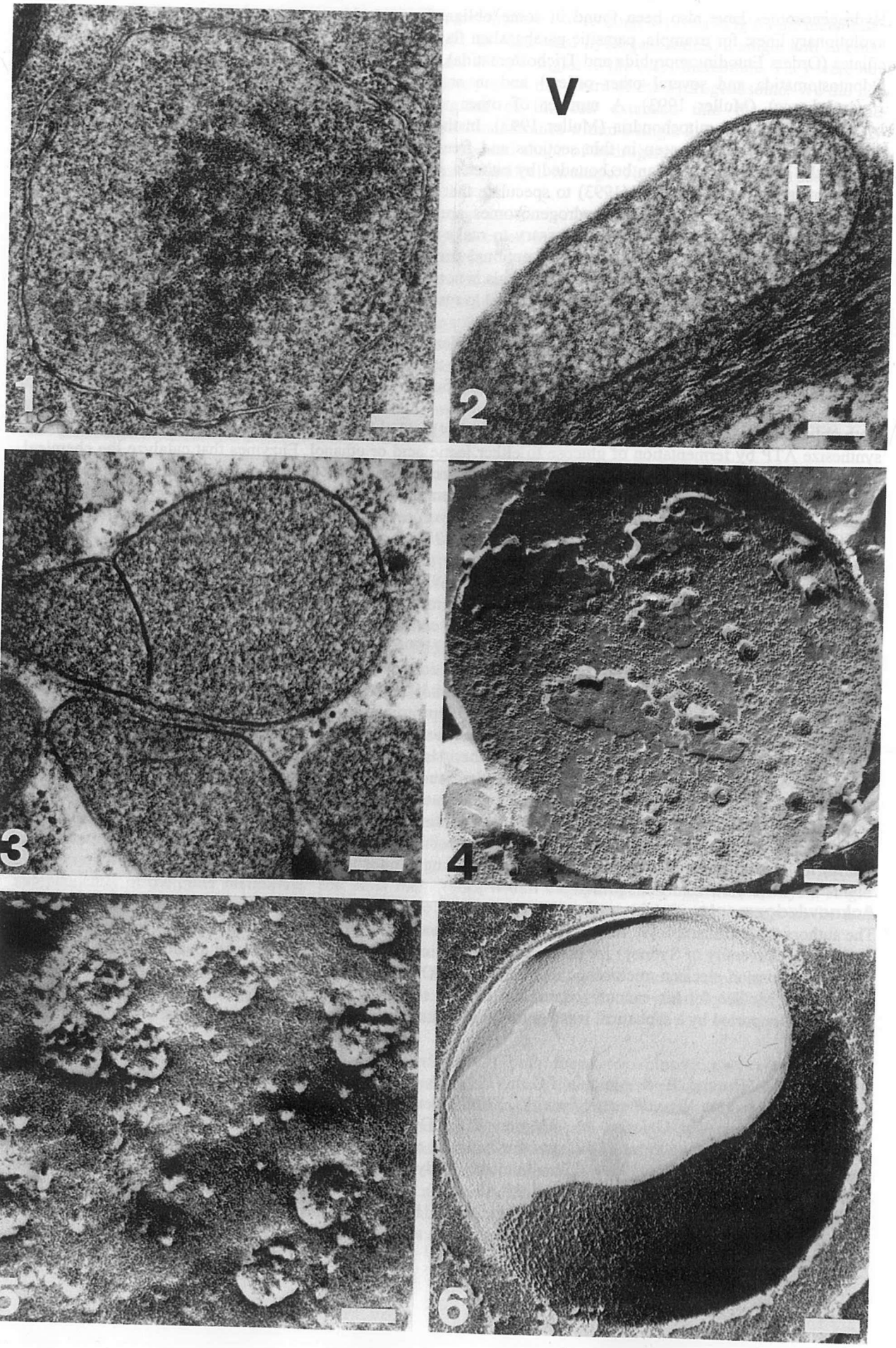
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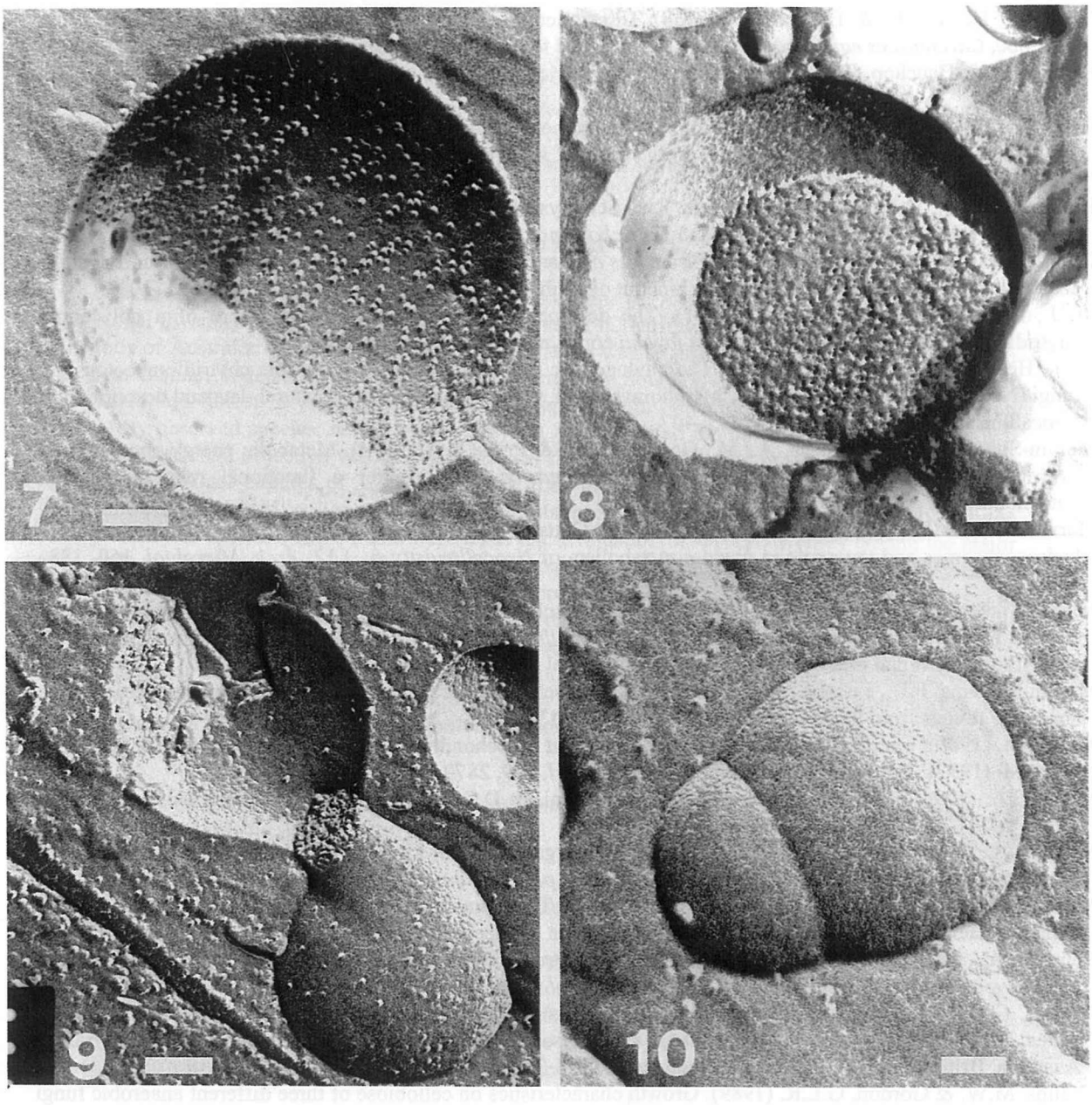

