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Hydrogenosomes in the rumen protozoon *Dasytricha ruminantium* Schuberg

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This paper reports for the first time the presence in the anaerobic rumen ciliate *Dasytricha ruminantium* (Schuberg) of microbody-like organelles, about 0.5 μm diameter, with a granular matrix and an equilibrium density of approx. 1.18 g/ml. These organelles can be isolated in a fraction sedimented at 10^5 ***g***-min that contains 67% of the total pyruvate synthase (EC 1.2.7.1), 66% of the hydrogenase (EC 1.18.3.1) and 20% of the lactate dehydrogenase (EC 1.1.1.27). Thus in several respects this fraction is enzymically similar to those containing hydrogenosomes in some other parasitic anaerobic protozoa (the trichomonads). However, in contrast with the hydrogenosomes of trichomonads, the oxygen-tolerant enzyme malate dehydrogenase (decarboxylating) (EC 1.1.1.40) is not particulate, but occurs only in the cytosol. These results enable the proposal of a scheme for the pathway of product formation (acetate, lactate, CO_2 and H_2) from carbohydrates.

Functional mitochondria are regarded as primary characteristics of eukaryotic cells, and their absence is usually taken as a sign of excessive irreversible differentiation (e.g. mammalian erythrocytes) or as a result of transient metabolic adaptations (e.g. anaerobically grown yeasts). It is therefore of great interest that it has been shown that certain anaerobic protozoal species lack mitochondria and all mitochondrial functions [e.g. trichomonads (Lindmark & Müller, 1973, 1974; Lloyd *et al.*, 1979) and *Entamoeba* sp. (von Brand, 1973)]. In some of these organisms at least one oxidative function is carried out by a unique organelle, termed the hydrogenosome (Lindmark & Müller, 1973). In others, e.g. *Entamoeba* sp., although the organelle is absent, the typical hydrogenosomal enzymes are present in the cytosol (Reeves *et al.*, 1977). The rumen holotrich protozoon, *Dasytricha ruminantium*, produces H_2 as well as CO_2, acetate, butyrate and lactate by the fermentation of sugars (Williams & Harfoot, 1976), but the mechanism of hydrogen production is unknown. The availability of techniques which enable large-scale isolation of virtually bacteria-free preparations of *D. ruminantium* enables investigation of the energy metabolism of this rumen ciliate protozoon.

We report that, in this organism, as in trichomonads, compartmentation of enzymes involved in the formation of H_2 and acetate from pyruvate has occurred. However, the hydrogenosomes of the rumen ciliate show several unexpected and unique features.

Materials and methods

Isolation of organism

D. ruminantium was isolated from rumen contents by filtration through 30 μm micropore nylon bolting cloth (Simon Industries, Stockport, Cheshire, U.K.) as described previously (Williams & Yarlett, 1980); buffers and all other isolation procedures were as described by Williams & Harfoot (1976). Samples were obtained from two sources, rumen contents from sheep maintained on a daily diet of cubed molassed sugar-beet pulp (0.6 kg) and chopped hay (0.3 kg), and rumen contents from a cow fed on a daily diet of cubed molassed sugar beet (0.6 kg) and chopped hay (6 kg). Water was given *ad lib.* Samples of rumen liquor were withdrawn approx. 2 h after the sugar-beet pulp ration had been consumed (i.e. 2 h after feed).

Disruption techniques

The washed cell pellet was disrupted under N_2 in a buffer consisting of 0.25 M-sucrose/0.72 mM-EDTA/10 mM-Tris/HCl (pH 7.4), by using a motor-driven 10 ml ground-glass homogenizer (Jencons), at 20 strokes/min for 3 min. The whole homogenate was

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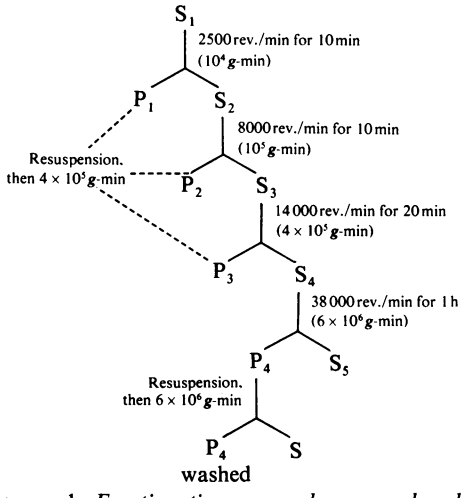


Scheme 1. *Fractionation procedure employed for D. ruminantium*

centrifuged at 5000 *g*-min (16 × 15 ml rotor, MSE 18) at 4°C under N_2 to remove unbroken cells. The supernatant (S_1) obtained was carefully decanted under N_2 and taken through the fractionation scheme (Scheme 1). Assays of enzymes in unfractionated extracts were in 0.1 M-potassium phosphate buffer (pH 7.2).

