Influence of Culture E_h on the Growth and Metabolism of the Rumen Bacteria *Selenomonas ruminantium*, *Bacteroides amylophilus*, *Bacteroides succinogenes* and *Streptococcus bovis* in Batch Culture

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One facultatively and three strictly anaerobic rumen bacteria were grown in pH-controlled anaerobic batch cultures in which the E_h of the medium was regulated by the addition of titanium (III) citrate solution at values below -50 mV and potassium ferricyanide above -50 mV. Growth occurred over a wide range of E_h , with the maximum limit being $+360$, $+250$, $+175$ and $+414$ mV for *Selenomonas ruminantium*, *Bacteroides amylophilus*, *Bacteroides succinogenes* and the aerotolerant *Streptococcus bovis*, respectively. Changes in E_h had little influence on the growth yield or ratios of fermentation end-products in these bacteria over a fairly wide range, although the specific growth rate of all species tended to decline at E_h values above 0 mV. Abnormal, elongated forms of *Sel. ruminantium* and *B. succinogenes* predominated at high E_h . It was concluded that O_2 , and not a high E_h , is the toxic factor in 'oxidized' anaerobic growth medium, and that it would not normally be necessary to regulate E_h closely in experiments where the growth and metabolism of these bacteria is under study, provided that O_2 -free conditions were maintained.

INTRODUCTION

The growth of strictly anaerobic bacteria, including most species from the rumen, is often said to require the exclusion of O_2 from the gas phase and culture medium, and additionally a low redox potential (E_h) in the medium (Hungate, 1969; Jones & Pickard, 1980). These conditions, which simulate the native habitat of the organisms, such as rumen fluid, are obtained by the reductive removal of any O_2 present as an impurity in the CO_2 or other gas phase (Hungate, 1969) and by the addition of reducing agents to the growth medium (Hungate, 1969; Brock & O'Dea, 1977; Jones & Pickard, 1980). The reducing agents effect both the removal of oxygen and the lowering of E_h . As a consequence, it is sometimes difficult to tell whether the growth-enhancing effect of reducing agents is due to the removal of toxic O_2 or to the creation of a favourable E_h .

Walden & Hentges (1975) examined the effects of O_2 and E_h separately on the growth of three species of strict anaerobes isolated from human faeces, and found that these bacteria grew well at an E_h of $+325$ mV in the absence of O_2 but not at all at an E_h of -50 mV in the presence of O_2 . *Clostridium acetobutylicum* had previously been shown to behave in a similar manner (O'Brien & Morris, 1971). Thus it would appear that it is molecular O_2 , or one of its derivatives (Morris, 1979), rather than a high E_h which inhibits the growth of strict anaerobes in 'oxidized' medium. The E_h of rumen fluid can vary from -145 mV to -260 mV (Barry *et al.*, 1977; Marounek *et al.*, 1982) and similarly the E_h of culture medium depends on the reducing agent used (Jones & Pickard, 1980). We therefore undertook the present study to examine the influence of changing E_h on the growth and metabolism of some common rumen bacteria under oxygen-free conditions.

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METHODS

Organisms. *Selenomonas ruminantium* Z108, *Streptococcus bovis* ES1, *Bacteroides amylophilus* WP91 and *Bacteroides succinogenes* BL2 were isolated from rumen fluid at this Institute. These bacteria were maintained in medium 2 of Hobson (1969), and are held in the Institute's culture collection.

Culture media. *Sel. ruminantium* Z108 and *Strep. bovis* ES1, isolated for their ability to grow in medium free from amino N, were grown in the same simple defined medium as that used previously (Wallace, 1978), containing glucose as sole source of carbon and ammonia as sole source of nitrogen, except that 0.025% (w/v) cysteine hydrochloride and 0.025% (w/v) $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ replaced sodium dithionite as reducing agent and 0.5 mM-*n*-valeric acid was added to the medium. The last ingredient was essential for the growth of this strain of *Sel. ruminantium*. *Bacteroides amylophilus* WP91 was grown in a defined medium containing maltose, the same as that used for the growth of this species by Hobson & Summers (1967) except that the medium was reduced by a mixture of 0.025% (w/v) cysteine hydrochloride and 0.025% (w/v) $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ instead of cysteine alone. A trace metals solution containing nitrilotriacetic acid (Clark & Holms, 1976) was also added, mainly to prevent precipitation of salts. *Bacteroides succinogenes* BL2 was grown in the liquid form of medium 2 of Hobson (1969) modified so that glucose was the only sugar present, the concentration of clarified rumen fluid was 30% (v/v) and so that the reducing agents were again 0.025% (w/v) cysteine hydrochloride and 0.025% (w/v) $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ rather than cysteine alone. Sugars, vitamins and reducing solutions were sterilized by filtration, and the remainder of the ingredients were autoclaved at 121 °C for 15 min.

