Butyrate formation from glucose by the rumen protozoon *Dasytricha ruminantium*

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Production of butyrate by the holotrich protozoon *Dasytricha ruminantium* involves the enzymes of glycolysis, pyruvate:ferredoxin oxidoreductase, acetyl-CoA:acetyl-CoA C-acetyltransferase, 3-hydroxybutyryl-CoA dehydrogenase, 3-hydroxyacyl-CoA hydro-lyase, 3-hydroxyacyl-CoA reductase, phosphate butyryltransferase and butyrate kinase. Subcellular fractionation by differential and density-gradient centrifugation on sucrose gradients indicated that all those enzymes except pyruvate:ferredoxin oxidoreductase were non-sedimentable at $6 \times 10^6 g\text{-min}$. Butyrate kinase and phosphate butyryltransferase were associated with the large- and small-granule fractions. Thus, although metabolic reactions necessary for butyrate production proceed predominantly in the cytosol, hydrogenosomes play a key role in the conversion of pyruvate into acetyl-CoA.

The rumen holotrich protozoon *Dasytricha ruminantium* produces acetate, butyrate, lactate, carbon dioxide and hydrogen by the fermentation of various soluble sugars (Williams & Harfoot, 1976). Despite the importance of these metabolites to ruminant nutrition, little is known about the intermediary metabolism of this ciliate. Several hydrolases have been shown to be important in carbohydrate assimilation (Williams, 1979b); however, their subcellular localization remains unknown. In common with a number of other rumen ciliates, the production of acetate and hydrogen by *D. ruminantium* is localized within a specialized organelle, the hydrogenosome (Yarlett *et al.*, 1981, 1983, 1984; Snyers *et al.*, 1982). The absence of detectable levels of glucose-6-phosphate dehydrogenase (Yarlett *et al.*, 1981), a prerequisite for the generation of pyruvate via 6-phosphogluconate, led to the conclusion that pyruvate formation by this ciliate occurred solely via glycolysis; however, a functional glycolytic pathway has not been demonstrated.

In the present study we identified all the enzymes responsible for the formation of butyrate from glucose, and subcellular-localization studies revealed them to be of a non-sedimentable nature. The detection of sedimentable hydrolases indi-

cated the presence of characteristic lysosomal-like structures. These results, together with those of previous publications (Yarlett *et al.*, 1981, 1982), extend the knowledge of intermediary metabolism by the rumen ciliate *D. ruminantium*.

Methods and materials

Isolation of the organism

D. ruminantium was isolated from rumen contents by filtration as described by Williams & Yarlett (1982).

Disruption technique

The washed cell pellet was disrupted under N_2 in a buffer consisting of 0.23 M-sucrose/0.72 mM-EDTA/10 mM-Tris/HCl, pH 7.4, by using a motor-driven 10 ml ground-glass (Jencons) homogenizer, at 20 strokes/min for 3 min. The whole homogenate was centrifuged at 5000 $g\text{-min}$ (16×15 ml; MSE 18) at 4°C under N_2 to remove unbroken cells. The supernatant obtained was fractionated by differential centrifugation at $10^4 g\text{-min}$ (P1), $10^5 g\text{-min}$ (P2), $4 \times 10^5 g\text{-min}$ (P3) and $6 \times 10^6 g\text{-min}$ (P4) and a non-sedimentable fraction (S).

Enzyme assays

The glycolytic enzymes hexokinase (EC 2.7.1.1), aldolase (EC 4.1.2.13), phosphoglycerate mutase