Treatment with ultrasound (20 kHz, 50 W, 10 s; Branson, Danbury, CT, U.S.A.) under N_2 gave specific activities similar to those obtained after homogenization for all enzymes assayed. Disintegration in a Polytron blender (50 Hz, 20 s), chemical breakage using indole [0.1%; 3 parts (v/v) of indole: 1 part of cell suspension, incubated for 15 min] and digitonin (40 mg/ml in dimethyl formamide, added to cells to give a 6 mg/g cell suspension, incubated for 5 min and homogenized at 15 strokes/min for 5 min) were occasionally employed as alternative breakage techniques.

Density gradients

The gradients were prepared from 15% and 60% (w/w) sucrose containing 0.7 mM-EDTA/30 mM-2-mercaptoethanol/10 mM-Tris/HCl (pH 7.4). The 10^5 *g*-min fraction was carefully layered on the gradient and centrifuged in a 3 × 25 ml swing-out rotor for 1 h at 27000 rev./min (10^6 *g*-min). Fractions (2 ml) were collected under N_2 by puncturing the base of the tube. The density of the sucrose was measured with a refractometer (10–50%); dilutions were used to measure the higher densities.

Enzyme assays

Malate dehydrogenase (decarboxylating) (EC 1.1.1.40), malate dehydrogenase (EC 1.1.1.37), succinate thiokinase (EC 6.2.1.4), pyruvate synthase (EC 1.2.7.1) and hydrogenase (EC 1.18.3.1) were assayed as described by Lindmark & Müller (1973, 1974). Catalase (EC 1.11.1.6) was assayed as described by Baudhuin *et al.* (1964), superoxide dismutase (EC 1.15.1.1) as described by McCord & Fridovich (1969), formate dehydrogenase (particulate) (EC 1.2.1.2) as described by Friedrich *et al.* (1979) and diaminopimelic acid as described by Czerkawski (1974). Amylopectin was determined by treatment of the fractions with 0.5 M-NaOH; after warming to 50°C for 5 min the solution was cooled and neutralized with 0.5 M-HCl. Iodine reagent contained iodine (0.2 g/l) and KI (2.0 g/l); 0.4 ml of this was added to 1.0 ml of the test solution and A_{550} was read. Adenylate kinase (EC 2.7.4.3) was assayed as detailed by Boehringer (Biochemical Catalogue, 1970). NADH peroxidase was assayed as described by Prins & Prast (1973).

Acid hydrolases were assayed by determining the rate of *p*-nitrophenol release from the specific substrate (0.015 M in 0.1 M-acetate buffer, pH 4.2, or 0.1 M-phosphate buffer, pH 5.5) at 39°C. The *p*-nitrophenol was measured spectrophotometrically at 420 nm in alkaline solution (Barrett & Heath, 1977). All other enzyme assays were as described by Colowick & Kaplan (1966). Hydrogenase, pyruvate synthase and malate dehydrogenase (decarboxylating) were assayed in the presence of 0.1% Triton X-100. All other enzymes were assayed after two cycles of freezing and thawing.

Protein was measured by the method of Lowry *et al.* (1951).

Electron microscopy

Suspensions of washed organisms and materials in fractions were fixed in 3% (v/v) glutaraldehyde in 0.1 M-phosphate buffer/5 mM-$CaCl_2$/2.5% (w/v) sucrose for 1 h at 4°C. Post-fixation was with 2% (w/v) OsO_4 for 30 min; the fixed pellets were dehydrated through an ethanol series and embedded in Araldite. Sections were cut at a minimal 60 nm thickness and stained with 1% lead citrate. Examination was in a Philips electron microscope with 60 kV accelerating voltage.

Results

Enzymes involved in H_2 production were detected in extracts of the rumen protozoon *Dasytricha ruminantium*, including pyruvate synthase, hydrogenase and malate dehydrogenase (decarboxylating), as well as low activities of adenylate kinase. Neither succinate thiokinase nor acetate: succinate CoA-transferase was detected in the extracts. Other enzymes not detected include catalase, superoxide dismutase, glucose 6-phosphate dehydrogenase (EC

Table 1. *Activities of oxidoreductases and other enzymes in cell-free extracts of D. ruminantium*
Specific activities are expressed as munits (nmol/min per mg of protein, ±S.D. for six experiments where appropriate. Enzymes were assayed at their pH optima. Fractions are as in Scheme 1.