Page 96. Fig. 1. Thin section of the nucleus showing the double membrane with nuclear pores (NJ11). **Fig. 2.** Thin section of a hydrogenosome (H), membrane around hydrogenosome, vacuole (V) and vacuolar membrane (NJ11). **Fig. 3.** Thin sections of hydrogenosomes, one of which has an internal membrane (NJ12). **Fig. 4.** Freeze fracture replica of the nucleus showing the outer surfaces of the inner and outer membranes (NM1). **Fig. 5.** Freeze fracture replica of the nucleus with nuclear pores (NM1). **Fig. 6.** Freeze fracture replica of a hydrogenosome showing outer and inner surfaces of the membrane (NM1).

Fig. 7. Freeze fracture replica of a hydrogenosome showing inner surface of the membrane (NM1). **Fig. 8.** Freeze fracture replica of a spherical hydrogenosome showing the outer surface of the membrane and granules in the centre (NM1). **Fig. 9.** Freeze fracture replica of a dumbbell shaped hydrogenosome showing the inner and outer surfaces of the membrane and granules in the centre (NM1). **Fig. 10.** Freeze fracture replica of a hydrogenosome showing a groove on the outer surface (NM1).

Scale bars: Fig. 1, 230 nm; Fig. 2, 68 nm; Fig. 3, 120 nm; Fig. 4, 260 nm; Fig. 5, 68 nm; Fig. 6, 89 nm; Fig. 7, 154 nm; Fig. 8, 138 nm; Fig. 9, 159 nm; Fig. 10, 88nm.

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