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(EC 2.7.5.3), enolase (EC 4.2.1.11) and phosphofructokinase (EC 2.7.1.11) were assayed as described by Colowick & Kaplan (1962, 1970); glucosephosphate isomerase (EC 5.3.1.9), 3-phosphoglycerate kinase (EC 2.7.2.3), triosephosphate isomerase (EC 5.3.1.1) and pyruvate kinase (EC 2.7.1.40) were assayed by the method of Bergmeyer (1974). The butyrate-synthesizing enzymes, acetyl-CoA:acetyl-CoA C-acetyltransferase (EC 2.3.1.9), 3-hydroxybutyryl-CoA dehydrogenase (L-3-hydroxyacyl-CoA:NAD⁺ oxidoreductase) (EC 1.1.1.35), L-3-hydroxyacyl-CoA hydro-lyase (EC 4.2.1.17), 3-hydroxyacyl-CoA reductase (EC 1.3.99.2), phosphate butyryltransferase (butyryl-CoA:orthophosphate butyryltransferase) (EC 2.3.1.19) and butyrate kinase (ATP:butyrate phosphotransferase) (EC 2.7.2.7) were assayed as described by Miller & Jenesel (1979) by using the hydroxamate determination for butyrate kinase and phosphate butyryltransferase as described by Lipmann & Tuttle (1945). Enzyme activity is expressed as nmol/min per mg of protein. Spectrophotometric recording assays were performed at 37°C with a Gilford 250 spectrophotometer. Acid hydrolases were assayed at 39°C by determining the rate of *p*-nitrophenol release from the specific *p*-nitrophenyl derivative (0.015M) dissolved in 0.1M-phosphate buffer, pH 5.5; the acid phosphatase substrate (*p*-nitrophenyl acetate) was dissolved in 0.1M-sodium acetate/acetic acid buffer, pH 4.2. The *p*-nitrophenol was measured spectrophotometrically at 420nm in alkaline solution (Barrett & Heath, 1977). Protein was determined by the method of Lowry *et al.* (1951).

Results

Those enzymes involved in the glycolytic production of pyruvate and the synthesis of butyrate were detected in cell-free extracts of *D. ruminantium* (Table 1).

The production of butyrate from pyruvate proceeds via acetyl-CoA; the presence of pyruvate:ferredoxin oxidoreductase producing acetyl-CoA in *D. ruminantium* has previously been reported (Yarlett *et al.*, 1981). Enzymes involved in the further metabolism of acetyl-CoA to butyrate were assayed in homogenates and subcellular fractions. A slow acetyl-CoA-dependent oxidation of NADH by cell-free extracts of the ciliates suggested that the acetyl-CoA:acetyl-CoA C-acetyltransferase reaction was rate-limiting. 3-Hydroxybutyryl-CoA dehydrogenase was specific for NAD⁺. The reduction of hydroxyacyl-CoA to butyryl-CoA with NADH by cell-free extracts required the addition of FAD. In the absence of FAD the rate of NADH oxidation was undetectable. Initial rates due to NADH oxidase could be decreased to zero by incorporation of 2-mercaptoethanol in anaerobic assays.

Distribution profiles of enzymes after differential centrifugation of cell-free extracts revealed characteristic distribution patterns. The results of several such experiments are shown in Figs. 1–3. The enzymes pyruvate:ferredoxin oxidoreductase and hydrogenase were used as markers of hydrogenosomes, and typically sedimented at 10⁵g·min (Yarlett *et al.*, 1981). Distribution of acid hydrolases [indicators of lysosome-like organelles (Lloyd

Table 1. Activities of (a) glycolytic and (b) butyrate-forming enzymes in cell-free extracts of *D. ruminantium*. Specific activities are expressed as munits (nmol/min per mg of protein) ± s.d. for duplicate experiments where appropriate. Enzymes were assayed at the indicated pH.

Enzyme	pH	Specific activity in whole homogenate (munits)
(a) Glycolytic		
Hexokinase	9.0	47
Glucosephosphate isomerase	7.6	153 ± 34
Phosphofructokinase	8.5	583
Aldolase	7.5	9.0 ± 0.8
Triosephosphate isomerase	7.6	9.0
3-Phosphoglycerate kinase	7.6	66.0 ± 0.7
Phosphoglycerate mutase	7.0	1972 ± 272
Enolase	7.8	73
Pyruvate kinase	6.0	34
(b) Butyrate-forming		
Acetyl-CoA:acetyl-CoA C-acetyltransferase	6.5	1
3-Hydroxybutyryl-CoA dehydrogenase	6.5	5
L-3-Hydroxyacyl-CoA hydro-lyase	8.0	4
3-Hydroxyacyl-CoA reductase	7.0	3
Phosphate butyryltransferase	8.0	27
Butyrate kinase	7.4	58 ± 4

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

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 6×10^6 g-min. Percentage recoveries [based on enzyme units in cell-free extracts from two separate experiments, (a) and (b)] were: (a) pyruvate:ferredoxin oxidoreductase, 76%; malate dehydrogenase (decarboxylating), 58%; β -glucosidase, 97%; acid phosphatase, 47%; and protein, 86%; (b) α -D-glucosidase, 82%; α -D-galactopyranosidase, 98%; α -L-arabinofuranosidase, 135%; β -D-glucuronidase, 114%; and protein, 112%.