Enzyme	pH	Specific activity in whole homogenate (munits)		Specific activity in fraction (munits)
Malate dehydrogenase (decarboxylating)	7.4	33 ± 6	S	132 ± 27
Pyruvate synthase	7.4	53.8 ± 23.8	P_2	243.6 ± 160
Hydrogenase	7.4	41.1 ± 26	P_2	231 ± 170
Aldolase	7.5	8.3 ± 4.2	S	55 ± 12
NADH peroxidase	7.0	23 ± 12	S	23 ± 12
Lactate dehydrogenase	7.0	210	P_3	1575
			P_2	315
Malate dehydrogenase	7.4	13	P_3	118
			P_4	192
			S	42
NADH oxidase	7.0	12.2 ± 2.5	S	13.0 ± 4
Adenylate kinase	7.2	4		
Serine dehydratase	8.0	434.6		

Table 2. *Specific activities of hydrolases in cell-free extracts of D. ruminantium*
Extracts were assayed at their pH optima.

Enzyme	pH	Specific activity in whole homogenate (munits)		Specific activity in fraction (munits)
Acid phosphatase	4.2	28.5 ± 9.5	P_3	66 ± 17
β-D-Glucosidase	5.5	42 ± 6	P_3	266 ± 30
α-D-Glucosidase	5.5	1.6	P_3	5.1
α-L-Arabinofuranosidase	5.5	1.1	P_1	14
β-D-Glucuronidase	4.2	2.5	P_4	56.7 (P_3 20.8)
α-D-Galactosidase	4.2	0.3	P_1	0.9

1.1.1.49), formate dehydrogenase and succinate dehydrogenase (EC 1.3.99.1). Specific activities of the oxidoreductases and a number of other enzymes assayed at their pH optima in cell-free extracts are listed in Table 1. Malate dehydrogenase (decarboxylating) was distinguished from the non-decarboxylating enzyme by its dependence on Mn^{2+}; it was shown that 0.15 μM-EDTA gave 65% inhibition of the enzyme. This enzyme is insensitive to oxygen, unlike pyruvate synthase and hydrogenase, which are completely inactivated by exposure to atmospheric oxygen for 30s. The specific activities of hydrolases are listed in Table 2. Alkaline phosphatase (EC 3.1.3.1) and β-D-galactosidase (EC 3.2.1.23) were not detectable.

Differential centrifugation of cell-free extracts of *D. ruminantium* revealed characteristic distribution patterns for different enzymes of groups of enzymes; Figs. 1 and 2 summarize the results of several such experiments using the fractionation scheme shown in Scheme 1. The enzymes pyruvate synthase and hydrogenase were always predominantly associated with the same large-particle fraction, P_2, sedimenting at 10^5 *g*-min. Aldolase served as a cytosolic marker; malate dehydrogenase (decarboxylating) also appeared in the cytosol in this study. The distributions of acid *p*-nitrophenyl phosphatase (EC 3.1.3.2), β-D-glucosidase (EC 3.2.1.21) and α-D-galactosidase (EC 3.2.1.22) markers for acid-hydrolase-containing organelles (Lloyd & Cartledge, 1974) indicate the presence of at least two populations of these organelles. Tables 1 and 2 indicate the purification of enzymes in fractions obtained.

NADH oxidase was found to be insensitive to antimycin A (120 μg of antimycin A/mg of protein) and cyanide (8.3 mM), but the activity was decreased by 62.5% by using anaerobic buffers and was eliminated completely by including 250 mM-2-mercaptoethanol in the buffer. NADH peroxidase was also found to be insensitive to antimycin A (85.5 μg of antimycin A/mg of protein) and cyanide (10 mM), but again activity was eliminated by the inclusion of 250 mM-2-mercaptoethanol in anaerobic buffers.