Batch culture apparatus. The culture vessel was a round-bottomed glass tube of working volume 300 ml, closed by a rubber bung with ports for gas entry and exit, pH and redox electrodes, sampling, and input of pH and E_h regulating solutions, and was maintained at 39 °C in a water bath. The culture was mixed by bubbling with CO_2 , at a rate of approximately 0.4 l min^{-1} , from which O_2 had been removed by passing the gas through a column (18 × 200 mm) of copper turnings maintained at 350 °C (Hungate, 1969). The CO_2 was passed through a sterile filter before entering the vessel, and gas was allowed to efflux via a bunsen valve. The pH of the culture (6.5) was controlled automatically by an EIL model 91B pH controller (EIL, Richmond, Surrey, UK) connected to a peristaltic pump (Minipump; Schuco Scientific, London, UK) which delivered 2 M-NaOH to the vessel.

The bacteria were studied in a series of batch cultures, each regulated at a different E_h . The E_h of the cultures was measured using a combination redox electrode (CT Pt 10/RP; Russell pH, Auchtermuchty, Fife, UK) consisting of a Pt electrode and a KCl/Ag/AgCl reference system. This electrode was connected to an EIL 91B pH controller, and E_h was controlled automatically at values higher than -50 mV by the addition of filter-sterilized 0.2 M-potassium ferricyanide solution, delivered by a Minipump. Lower E_h values were maintained manually by additions via a syringe of autoclaved 0.1 M-titanium (III) citrate (Zehnder & Wuhrmann, 1976), a method which resulted in fluctuations in E_h of no more than 30 mV during the course of an experiment. E_h values are expressed relative to the standard hydrogen electrode, and the combination electrode was calibrated before and after each experiment by immersing it for 30 min in 0.1 M-potassium phosphate (pH 7.0) saturated with quinhydrone. The pH of the quinhydrone solution was measured, and the E_h of the solution was calculated using an r_h value for quinhydrone of 23.2 and assuming a reference electrode potential of 235 mV.

Inoculation and sampling. Both procedures were carried out under O_2 -free CO_2 . The inoculum was an 18 h culture (50 ml) of bacteria in the same growth medium, except for *B. succinogenes*, which was a 40 h culture. This was added to 250 ml of sterile medium in the growth vessel. Samples (5 ml) were removed periodically by syringe, the turbidity was taken immediately, then the sample was frozen at -50 °C, and stored at this temperature until it could be analysed.

Analytical methods. The turbidity of cultures at 640 nm was determined using a Cecil CE595 double-beam spectrophotometer. Suspensions were diluted to an absorbance of <0.6, where the absorbance was directly proportional to cell density. The relationship between turbidity and dry weight for each species was determined by the method of Jenkinson *et al.* (1979), except that washed suspensions were dried at 105 °C for 18 h before weighing. Cultures were examined microscopically at regular intervals to confirm their purity.

The concentration of substrates and products in the culture medium was determined by thawing the stored sample of culture, and removing bacteria by centrifugation (12000 g, 20 s). All assays were done using the supernatant fluid. Glucose was measured by an automated glucose oxidase method (Morley *et al.*, 1968). Maltose was estimated from the glucose released by acid hydrolysis as follows. A portion of supernatant fluid (0.75 ml) was mixed with 0.25 ml of 6 M-HCl, and incubated at 100 °C for 2 h. This solution was cooled and neutralized with 6 M-KOH, then mixed with 1.75 ml of 0.5 M-potassium phosphate buffer pH 7.4 and analysed as for glucose. Formate was determined enzymically, from the change in A_{340} of a sample incubated for 2 h at 39 °C in a reaction mixture (pH 7.0) containing 0.167 M-potassium phosphate, 0.5 mM-NAD and 0.1 mg formate dehydrogenase ml^{-1} . The other volatile fatty acids were determined by GLC (Fell *et al.*, 1968), except when acetate was the only volatile fatty acid produced, when it was measured using acetate kinase (Holz & Bergmeyer, 1974). Lactate was oxidized to acetaldehyde and measured by using microdiffusion chambers (Conway, 1957). Succinate was assayed by an enzymic method (Clark & Porteous, 1964). With samples containing ferricyanide, which interfered with the

enzymic assay, succinate was first extracted into ether by shaking 1 ml of sample with 8×2 ml ether for 5 min. The ether was removed by evaporation and the succinate concentration in the redissolved material was determined as before. Other fermentation products were analysed using the methods described by Dawes *et al.* (1971).