Fig. 2. Distribution of butyrate-forming enzymes in fractions obtained by differential centrifugation of a cell-free extract of *D. ruminantium*

For details of presentation, see Fig. 1. Percentage recoveries (based on enzyme units in the cell-free extract) were: 3-hydroxyacyl-CoA reductase, 100%; phosphate butyryltransferase, 62.5%; butyrate kinase, 99%; acetyl-CoA:acetyl-CoA C-acetyltransferase, 87%; 3-hydroxybutyryl-CoA dehydrogenase, 45%; L-3-hydroxyacyl-CoA hydrolyase, 79%; and protein, 102%.

& Cartledge, 1974)] were enriched in fractions sedimenting at 4×10^5 g-min. The distribution patterns obtained for those enzymes indicate that the structural integrity of subcellular organelles

was maintained throughout the experimental procedures. Malate dehydrogenase (decarboxylating) was non-sedimentable (at 6×10^6 g-min), and was used as an indicator of cytosolic enzyme location; the distributions of the enzymes responsible for the formation of pyruvate and butyrate were also of a non-sedimentable nature, indicating their cytosolic localization in the cell. The heterogeneous distribution of phosphate butyryltransferase and butyrate kinase (19.4% particulate, 43.1% non-sedimentable; and 66.2% particulate, 33% non-sedimentable respectively), may be the result of a real association of the enzyme with hydrogenosomes, a loose association with the organelle, or it may represent broad substrate

Fig. 3. Distribution of glycolytic enzymes in fractions obtained by differential centrifugation of a cell-free extract of *D. ruminantium*

For details of presentation, see Fig. 1. Percentage recoveries (based on enzyme units in the cell-free extract) were: aldolase, 42%; enolase, 58%; triosephosphate isomerase, 104%; hexokinase, 81%; phosphofructokinase, 69%; glucosephosphate isomerase, 121%; phosphoglycerate mutase, 101%; 3-phosphoglycerate kinase, 113%; and protein, 103%.

specificity of phosphate acetyltransferase and acetate kinase. Purified phosphate butyryltransferase and butyrate kinase from *Clostridium butyricum* were shown to be specific for their respective substrates (Twarog & Wolfe, 1962; Valentine & Wolfe, 1960).

Hydrogenosomes showed a mean equilibrium density of $1.18 \text{ g} \cdot \text{ml}^{-1}$ in sucrose (Figs. 4a and 4b), those acid hydrolase enzymes sedimenting at $4 \times 10^5 \text{ g} \cdot \text{min}$ had a mean equilibrium density in sucrose of $1.13 \text{ g} \cdot \text{ml}^{-1}$ (Figs. 4c–4e).

Fig. 4. Distribution of enzymes (pyruvate:ferredoxin oxidoreductase, hydrogenase, β -D-glucosidase, acid phosphatase and α -D-glucosidase) after equilibrium density-gradient centrifugation in a sucrose gradient of fractions obtained by differential centrifugation

The direction of sedimentability was from left to right. The specific activities of enzymes at their maxima and percentage recoveries (in parentheses) were as follows: (a) pyruvate:ferredoxin oxidoreductase, 4000 (73%); (b) hydrogenase, 4533 (78%); (c) β -D-glucosidase, 809 (83%); (d) acid phosphatase, 250 (66%), and (e) α -D-glucosidase, 74 (75%). Recovery of protein was (a, b) fraction sedimentable at $10^5 \text{ g} \cdot \text{min}$; (c–e) fraction sedimentable at $4 \times 10^5 \text{ g} \cdot \text{min}$. Enzyme activity is expressed in munits/ml, where 1 unit = $1 \mu\text{mol} \cdot \text{min}^{-1}$.

Discussion

Fermentative metabolism in the rumen ciliate *D. ruminantium* involves the utilization of mono-saccharides and leads to the formation of several volatile fatty acids (Howard, 1963). The mechanism of hydrogen and acetate production from pyruvate by this rumen ciliate was studied previously (Yarlett *et al.*, 1981) and it was shown that, in common with the situation in several other anaerobic protozoa, hydrogen was produced by a mechanism similar to that found in the saccharolytic clostridia, and was localized within an organelle termed the 'hydrogenosome' (Müller, 1980).