The activity of hydrogenosomal enzymes showed a mean equilibrium density of 1.18 g/ml in sucrose

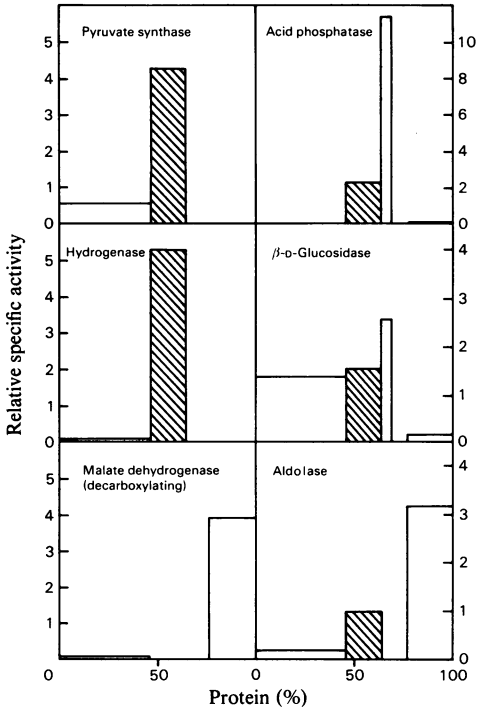


Fig. 1. *Distribution of enzymes in fractions obtained by differential centrifugation of a cell-free extract of D. ruminantium*

Disruption was with a motor-driven homogenizer. Relative specific activities (the ratios of specific activities in fractions to 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 $6 \times 10^6 g$-min. Percentage recoveries (based on enzyme units in the cell-free extract) were: hydrogenase, 67%; pyruvate synthase, 76%; malate dehydrogenase (decarboxylating), 58%; acid phosphatase, 47%; β-glucosidase, 97%; aldolase, 61%; protein, 86%. Material from the shaded area was subjected to isopycnic density centrifugation (Figs. 3 and 4).

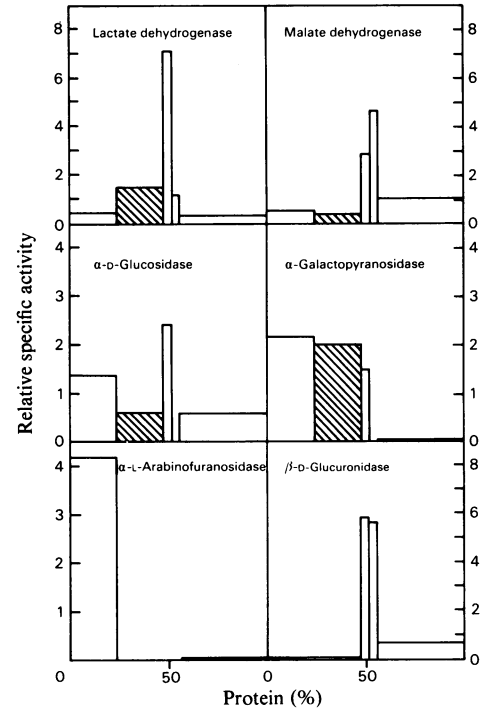


Fig. 2. *Distribution of marker enzymes after differential centrifugation of a cell-free extract of D. ruminantium*

The extract was prepared by treatment with ultrasound. Relative specific activities 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 $6 \times 10^6 g$-min. Percentage recoveries were: lactate dehydrogenase, 130%; α-galactopyranosidase, 98%; malate dehydrogenase, 129%; β-glucuronidase, 114%; α-D-glucosidase, 81.7%; α-L-arabinofuranosidase, 135%; protein, 112%. Material from the shaded area was subjected to isopycnic density centrifugation (Figs. 3 and 4).