The O_2 concentration of the growth medium was measured by an O_2 electrode and meter (model 9402, EIL) linked to a chart recorder (LKB 2210). This was calibrated by using a saturated sodium dithionite solution as zero O_2 concentration and assuming an O_2 concentration in air-saturated water at $30^\circ C$ of 6.6 mg l^{-1} (Hodgman *et al.*, 1955). Although an imprecise means of calibration, this method was adequate to show that the O_2 concentration in the growth vessel was exceedingly low.

Calculations. Specific growth rate (μ) was the maximum rate achieved, measured on a semi-logarithmic plot of cell density against time. The molar growth yield was determined by the end-point method (Mallette, 1969) where the difference in cell density between inoculation and the onset of stationary phase was related to the difference between measured substrate concentrations at these times. Carbon recovery was based on known fermentation pathways, such as outlined by Hobson & Summers (1972), assuming a carbon content of 37% for *B. amylophilus* (Jenkinson & Woodbine, 1979) and 45% for the other three bacteria (the average of the C content of mixed rumen bacteria measured by Isaacson *et al.*, 1975). The assumed C content would have to be considerably in error to influence the overall carbon recovery, particularly as it is necessarily incomplete, due to the use of a CO_2 gas phase in these experiments.

RESULTS

Regulation of E_h

The redox potential (E_h) of the growth medium in batch cultures of rumen bacteria was regulated at low ($< -50 \text{ mV}$) values by titanium(III) citrate and at higher values by $K_3Fe(CN)_6$ solutions. The composition of the medium for the four species ranged from a completely defined simple medium for the growth of *Sel. ruminantium* and *Strep. bovis* to a complex medium containing rumen fluid for *B. succinogenes*. Since the latter medium was more effectively E_h -buffered by the abundant organic material present, much more of each redox-regulating solution was required to effect E_h control. For example, growth of *Sel. ruminantium* at -274 mV required the addition of $0.58 \text{ mmol titanium(III) citrate (l medium)}^{-1}$ during the experiment, whereas growth of *B. succinogenes* at -267 mV required the addition of $3.60 \text{ mmol titanium(III) citrate l}^{-1}$. The maximum additions of redox titrators at the limits of growth of each species are given in Table 1. E_h control by titanium(III) citrate was not practicable at values lower than those shown for the different growth media. The upper limit of E_h control by $K_3Fe(CN)_6$ was about $+414 \text{ mV}$.

Oxygen in the growth medium was measured in a *B. succinogenes* culture using an O_2 electrode. The addition of $1.33 \text{ mmol titanium(III) citrate}$ as a 0.1 M solution did not result in the addition of any O_2 to the medium. Stepwise addition of $12 \text{ mmol } K_3Fe(CN)_6$ as a 0.2 M solution over a period of 2 h, which gave a final E_h of $+380 \text{ mV}$, caused a barely detectable (less than $0.1 \mu M$) increase in the oxygen concentration of the medium. This quantity of $K_3Fe(CN)_6$ greatly exceeded that actually used in any experiments with *B. succinogenes* (Table 1, Fig. 4).

Table 1. Additions of titanium(III) citrate and $K_3Fe(CN)_6$ at the lower and upper limits of growth of rumen bacteria in batch culture

The E_h of batch cultures (pH 6.5) was regulated by the addition of $0.2 \text{ M-} K_3Fe(CN)_6$ solution at $E_h > -50 \text{ mV}$ or $0.1 \text{ M-titanium(III) citrate}$ at lower E_h . The final concentrations of these compounds in the growth medium at the limits of E_h at which growth was observed were calculated from the volume of the culture and the volume of reagent delivered.

Species	Medium	Lower limit		Upper limit	
		E_h (mV)	Ti(III) citrate (mM)	E_h (mV)	$K_3Fe(CN)_6$ (mM)
<i>Strep. bovis</i>	Simple defined	-344	1.32	+414	85.00
<i>Sel. ruminantium</i>	Simple defined	-361	1.32	+354	5.00
<i>B. amylophilus</i>	Simple + 0.1% (w/v) tryptose	-313	2.16	+226	4.33
<i>B. succinogenes</i>	Complex	-267	3.60	+187	8.33

Fig. 1

Fig. 1. Influence of E_h on the growth and metabolism of *Strep. bovis* growing on glucose in batch culture. The redox potential (E_h) was regulated by the addition of titanium(III) citrate at $E_h < -50$ mV and $K_3Fe(CN)_6$ at $E_h > -50$ mV. ○, Growth yield ($Y_{glucose}$); ●, specific-growth rate (μ); □, ethanol; ■, acetate; △, lactate.

