The Presence and Function of Cytochromes in *Selenomonas ruminantium*, *Anaerovibrio lipolytica* and *Veillonella alcalescens*

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(Received 3 July 1973; revised 19 September 1973)

SUMMARY

Strains of *Selenomonas ruminantium*, *Anaerovibrio lipolytica* and *Veillonella alcalescens* contained cytochrome *b*. Peaks corresponding to cytochromes *a* and a carbon monoxide-binding pigment were also observed. By means of dual-wavelength experiments with crude membrane fractions it was established that cytochrome *b* functioned in anaerobic electron transport to fumarate. In *V. alcalescens* and one strain of *S. ruminantium* which reduced nitrate, anaerobic electron transport to nitrate was found. Glycerol 1-phosphate and NADH were active as hydrogen donors for cytochrome *b* reduction in glycerol-grown *A. lipolytica*, lactate and pyruvate were active in lactate-grown *V. alcalescens*, and NADH was active in lactose-grown *S. ruminantium*. Oxidative phosphorylation associated with these electron transfer systems might explain the high molar growth yields previously found for these micro-organisms. Fermentation products were measured in supernatant fluids of cultures grown in the presence and absence of nitrate. Nitrate did not influence the fermentation of lactose to lactate by *S. ruminantium*, and inhibited propionate formation by *V. alcalescens*.

INTRODUCTION

Propionic acid bacteria contain membrane-bound cytochrome-linked enzyme systems which catalyse electron transfer from lactate, glycerol 1-phosphate and NADH to fumarate (de Vries, van Wijck-Kapteyn & Stouthamer, 1972, 1973; Sone, 1972). The high molar growth yields of these bacteria may indicate that ATP is formed in anaerobic electron transport (de Vries *et al.* 1973).

Selenomonas ruminantium, *Anaerovibrio lipolytica* and *Veillonella alcalescens* are strictly anaerobic bacteria which, like the propionic acid bacteria, form propionate via the succinate pathway (Paynter & Elsdon, 1970; Hobson & Summers, 1967; Johns, 1951). *V. alcalescens* and certain strains of *S. ruminantium* form nitrite from nitrate, whereas *A. lipolytica* does not reduce nitrate (Hungate, 1966; Hobson & Mann, 1961). Hobson (1965) and Hobson & Summers (1967, 1972) reported high molar growth yields for *S. ruminantium* growing with glucose and for *A. lipolytica* growing with fructose or glycerol in carbohydrate-limited continuous cultures. They concluded that the formation of propionate was associated with the formation of additional ATP. The reactions which could be linked to ATP formation were discussed by Hobson & Summers (1972). In the present work we have shown membrane-bound, cytochrome *b*-linked electron transport systems capable of

using fumarate or nitrate as a hydrogen acceptor in these micro-organisms. The influence of nitrate on the fermentation pattern of *S. ruminantium* and *V. alcalescens* has also been studied.

METHODS

Bacteria and media. *Selenomonas ruminantium* strains 6, 21 and GFA were isolated from the rumen. *S. ruminantium* strains 6 and 21 have been described previously (Hobson & Mann, 1961; Hobson, 1965). Strain GFA (germ-free animal strain) has been used in gnotobiotic lamb work (P. N. Hobson, unpublished results). The strains were maintained as stab cultures in the basal medium of Paynter & Elsdon (1970), with agar (Oxoid; 15 g/l) and with lactose (10 g/l) instead of sodium lactate.

Anaerovibrio lipolytica 5S and LV2 were used. Strain 5S has been previously used (Hobson, 1965; Hobson & Summers, 1967). The strains were maintained as stab cultures in a medium containing (g/l distilled water): casein hydrolysate (Oxoid), 7.5; yeast extract (Difco), 6.0; agar (Oxoid) 1.5; K_2HPO_4 , 0.45; KH_2PO_4 , 0.45; $NaHCO_3$, 6.0; cysteine HCl, 0.5; and glycerol, 13.4. $NaHCO_3$, cysteine HCl and glycerol were added as a filter-sterilized solution.