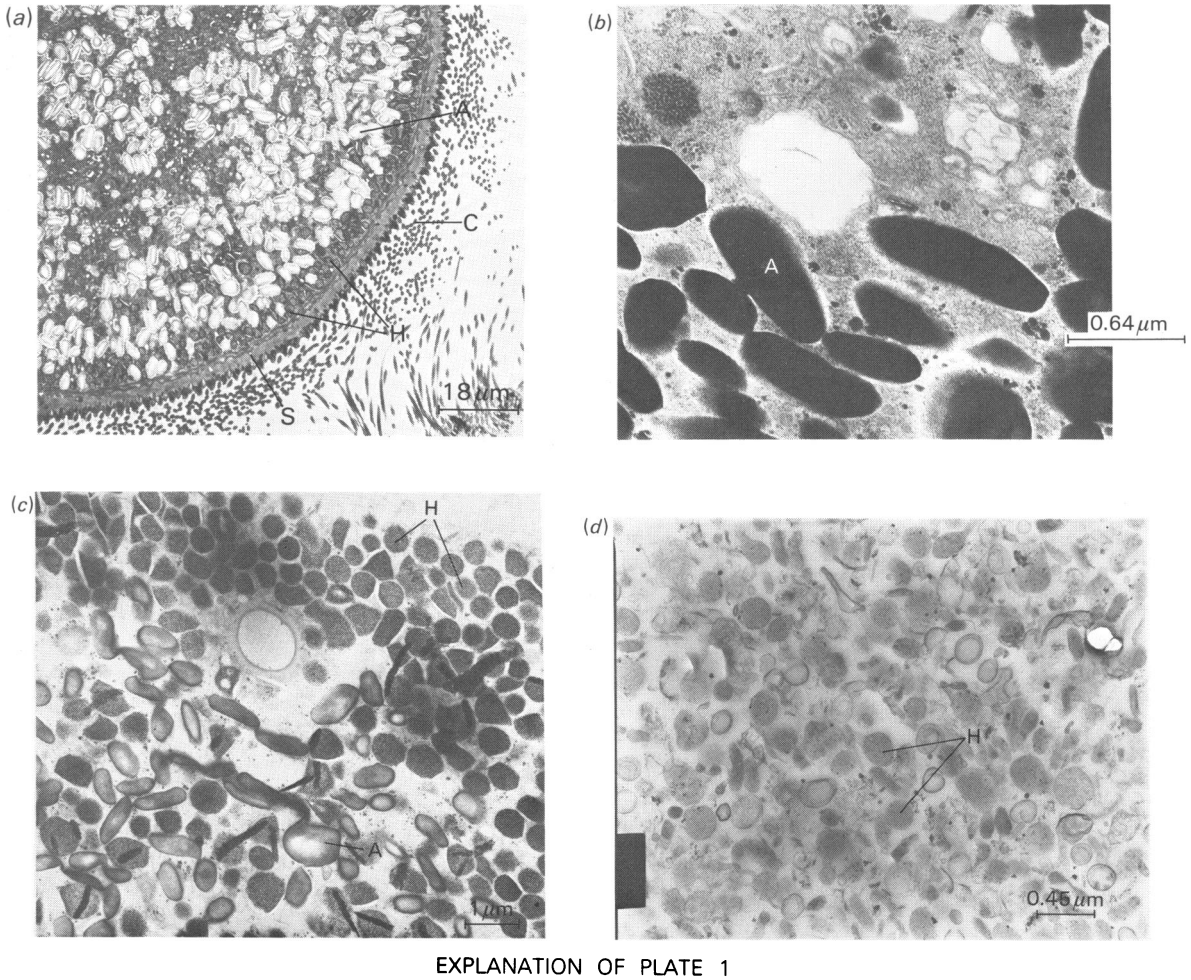
(Figs. 3 and 4), and this band contained 24.2% of the lactate dehydrogenase, 37.5% of the NADH oxidase and 35.6% of the NADH peroxidase. These values correspond to 8.7, 3 and 2% respectively of the total activities in the homogenate.

Electron microscopy

Plate 1(*a*) shows a thin section of a whole organism. The largest subcellular structures are ovoid, electron-translucent amylopectin bodies, which range in size from $1.2\,\mu m \times 0.5\,\mu m$ to $0.5\,\mu m \times 0.2\,\mu m$. Lying between these and the sub-pellicular layer a second smaller class of organelle is evident. These two types of organelle are also characterized in fractionated extracts and occur predominantly in fractions P_1 and P_2 respectively [Plates 1(*b*) and 1(*c*)]. Smaller membranous vesicles also occur in both fractions. The organelles which predominate in fraction P_2 resemble both in size and in granular appearance those microbodies previously observed in trichomonads, which are known to be sites of hydrogen production (Müller, 1980); Plate 1(*d*) shows these organelles from the gradient.

Assessment of extent of bacterial contamination

Experiments were carried out to determine if there was any contribution to enzyme activities by



EXPLANATION OF PLATE 1

Electron micrographs of sections of Dasytricha ruminantium and of derived subcellular fractions

(*a*) Intact organism showing cilia (C), sub-pellicular structures (S), hydrogenosomes (H) and amylopectin granules (A); (*b*) fraction P_1, enriched in amylopectin granules (A); (*c*) fraction P_2, enriched in hydrogenosomes (H), but also containing some amylopectin granules (A); (*d*) particles sedimented in aqueous sucrose to equilibrium density ($\rho = 1.18$ g/ml) from fraction P_2, showing hydrogenosomes (H).