Fig. 2

Fig. 2. Influence of E_h on the growth and metabolism of *B. amylophilus* growing on maltose in batch culture. Titanium(III) citrate and $K_3Fe(CN)_6$ solutions were used to regulate E_h . ○, Growth yield ($Y_{maltose}$); ●, specific growth rate (μ); □, lactate; ■, acetate; △, formate; ▲, succinate.

Streptococcus bovis

The redox potential of the medium had little effect on the growth characteristics of *Strep. bovis* (Fig. 1). Over a range from -340 to $+414$ mV the specific growth rate (μ) varied slightly, with a peak of 0.6 h^{-1} at 0 mV, but there was little influence on the growth yield ($22\text{--}25$ g per mol glucose) or fermentation stoichiometry. Lactate was the major fermentation product, with acetate only appearing in the medium at E_h values above 0 mV and at only about 0.1 mol acetate per mol glucose. The recovery of C varied from 87 to 110% over this range of E_h .

Bacteroides amylophilus

The molar growth yield of *B. amylophilus* on maltose (76 g mol^{-1}) was not affected by E_h over the range -320 to $+250$ mV, despite the fall in μ which took place at values higher than 0 mV, from a plateau value of 0.6 h^{-1} at lower E_h (Fig. 2). Approximately equal quantities of formate, acetate and succinate (1.5 mol per mol maltose) were produced over the full range of E_h , except at more than 200 mV, where lactate (up to about 0.5 mol per mol glucose) was increasingly produced at the expense of succinate. The recovery of maltose carbon was $>90\%$ in all but one culture, and averaged 105% .

Selenomonas ruminantium

Selenomonas ruminantium grew at about 0.45 h^{-1} between -360 and $+100$ mV (Fig. 3). Thereafter μ fell, until growth was no longer possible at $+360$ mV. The growth yield was not

Fig. 3

Fig. 3. Influence of E_h on the growth and metabolism of *Sel. ruminantium* growing on glucose in batch culture. Titanium(III) citrate and $K_3Fe(CN)_6$ solutions were used to regulate E_h . $\circ$, Growth yield ($Y_{glucose}$); $\bullet$, specific growth rate (μ); $\square$, lactate; $\blacksquare$, acetate + propionate.

Fig. 4

Fig. 4. Influence of E_h on the growth and metabolism of *B. succinogenes* growing on glucose in batch culture. Titanium(III) citrate and $K_3Fe(CN)_6$ solutions were used to regulate E_h . $\circ$, Growth yield ($Y_{glucose}$); $\bullet$, specific growth rate (μ); $\square$, succinate; $\blacksquare$, acetate; $\triangle$, formate.

affected by E_h over the whole range of E_h , varying between 28 and 32 g mol⁻¹. Lactate was the most abundant product, at 1.1–1.5 mol per mol glucose, with acetate, propionate and succinate as minor products. Acetate and propionate were produced in similar amounts, at about 0.15 mol per mol glucose, whereas succinate production was lower (<0.04 mol per mol glucose). Light microscopy showed that at E_h values where μ was depressed, and particularly as the maximum E_h was approached, the cells of *Sel. ruminantium* became some ten or more times longer than those usually observed for these Gram-negative curved rods (2–3 μ m).

Bacteroides succinogenes

Bacteroides succinogenes grew within a more restricted range of E_h (–290 to +175 mV) than the other bacteria examined (Fig. 4). The main products of the fermentation of glucose were acetate (about 0.4–0.7 mol per mol glucose), succinate (0.4) and formate (0.1), thus accounting for a C recovery of about 80%. These products changed little with changing E_h . Other potential products were assayed for, but not found, including propionate and other volatile fatty acids, lactate, ethanol, pyruvate, isopropanol, acetone, diacetyl and acetoin. The maximum specific growth rate in this medium was 0.33 h⁻¹ and the maximum yield was about 42 g per mol glucose (Fig. 4). Again, greatly elongated forms of bacteria were seen at the highest E_h values.