All media were prepared and incubated under CO_2 , and inoculations were carried out under a stream of CO_2 made oxygen-free by passing it through a tube filled with copper turnings and heated at 400 °C (Hobson & Mann, 1961).

Veillonella alcalescens was obtained from the Dental Institute of the Free University, Amsterdam, The Netherlands. The strain was isolated from dental plaque, and identified by morphological and biochemical characteristics (Rogosa, 1964, 1971). The strain was maintained as a stab culture in small bottles completely filled with the basal medium used for propionic acid bacteria (de Vries *et al.* 1972) supplemented with sodium lactate (10 g/l) and cysteine (0.5 g/l). All stock cultures were incubated at 37 °C and transferred every two weeks.

Fermentor cultures. Fermentor cultures were grown in the Chemap Vibro-glass-fermentor (Chemap AG, Männedorf ZH, Switzerland) with a pH controller (de Vries *et al.* 1973) in the media described above. *Selenomonas ruminantium* was also cultured with lactose replaced by glycerol (10 g/l) and galactose (0.5 g/l). *S. ruminantium* was cultured at pH 6.5, *Anaerovibrio lipolytica* and *Veillonella alcalescens* were cultured at pH 6.8. In some experiments potassium nitrate was added to a final concentration of 5 g/l. Cultures were kept anaerobic with a stream of oxygen-free CO_2 (*A. lipolytica*) or $N_2 + 5\% CO_2$ (*S. ruminantium* and *V. alcalescens*). Growth was assayed turbidimetrically at 660 nm (1 cm cuvettes). Bacterial suspensions and crude membrane fractions were prepared from cultures harvested at extinctions between 0.50 and 0.90.

Preparation of bacterial suspensions, extracts and crude membrane fractions. Suspensions, extracts and membrane fractions were prepared as described by de Vries *et al.* (1972, 1973). All buffer solutions were supplemented with cysteine (final concentration 0.5 g/l). Membranes were suspended in 0.04 M-potassium phosphate buffer containing 0.005 M- $MgCl_2$ and cysteine (0.5 g/l), pH 7.0. During the preparation of the crude membrane suspensions from *Anaerovibrio lipolytica* any exposure to oxygen was avoided by filling centrifuge tubes under a stream of CO_2 and by centrifuging cultures and extracts under CO_2 . Cells of *A. lipolytica* were not washed. Dry weight of bacterial suspensions was measured by filtration. Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as standard.

Measurement of spectra and reduction kinetics of cytochrome b. Reduced minus oxidized

difference spectra and carbon monoxide difference spectra of bacterial suspensions were recorded with an Aminco Chance spectrophotometer (American Instruments Co., Washington, U.S.A.). Cytochrome *b* was estimated from the dithionite-reduced minus oxygen-oxidized difference spectra using $C = 17500 \text{ l/mol/cm}$ (Deeb & Hager, 1964). The reduction of cytochrome *b* by membrane suspensions was followed with the Aminco Chance spectrophotometer set in dual-wavelength position (560 and 578 nm). Amounts (50 μl) of 0.2 M-NADH, 0.8 M-L-lactate, glycerol, glycerol 1-phosphate, pyruvate, fumarate or potassium nitrate were added to cuvettes containing about 3 ml of the membrane suspension.

Determination of fermentation products and nitrite. Fermentation products were measured in supernatant fluids obtained from cultures grown with an excess of substrate and harvested at different extinction values (0.40 to 0.90). Acetate, propionate and succinate were measured as described previously (de Vries *et al.* 1972). L-Lactate and D-lactate were measured enzymically (Bergmeyer, 1970). Nitrite was measured as described by van 't Riet, Stouthamer & Planta (1968). Corrections were made for fermentation products formed in the absence of added substrate. Values for glycerol were corrected for fermentation products (mainly L-lactate) formed from galactose which was added to prevent lysis.