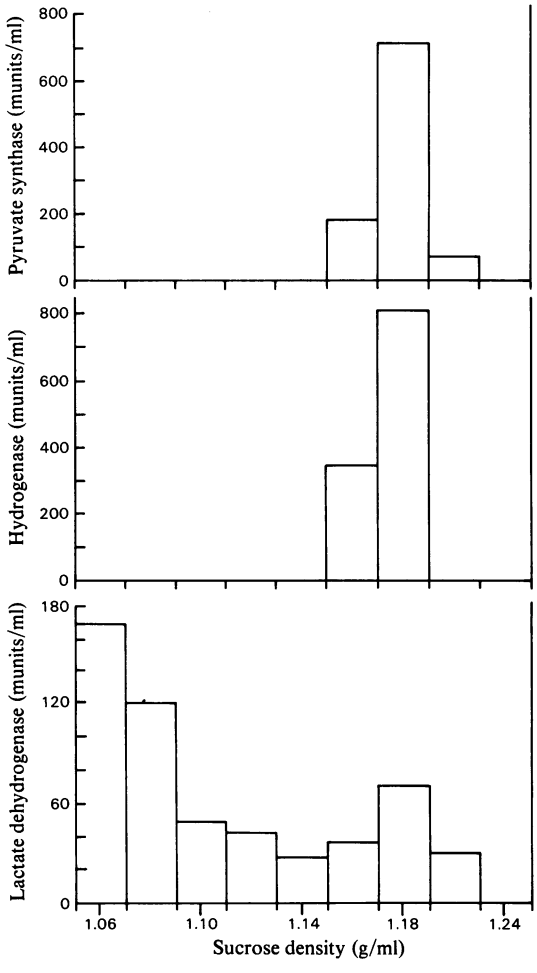


Fig. 3. *Distribution of enzymes (pyruvate synthase, hydrogenase and lactate dehydrogenase) after equilibrium density-gradient centrifugation in a sucrose gradient of the 10^5 g-min fraction*

Direction of sedimentability was from left to right. The specific activities of enzymes (nmol/min per mg of protein) at their maxima, and percentage recoveries (in parentheses), were as follows: pyruvate synthase, 4000 (73%); hydrogenase, 4533 (78%); lactate dehydrogenase, 400 (92%). Recovery of protein was 81%.

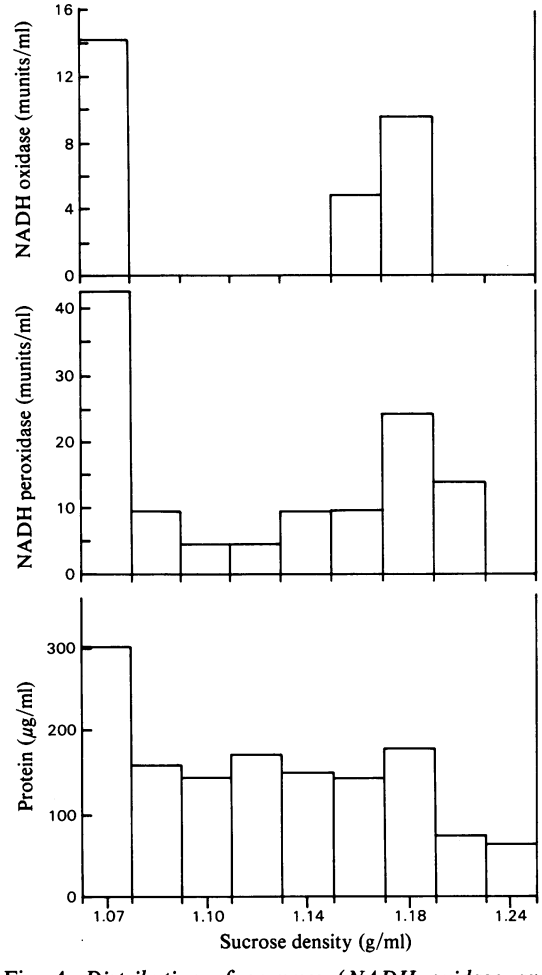


Fig. 4. *Distribution of enzymes (NADH oxidase and NADH peroxidase) and protein after equilibrium density-gradient centrifugation in a sucrose gradient of the 10^5 g-min fraction*

Direction of sedimentability was from left to right. The specific activities of enzymes at their maxima, and percentage recoveries (in parenthesis) were as follows: NADH oxidase, 53.3 (75%); NADH peroxidase, 133 (89%); protein, 81%.

ingested or attached bacteria. Diaminopimelic acid could not be detected in fractions; the lower limit of sensitivity of the assay is 0.5 μg/ml, which corresponds to approx. 2.5×10^7 bacteria/ml. The absence of detectable alkaline phosphatase also suggests the absence of bacterial contamination (Forsberg & Cheng, 1980).