The synthesis of butyryl-CoA from acetyl-CoA by cell-free extracts of this ciliate involves the enzymes acetyl-CoA:acetyl-CoA C-acetyltransferase, 3-hydroxybutyryl-CoA dehydrogenase, 3-hydroxyacyl-CoA hydro-lyase and 3-hydroxyacyl-CoA reductase. The subsequent production of butyrate from butyryl-CoA occurs via the intermediate butyryl phosphate and generates ATP, involving the enzymes phosphate butyryltransferase and butyrate kinase. Hence the formation of butyrate from acetyl-CoA by *D. ruminantium* is also essentially the same as that for the saccharolytic clostridia and *Butyrivibrio fibrisolvens* (Miller & Jenesele, 1979).

Several species of rumen protozoa have been shown to be active producers of butyrate (Heald & Oxford, 1953; Abou Akkada & Howard, 1960), and all the aforementioned enzymes have also been detected in *Isotricha prostoma* (Prins, 1977).

In many eukaryotic cells, β -oxidation of fatty acids to yield acetyl-CoA may be compartmentalized within specialized organelles, peroxisomes or glyoxysomes (Müller, 1975), or mitochondria (Lloyd, 1974). However, in *D. ruminantium* those enzymes leading to the formation of butyryl-CoA have a cytosolic location within the cell. Phosphate butyryltransferase and butyrate kinase occur in both the particulate and non-sedimentable fractions. Several enzymes, usually organelle-bound in other organisms, are localized in the cytosol of *Tritrichomonas foetus*. These include all enzymes participating in the formation of succinate (Müller & Lindmark, 1974), catalase (Müller, 1969), and most of the NADH oxidase activity (Müller, 1969). In *D. ruminantium*, cytochromes have not been demonstrated (Yarlett *et al.*, 1981); neither have they been shown to be present in *T. foetus* (Ryley, 1955).

The fermentation of monosaccharides by anaerobes can occur by several different known pathways, but it was previously shown that *D. ruminantium* did not possess the enzymes necessary for a route alternative to glycolysis. In the present

study, all the glycolytic enzymes were detected, and subcellular studies indicated that they were predominantly non-sedimentable. The major route of ATP synthesis in *D. ruminantium* occurs via glycolysis. In *Trypanosoma brucei* it has been shown that the glycolytic conversion of glucose into 3-phosphoglyceric acid is associated with a microbody-like organelle termed the 'glycosome' (Oppendoes & Borst, 1977; Oduro, 1977; Taylor *et al.*, 1980). The trypanosomes are completely dependent upon glycolysis for ATP synthesis (Bowman & Flynn, 1976), and it has been suggested (Oppendoes & Borst, 1977) that compartmentation of glycolytic enzymes within the glycosome has evolved to allow the high optimal concentrations of enzymes, substrates and cofactors to be maintained. *D. ruminantium*, in common with the trichomonads (Taylor *et al.*, 1980), obtains 5 ATP molecules per molecule of glucose oxidized from the further conversion of pyruvate into acetate, and these later steps are localized within the hydrogenosome. The non-sedimentable nature of enzymes in *D. ruminantium* reported in the present study, and the non-sedimentable nature of enzymes in *Tritrichomonas foetus* (Taylor *et al.*, 1980) suggest that the glycosome may be confined to the trypanosomes.

Several hydrolases are known to be involved in the utilization of plant storage polysaccharides by holotrich ciliates; these include α -glucosidase, β -fructofuranosidase (Howard, 1959) and several glycosidases (Williams, 1979a; A. G. Williams, B. Ellis & G. S. Coleman, unpublished work). Sedimentable hydrolase activity (β -glucosidase, α -glucosidase, phosphatase and *N*-acetylglucosaminidase) is associated in *Tetrahymena pyriformis* with lysosomal structures (Müller *et al.*, 1966; Müller, 1972), which have an apparent isopycnic density in sucrose gradients of $1.26 \text{ g} \cdot \text{ml}^{-1}$. Sedimentable hydrolase activity has also been demonstrated in the rumen Ophryoscolecidae (Hellings *et al.*, 1981), and sucrose-gradient studies revealed isopycnic values of $1.24\text{--}1.26 \text{ g} \cdot \text{ml}^{-1}$. More than 80% of the recovered hydrolase activities in *D. ruminantium* were found to be sedimentable.

The results of the present study show that although metabolic reactions necessary for butyrate production proceed predominantly in the cytosol, hydrogenosomes play a key role in the conversion of pyruvate into acetyl-CoA, and show further similarities between some anaerobic protozoa and the saccharolytic clostridia.

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