Chemicals. NADH, L-lactate dehydrogenase and D-lactate dehydrogenase were obtained from C. F. Boehringer und Soehne GmbH, Mannheim, Germany. Sodium DL-glycerol 1-phosphate and sodium L-lactate were obtained from Serva, Entwicklungslabor, Heidelberg, Germany. Sodium fumarate was obtained from BDH Ltd, Poole, Dorset. Glycerol, lactose, galactose, sodium pyruvate and potassium nitrate were obtained from E. Merck, Darmstadt, Germany. 2-*n*-Heptyl-4-hydroxy-quinoline-*N*-oxide (HOQNO) was obtained from Sigma Chemical Co., St Louis, Missouri, U.S.A.

RESULTS

Selenomonas ruminantium

Each of the three strains of *Selenomonas ruminantium* lysed when cultured in the basal medium with glucose. This did not occur if lactose or galactose replaced glucose. Similar observations were made by Dirrar & Collins (1972) for *Lactobacillus plantarum*. E. B. Collins suggested that *L. plantarum* has difficulty synthesizing galactosamine from glucose. All strains of *S. ruminantium* formed lactate as the main fermentation product from lactose. Small and variable amounts of acetate, propionate and succinate were also found. These results are similar to those of Hobson (1965) who showed that lactate was the main fermentation product in batch cultures of *S. ruminantium* growing with glucose. Despite the addition of small amounts of galactose to the growth medium, growth of all strains of *S. ruminantium* on glycerol was poor and irregular and did not appreciably exceed growth on galactose without added glycerol. However, when *S. ruminantium* strain GFA was cultured with glycerol and nitrate, good growth was obtained, large amounts of nitrite accumulated in the growth medium and L-lactate was the main fermentation product (Table 1). During growth on lactose and nitrate L-lactate was also the main fermentation product. In this case, only traces of nitrite were formed (Table 1). D-Lactate was not found in cultures of *S. ruminantium* strain GFA.

Whole organisms of *Selenomonas ruminantium* strain 21 contained cytochrome *b* (peaks at 558, 529 and 424 nm) (Fig. 1, I). Cytochromes a_1 and a_2 did not seem to be present. Similar spectra were found for *S. ruminantium* strains 6 and GFA. A small amount of a carbon monoxide-binding pigment (peak at 421 nm, trough at 432 nm) was found in all

Table 1. *The influence of nitrate on the fermentation pattern of Selenomonas ruminantium strain GFA and Veillonella alcalescens*

Bacterium	Substrate*	Products†					Total amount of fermentation products (mm)
		L-Lactate	Acetate	Pro-pionate	Suc-cinate	Nitrite	
<i>Selenomonas ruminantium</i>	Lactose	88	5	5	2	—	12.7; 23.4
<i>S. ruminantium</i>	Lactose + KNO ₃	92	3	4	1	4	14.3; 22.4
<i>S. ruminantium</i>	Glycerol + KNO ₃	67	13	15	5	64	7.8; 19.2
<i>Veillonella alcalescens</i>	Lactate	—	48	52	0	—	27.7‡; 39.4‡
<i>V. alcalescens</i>	Lactate + KNO ₃	—	90	10	0	138	10.6; 29.6‡

* *Selenomonas ruminantium* was grown with an excess lactose (30 mm) or glycerol (110 mm), in the absence and presence of potassium nitrate (50 mm). *Veillonella alcalescens* was grown with sodium lactate (30 or 40 mm) or sodium lactate (30 mm) and potassium nitrate (100 mm).

† Values, expressed as the percentage of the total amount of fermentation products, represent the mean of the percentages found in two individual supernatant fluids from cultures grown to different extinction values and containing different total amounts of fermentation products (last column). Individual percentages did not differ significantly from each other.

‡ Lactate had been consumed completely.

Fig. 1

Fig. 2

Fig. 1. Dithionite-reduced versus oxygen-oxidized difference spectra of *Selenomonas ruminantium* 21 (I), *Anaerovibrio lipolytica* 5s (II) and *Veillonella alcalescens* (III). The suspensions contained 10.4, 2.8 and 7.6 mg dry wt bacteria/ml respectively.