A mixed population of rumen bacteria (from 600ml of rumen liquor, 10^{10}–10^{11} bacteria/ml suspended in 15ml of disruption buffer) was subjected to the motor-driven homogenizer and differential-centrifugation procedure described for *D. ruminantium*. Material sedimentable at 10^5 *g*-min contained pyruvate synthase, malate dehydrogenase (decarboxylating) and lactate dehydrogenase activities. Density-gradient centrifugation indicated that particles containing these enzymes were sedimented to the bottom of the tube (density >1.27g/ml). A small proportion (<2%) of the total lactate dehydrogenase activity remained heterogeneously

Table 3. *Sedimentability of pyruvate synthase in cell-free extracts of D. ruminantium prepared by different methods*
For details see the Materials and methods section.

	Particulate fraction (6×10^6 *g*-min)		Soluble fraction	
Breakage technique	Total enzyme units (%)	Recovery (%)	Total enzyme units (%)	Recovery (%)
Motor-drive homogenizer	171	65	—	—
Polytron homogenizer	258	161	—	—
Indole treatment	279	53	176	52
Digitonin treatment	183	109	2	3
Ultrasound treatment	251	95	12	12

spread through the gradient. The protozoal origin of enzymes was confirmed by using an alternative breakage technique. Indole lysis is specific for the disruption of protozoal cells and does not affect bacteria (Bailey & Howard, 1962). Release of enzymes thus confirms their protozoal origin. That pyruvate synthase was distributed between a particulate fraction and the supernatant indicates that this agent disrupts not only the limiting membrane of protozoa but also those of organelles. Table 3 shows the distribution of pyruvate synthase between fractions after different breakage procedures.

Discussion

The rumen protozoon *D. ruminantium* contains, like some other anaerobic protozoa, no organelles identifiable as mitochondria, no cyanide-sensitive respiration and no detectable cytochrome *c* [limits <2pmol of cytochrome *c*/mg of protein (N. Yarlett & D. Lloyd, unpublished work)]. The organelles described as mitochondria in the holotrich species *Dasytricha* and *Isotricha* by Grain (1966) have no typical mitochondrial ultrastructure, but are similar in appearance to the hydrogenosomes of the trichomonads.

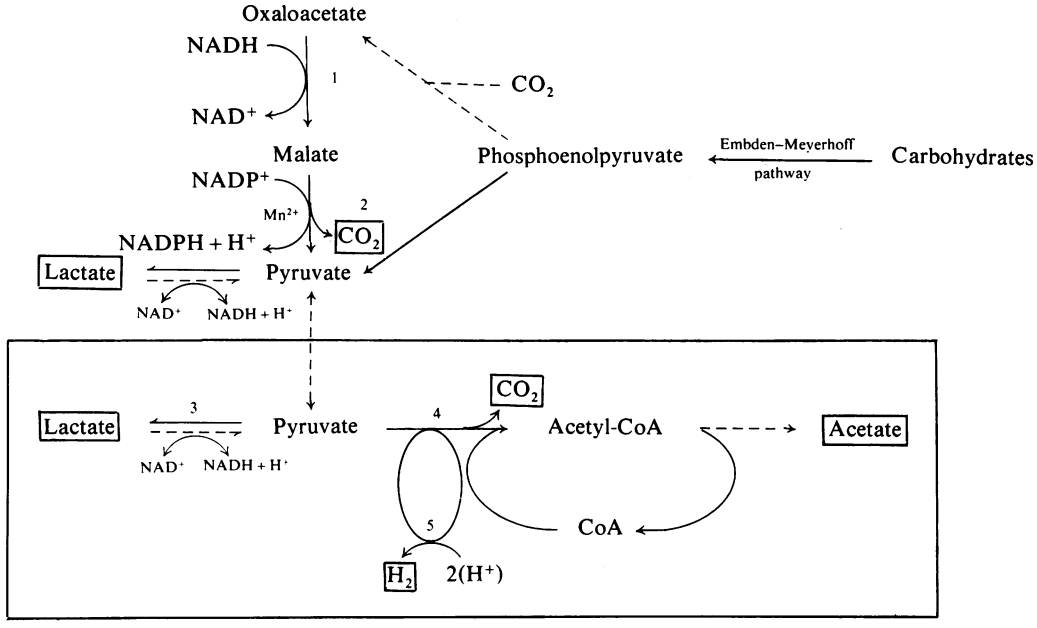
A microbody-like organelle of approx. 0.5–1.0μm diameter with a granular matrix is evident in sections of whole cells and can be enriched in a fraction sedimentable at 10^5 *g*-min. This fraction also contains a major percentage of the pyruvate synthase and hydrogenase activities, and therefore appears of similar enzymic characterization to fractions from trichomonads containing hydrogenosomes (Lindmark & Müller, 1973). A significant proportion (20%) of the lactate dehydrogenase is present in this fraction; this association has not been described for hydrogenosomes from other protozoa. Like the hydrogenosomes from *Trichomonas vaginalis* (Lindmark *et al.*, 1975), the organelle from *D. ruminantium* lacks succinate thiokinase and acetate:succinate CoA-transferase. In contrast with the findings with trichomonads, the malate dehydrogenase (decarboxylating) enzyme of *D. ruminantium* is non-sedimentable.