DISCUSSION

Growth of the rumen bacteria *Strep. bovis*, *B. amylophilus*, *Sel. ruminantium* and *B. succinogenes* occurred over a wide range of E_h in the absence of O₂, in a way similar to that found with intestinal anaerobes (Walden & Hentges, 1975) and *C. acetobutylicum* (O'Brien & Morris, 1971). It was furthermore shown that the growth yield and fermentation stoichiometry of these

bacteria were unaffected over this range, and that the rate of growth was depressed only at higher E_h values than are normally obtained in anaerobic media (Figs 1–4). The bacteria did vary, however, in the range of E_h within which growth occurred, with *B. succinogenes* being the most limited, and *Strep. bovis* the most tolerant of high E_h , but even at the highest E_h values for which growth was possible for each species it is unlikely that O_2 was the toxic factor. The maximum O_2 concentration introduced was likely to be substantially less than $0.1 \mu M$ in any experiment, well below the concentration observed in rumen fluid between feeds ($1.6 \mu M$; Scott *et al.*, 1983). In contrast it was not possible using titanium(III) citrate as reducing agent to determine if there was a lower limit of E_h at which growth occurred. However, values lower than those obtained here would seldom, if ever, be obtained *in vivo* or under laboratory conditions.

The finding that the fermentation end-products of these rumen bacteria did not change in relative proportion as E_h was altered contrasts with, for example, the changes in the fermentation stoichiometry with growth rate which have been found with *Strep. bovis* (Russell & Baldwin, 1979; Silley, 1982), *Sel. ruminantium* (Hobson, 1965; Scheifinger *et al.*, 1975; Wallace, 1978) and *B. amylophilus* (Jenkinson & Woodbine, 1979). This is similar to the findings of O'Brien & Morris (1971) with *C. acetobutylicum*, which was unaffected by increased E_h caused by the addition of ferricyanide but which ceased growth and butyrate production when O_2 was the cause of the increased E_h . The products observed in the present work were fairly similar to those observed by other workers using different strains of the same organisms, with only some minor quantitative differences. For example, the strains of *B. amylophilus* investigated by Caldwell *et al.* (1969) produced succinate, acetate and formate in the proportions 3:2:1, whereas the strain used here formed these acids in equimolar quantities, as did the strain used by Hobson & Summers (1967). The high lactate concentrations produced by *Sel. ruminantium* were also typical of batch cultures of this organism (Caldwell *et al.*, 1969), rather than the mixed fermentation at lower μ in chemostat cultures (Hobson, 1965; Scheifinger *et al.*, 1975; Wallace, 1978). Similarly, the main product of glucose fermentation by *Strep. bovis* was lactate, unlike the mixed fermentation products found at lower μ (Russell & Baldwin, 1979).

The molar growth yields of the bacteria were similar to those observed by others (reviewed by Hobson & Wallace, 1982). Of particular note are the low yields of *Strep. bovis* and *Sel. ruminantium* (22–25 and 28–32 g per mol glucose) from predominantly lactic fermentations, indicating that ATP synthesis occurred in these bacteria solely by substrate-level phosphorylation. The higher yield of *B. succinogenes* (42 g mol^{-1}) suggests that electron-transfer-linked ATP synthesis was involved, in agreement with the finding that cytochrome *b* occurs in this species (Dawson *et al.*, 1979). Thus interference of ferricyanide with electron-transfer carriers may account for the greater sensitivity of this organism to high E_h . The growth yield of *B. amylophilus* was also fairly high (76 g per mol maltose), despite its reported lack of cytochromes (Reddy & Bryant, 1977). As this organism was also more sensitive to high E_h /high ferricyanide concentration than bacteria which had lower yields, it may be that this particular strain possesses some electron-transfer-linked mechanisms of energy conservation.

In conclusion, the efficacy of reducing agents in promoting the growth of obligate anaerobes is probably due entirely to their oxygen-removing properties. Redox potential has little or no effect on the growth or metabolism of rumen bacteria over a fairly wide range. This last fact is a reassuring one for those working with anaerobic bacteria, as most laboratory batch and continuous cultures, although often pH-controlled, seldom have E_h regulation. Provided O_2 is excluded, a range of reducing agents can therefore be used, and E_h can fluctuate markedly, without having any effect on the physiological properties of the bacteria. As the intracellular E_h was not measured in these experiments, it is not possible to say whether this lack of effect is due to a real insensitivity of bacterial metabolism to changes in intracellular redox potential, or if the redox couples in the growth medium do not, for several possible reasons, equilibrate with those which prevail intracellularly.

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