Fig. 2. (Reduced plus CO) minus reduced difference spectra of *Selenomonas ruminantium* 21 (I), *Anaerovibrio lipolytica* (II) and *Veillonella alcalescens* (III). The suspensions contained 5.2, 2.8 and 7.6 mg dry wt bacteria/ml respectively.

Table 2. Cytochrome *b* content of a number of anaerobic and facultatively aerobic bacteria grown anaerobically

Bacteria	Growth substrate	Cytochrome <i>b</i> content (μ mol/g bacterial dry wt)
<i>Selenomonas ruminantium</i> 6	Lactose	0.19
<i>S. ruminantium</i> 21	Lactose	0.13
<i>S. ruminantium</i> GFA	Lactose	0.13
<i>S. ruminantium</i> GFA	Lactose + KNO ₃	0.13
<i>Anaerovibrio lipolytica</i> LV2	Glycerol	0.34
<i>A. lipolytica</i> 5S	Glycerol	0.34
<i>A. lipolytica</i> 5S	Fructose	0.44
<i>Veillonella alcalescens</i>	Lactate	0.05
<i>V. alcalescens</i>	Lactate + KNO ₃	0.11
<i>Propionibacterium freudenreichii</i>	Lactate	0.23*
<i>Proteus mirabilis</i>	Glucose	0.20†
<i>Haemophilus parainfluenzae</i>	‡	0.50‡
<i>Escherichia coli</i>	Glucose	0.16§

* de Vries *et al.* (1972).

† E. G. v.d. Beek, Free University, The Netherlands (personal communication).

‡ Sinclair & White (1970). The bacterium was grown anaerobically with fumarate as a hydrogen acceptor.

§ Newton, Cox & Gibson (1972).

strains (Fig. 2, I). The cytochrome *b* content of *S. ruminantium* (3 strains) is shown in Table 2.

Although the crude membrane fraction from *Selenomonas ruminantium* strain GFA was not prepared anaerobically, NADH readily reduced cytochrome *b* (Fig. 3, I). After the initial rapid reduction of cytochrome *b* by NADH, an 'aerobic steady state' was reached in which the reduction of cytochrome *b* by NADH was balanced by its oxidation by the oxygen present in the reaction mixture. After 4 min the oxygen became limiting and an 'anaerobic steady state' (78 % reduction as compared with reduction by dithionite) was reached at about 6 min after addition of NADH (Fig. 3, I). Fumarate oxidized cytochrome *b* to a steady state of 24 % reduction. About 7 min after addition of fumarate, all cytochrome *b* present returned to the oxidized state. When more NADH was added, the steady state with fumarate was prolonged accordingly. This indicates that the conversion of cytochrome *b* to the fully oxidized state was due to exhaustion of NADH from the suspension. In the presence of 2-*n*-heptyl-4-hydroxy-quinoline-*N*-oxide (HOQNO), an inhibitor of cytochrome *b* function (Cox *et al.* 1970), both the aerobic steady state and the steady state with fumarate were prolonged markedly (Fig. 3, II). Similar results were found for *S. ruminantium* strains 21 and 6. Strain GFA contained a cytochrome *b*-linked electron transport system from NADH to nitrate (Fig. 3, III).

Anaerovibrio lipolytica

Hobson (1965) showed that *Anaerovibrio lipolytica* formed propionate and succinate as the main fermentation products from glycerol. Glycerol-grown *A. lipolytica* 5S contained cytochrome *b* (Fig. 1, II; Table 2). Peaks (594 nm; 636 nm) corresponding to cytochromes *a* were also observed (Fig. 1, II). The carbon monoxide difference spectrum (Fig. 2, II) shows the presence of a carbon monoxide-binding pigment. Similar spectra were found for *A. lipolytica* LV2.

Fig. 3. Reduction and re-oxidation of cytochrome *b* with NADH and fumarate respectively by a crude membrane fraction (4.2 mg protein/ml) from *Selenomonas ruminantium* strain GFA in the absence (I) and presence (II) of 0.1 mM-HOQNO. Re-oxidation of cytochrome *b* with nitrate (III) by a crude membrane fraction (6.4 mg protein/ml) from *S. ruminantium* strain GFA grown in the presence of nitrate.