The equilibrium density of the hydrogenosomes from *D. ruminantium* was found to be 1.18g/ml, which is considerably less than the equilibrium density of hydrogenosomes from trichomonads, which is 1.26g/ml (Lindmark *et al.*, 1975).

Two distinct populations of acid-hydrolase-containing organelles are present, a large-particle population sedimenting at 10^4 *g*-min and containing α-L-arabinofuranosidase (EC 3.2.1.55) and α-D-galactosidase (EC 3.2.1.22), and a smaller-particle population sedimenting at 4×10^5 *g*-min characterized by the presence of acid phosphatase, β-D-glucosidase (EC 3.2.1.21) and α-D-glucosidase. A very-small-particle fraction sedimenting at 6×10^6 *g*-min may contain Golgi-derived vesicles, as indicated by the presence of β-D-glucuronidase (EC 3.2.1.31).

Hydrogen is a common end product of carbohydrate fermentation by many protozoa (Bragg & Reeves, 1962; Bauchop, 1971; von Brand, 1973); however, the mechanism of its production has only been described in very few species (Bauchop, 1971; Lindmark & Müller, 1973). In bacteria two distinct mechanisms of hydrogen formation exist, both involving the intermediate formation of pyruvate. In the Enterobacteriaceae pyruvate is converted into acetyl-CoA and formate via a thioclastic split; formate is normally further metabolized to H_2 and CO_2 by formate hydrogenlyase. Saccharolytic Clostridia also cleave pyruvate to acetyl-CoA, H_2 and CO_2, but formate is not an intermediate in the reaction. The absence of both forms of formate dehydrogenase (particulate and soluble) here confirms that, as in the trichomonads (Bauchop, 1971), H_2 is formed by a reaction similar to that found in the saccharolytic Clostridia.

It has been shown that monosaccharides or oligosaccharides can be fermented by gut anaerobes via a variety of pathways: (1) glycolysis; (2) three pathways involving 6-phosphogluconate; (3) a unique mechanism found only operating in the Bifidobacteria (Sokatch, 1969). The second system requires glucose 6-phosphate dehydrogenase for the generation of 6-phosphogluconate; as this enzyme was not detectable in the present study, it is likely



Scheme 2. *Proposed pathway of fermentation in D. ruminantium*
(1) Malate dehydrogenase; (2) malate dehydrogenase (decarboxylating); (3) lactate dehydrogenase; (4) pyruvate synthase; (5) hydrogenase. Enzyme activities associated with fraction P_2 are shown in a box.

that glucose is metabolized solely via the Embden–Meyerhoff–Parnas scheme of glycolysis, the enzymes of which have been detected in homogenates of *D. ruminantium* by A. Williams, N. Yarlett & C. Rainger (unpublished work).

Superoxide dismutase and catalase were not detectable, but a functional NADH oxidase may play a role in protection against dioxygen. That two oxygen-sensitive enzymes (pyruvate synthase and hydrogenase) are particulate, whereas the oxygen-tolerant enzyme malate dehydrogenase (decarboxylating) is non-sedimentable, suggests that compartmentation may also afford some protection.

The results of the study strongly suggest the presence of a hydrogenosomal organelle in the rumen protozoon *D. ruminantium*, and permits the formulation of Scheme 2.

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