Fig. 4. Reduction of cytochrome *b* with glycerol 1-phosphate (I) and NADH in the absence (II) and presence (III) of 0.025 mM-HOQNO by a crude membrane fraction (3.2 mg protein/ml) from *Anaerovibrio lipolytica* 5s. Re-oxidation of cytochrome *b* by fumarate.

Anaerovibrio lipolytica 5s was selected for further investigations. Crude membrane fractions only showed activity when organisms were not washed and any exposure of fractions to oxygen was avoided. Results of a typical experiment are shown in Fig. 4. Before the addition of substrates the reduction level of cytochrome *b* was about 23 %

Fig. 5. Reduction of cytochrome *b* with NADH (I), lactate (II) and pyruvate (IV), and re-oxidation of cytochrome *b* with fumarate (II) and nitrate (IV) by crude membrane fractions (about 4 mg protein/ml) from *Veillonella alcalescens* grown in the absence (I, II, III) and presence (IV) of nitrate. Line III represents cytochrome *b* reduction in the presence of 0.025 mM-HOQNO.

(as compared with dithionite). NADH reduced cytochrome *b* to 66 %, and fumarate oxidized cytochrome *b* to a steady-state value of 23 % reduction (Fig. 4, II). Glycerol 1-phosphate reduced cytochrome *b* with a lower rate than NADH (Fig. 4, I). Glycerol did not reduce cytochrome *b*. HOQNO retarded the reduction of cytochrome *b* by NADH (Fig. 4, III).

Veillonella alcalescens

Growing cultures of *Veillonella alcalescens* formed equimolar amounts of acetate and propionate from lactate (Table 1). Similar results have been reported with bacterial suspensions (Johns, 1951). With nitrate added only traces of propionate were formed and acetate was the main fermentation product (Table 1).

Cytochromes *b*, *a*₁ and *a*₂ were found in *Veillonella alcalescens* (Fig. 1, III). The amount of cytochrome *b* was lower than that present in *Selenomonas ruminantium* and *Anaerovibrio lipolytica* (Table 2). A small amount of a carbon monoxide-binding pigment was found (Fig. 2, III). Crude membrane fractions from *V. alcalescens* reduced cytochrome *b* in the presence of lactate and pyruvate (Fig. 5, II and IV respectively). HOQNO retarded cytochrome *b* reduction (Fig. 5, III). In the presence of NADH cytochrome *b* reduction was very slow (Fig. 5, I). The extent of oxidation of cytochrome *b* with fumarate and nitrate was smaller in *V. alcalescens* than in *S. ruminantium* and *A. lipolytica*.

DISCUSSION

Selenomonas ruminantium, *Anaerovibrio lipolytica* and *Veillonella alcalescens* contain substantial amounts of cytochrome *b* and small amounts of other cytochromes. The cytochrome *b* levels of these anaerobic bacteria and of *Propionibacterium freudenreichii* are not very different from those found in certain facultatively aerobic bacteria (Table 2). Cytochromes have been reported in a number of other anaerobic bacteria which form

propionate (or succinate) via the succinate pathway: in species of *Bacteroides* (White, Bryant & Caldwell, 1962; Rizza, Sinclair, White & Cuorant, 1968), in *Vibrio succinogenes* (Jacobs & Wolin, 1963), in propionic acid bacteria (Chaix & Fromageot, 1942; de Vries *et al.* 1972; Sone, 1972), and in *Desulfovibrio gigas* (Hatchikian & Le Gall, 1972). In these bacteria cytochrome *b* (or *c*) was shown to be involved in anaerobic electron transport to fumarate. Our dual-wavelength experiments with membrane suspensions showed a similar function for cytochrome *b* in *S. ruminantium*, *A. lipolytica* and *V. alcalescens*. In *S. ruminantium* and *V. alcalescens* cytochrome *b* was also involved in anaerobic electron transport to nitrate.

In *Selenomonas ruminantium*, fermenting lactose, NADH was a hydrogen donor for cytochrome *b* reduction. Special anaerobic techniques were not required during the preparation of the membrane suspensions. *Selenomonas ruminantium* formed L-lactate as the main fermentation product from lactose both in the absence and presence of nitrate. Thus the cytochrome *b*-linked electron transport systems to fumarate and nitrate present in membrane fractions did not function in batch cultures with lactose, and NADH formed in the glycolytic system was used preferentially in the conversion of pyruvate to lactate. *Selenomonas ruminantium* was shown to convert glycerol to propionate (Hobson & Mann, 1961). However, we could not obtain growth on glycerol. P. N. Hobson (personal communication) has also observed that the strains have lost the ability to ferment glycerol. In our work one strain of *S. ruminantium* grew on glycerol when nitrate was added. Small amounts of propionate and large amounts of L-lactate and nitrite were formed. Apparently, consumption of excess hydrogen equivalents in nitrate reduction facilitated conversion of glycerol. Because the fermentation balance of glycerol without added nitrate could not be measured in the present investigation, conclusions on the influence of nitrate on propionate formation by *S. ruminantium* could not be drawn.

In *Anaerovibrio lipolytica* grown on glycerol, NADH and glycerol 1-phosphate were hydrogen donors for cytochrome *b* reduction. Apparently, glycerol is fermented by this bacterium via glycerol-kinase, as reported for propionic acid bacteria (de Vries *et al.* 1973). Membrane suspensions of *A. lipolytica* only showed activity when prepared under strictly anaerobic conditions. This indicates that this bacterium does not possess an oxygen-removing system.

de Vries *et al.* (1973) explained the high molar growth yield of *Propionibacterium freudenreichii* (65 g bacteria/mol glucose) by the formation of two moles of ATP in the anaerobic electron transport from NADH to fumarate. This explanation could also be valid for the high molar growth yields of *Selenomonas ruminantium* (62 g bacteria/mol glucose) and *Anaerovibrio lipolytica* (60 g bacteria/mol fructose) in carbohydrate-limited continuous cultures (Hobson, 1965; Hobson & Summers, 1967). From the molar growth yield of *A. lipolytica* on glycerol (22 g bacteria/mol glycerol) Hobson (1965) concluded that two moles of ATP were formed per mole of glycerol fermented. One mole of ATP must be formed in the conversion of glycerol to pyruvate, the other one could be formed in anaerobic electron transfer from glycerol 1-phosphate to fumarate as previously shown for propionic acid bacteria (de Vries *et al.* 1973).

Veillonella alcalescens ferments lactate to propionate, acetate, H₂ and CO₂ via lactate-malate transhydrogenase and a clostridial-type thioclastic system (Johns, 1951; Allen, 1969). Thus NADH is not formed in the fermentation pathway. This is in accordance with our observation that NADH was only a weak hydrogen donor for cytochrome *b* reduction. Instead, lactate and pyruvate functioned as hydrogen donors. The extent of oxidation of cytochrome *b* by membrane suspensions from *V. alcalescens* with fumarate

was only small. This indicates that membrane suspensions contained only weak fumarate reductase activity. The absence of propionate formation and the accumulation of acetate and nitrite in cultures of *V. alcalescens* growing with lactate and nitrate, indicate that the succinate pathway does not function under these conditions and that the hydrogen equivalents formed are preferentially consumed in nitrate reduction. Inderlied & Delwiche (1973) showed that nitrate reductase of *V. alcalescens* is membrane-bound and has characteristics of both assimilatory and dissimilatory nitrate reduction. The presence and function of cytochromes in this micro-organism were not studied by these authors. We are presently undertaking growth experiments with *V. alcalescens* to show whether electron transfer to fumarate and nitrate is coupled to oxidative phosphorylation.

We are grateful to Dr P. N. Hobson (The Rowett Research Institute, Aberdeen, Scotland) for the gift of *Selenomonas ruminantium* and *Anaerovibrio lipolytica* and for helpful discussion and advice. We also thank Professor Dr A. H. Stouthamer (Free University, Amsterdam, The Netherlands) for his interest and advice throughout our work.

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