Some Effects of Phosphate and Volatile Fatty Acid Salts on the Growth of Rumen Bacteria

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SUMMARY

Bacterial cultures in media with a low (0.38 %, w/v) initial glucose concentration showed much smaller pH changes during growth than cultures grown in media with excess (1.25 %, w/v) glucose. Increasing the concentration of the phosphate or volatile fatty acid salt (VFA salt, i.e. acetate, propionate or butyrate) in the media had no beneficial effect on cultures of *Streptococcus bovis* or *Lactobacillus plantarum* which had only minor falls in pH during growth (final pH $\geq$ 6.0), but increased the dry weight yield (but not the molar growth yield) of cultures which had major pH falls (final pH $\leq$ 4.6) during growth. The improvements in dry weight yield could be correlated with increases in the pH buffering capacity of media caused by the increased phosphate or VFA salt concentration.

The response of *Bacteroides ruminicola* to increases in the phosphate concentration of media was qualitatively similar to that of *S. bovis*. However, VFA salts (particularly acetate) always decreased both dry weight and molar growth yield. The effects of VFA salts on three other rumen bacteria, *Butyrivibrio fibrisolvens*, *Megasphaera elsdenii* and *Veillonella alcalescens*, were varied. The possible mechanisms and ecological implications of the effects of these compounds are discussed.

INTRODUCTION

The complex nature of the nutrient requirements of rumen bacteria makes the formulation of fully defined media laborious and costly. A number of workers, therefore, have developed semi-defined media, containing protein hydrolysates and yeast extract but lacking centrifuged rumen fluid, for the study of the metabolism of rumen bacteria (Hobson, 1969).

Although an accurate comparison of the composition of media and of rumen fluid is hindered by the variable nature of rumen fluid, some semi-defined media contain only small amounts of the phosphates and volatile fatty acid (VFA) salts which are important in determining the pH buffering capacity of rumen fluid (Turner & Hodgetts, 1955). The effect of pH control in increasing the yields of bacteria has been described (see, for example, Kempe, Halvorson & Piret, 1950 and Sargeant, 1968), but apart from effects on pH, both classes of compound have other effects on micro-organisms. Phosphates are essential in small quantities, but may be toxic in high concentration (Good *et al.* 1966), whilst VFA salts have both inhibitory (Meynell, 1963; Hentges, 1967) and stimulatory (Bryant & Robinson, 1962; Roche *et al.* 1973) effects on different micro-organisms under various conditions. Individual VFA may also have either inhibitory or stimulatory effects on the same bacteria, according to the concentrations of acid used (Hobson, Mann & Smith, 1963).

The phosphate concentration of the semi-defined media reviewed by Hobson (1969) is around 6 mM, whereas according to Garton (1951) comparable values for rumen fluid lie between 10 and 55 mM. Similarly, VFA are present only as trace quantities in many semi-defined media, whereas the VFA concentration of rumen fluid may be between 30 and 200 mM, and is commonly 90 to 110 mM (Hungate, 1966).

Table 1. *Concentrations of phosphate (K^+) and VFA (Na^+) salts in media*

Medium no.	Concn (mm)		Medium no.	Concn (mm)	
	Phosphate	VFA salts		Phosphate	VFA salts
1	5.8	—	5	17.4	50 (acetate)
2	17.4	—	6	17.4	20 (propionate)
3	29.0	—	7	17.4	20 (butyrate)
4	17.4	20 (acetate)	8	17.4	90 (50 acetate + 20 propionate + 20 butyrate)

The aim of the present study was to investigate the response of some rumen bacteria, grown in batch culture on semi-defined media, to increases in the phosphate and VFA salt concentrations of the media to levels within the rumen range. A preliminary study (Stewart, 1972) showed that only small effects were involved, but the results seemed likely to be relevant to a number of areas of current microbiological interest. For instance, the isolates used in the present study have been inoculated into the rumens of gnotobiotic lambs (Lysons *et al.* 1971; Mann & Stewart, 1974) as part of a continuing investigation into factors affecting the growth of rumen bacteria *in vivo*. It was therefore possible to compare the growth of these bacteria in the present study with that in the *in vivo* studies mentioned above.

METHODS

Bacteria. The bacteria were isolated by S. O. Mann and C. Grant at the Rowett Institute. The strains used were *Streptococcus bovis* s26; *Lactobacillus plantarum* LB17; *Bacteroides rumenicola* 46/5(2); *Butyrivibrio fibrisolvens* B834; *Megasphaera (Peptostreptococcus) elsdenii* LC1, and *Veillonella alcalescens* 692/1. Stock cultures were maintained on Mann's medium (No. 2 in the list of Hobson, 1969).

Media. Various amounts of phosphate (K^+ salts) and VFA (Na^+ salts) (media 1 to 8, Table 1), were added to a basal medium containing (g/100 ml): Bacto Casitone (Difco), 1.0; yeast extract (Difco), 0.25; cysteine-HCl, 0.05; $NaHCO_3$, 0.4; $(NH_4)_2SO_4$, 0.09; NaCl, 0.09; $MgSO_4$, 0.009; $CaCl_2$, 0.009; resazurin, 0.001; distilled water to 100 ml. The $NaHCO_3$, and the CO_2 gas phase (see below) form part of the normal buffering system in these media. Two sets of media were prepared, containing 0.38 and 1.25 % (w/v) D-glucose, respectively. Most semi-defined media used for growing rumen bacteria contain 5.8 mM-phosphate (Hobson, 1969). This was adopted as the basal level in the present study. Phosphate at 17.4 mM (as an equal-weight mixture of KH_2PO_4 and K_2HPO_4) was representative of many values in the literature for rumen phosphate concentration, and was used in the study of the effect of VFA salts. The proportions of VFA in the 90 mM-VFA mixture (medium 8) approximated to those found in the rumen, and the components of this mixture were studied separately (media 5, 6 and 7). The study of the effects of 20 mM-acetate (medium 4) allowed direct comparison between the effects of acetate and the other acids.

A solution of the Casitone, yeast extract, minerals and resazurin was sterilized by autoclaving at 121 °C for 15 min. A solution of the other medium components was sterilized by filtration through Millipore membrane filters (0.45 μ m pore size) and added to the autoclaved solution. Preparation and inoculation of media were done under oxygen-free CO_2 (Hungate, 1950). Media were dispensed in 10 ml amounts into 150 × 16 mm test tubes which were then closed with rubber stoppers. The initial pH of each medium was 6.70 ± 0.05 . The inoculum was 0.1 ml of a 24 h culture on medium 1, containing 1.25 % (w/v) glucose.

Experiments at constant pH. Batch cultures were set up under anaerobic conditions in vessels similar to the culture vessel described by Hobson (1965*a*), with provision for inoculation, CO₂ bubbling and continuous pH measurement; the addition of 2 M-NaOH was controlled by the manual operation of a stopcock. The flasks (working volume 120 ml) were inoculated with 10 ml of a 24 h culture on medium 1, containing 1.25 % (w/v) glucose. The introduction of this acidic inoculum, together with the continuous bubbling with CO₂ (for stirring, and to maintain anaerobiosis) resulted in a fairly low pH (6.3 to 6.4) before inoculation. During growth, cultures were maintained at pH 6.3 ± 0.1 .

Analyses and chemicals. The buffer capacities of media and of rumen fluid were estimated by rapid titration of 50 ml portions against 1 M-HCl using a Radiometer T11 titrator and model 26 pH meter (Radiometer, Copenhagen, Denmark). Rumen samples were from cannulated animals and were strained through light surgical gauze before filtration.

Dry weights of bacteria were estimated as described by Hobson (1965*b*) from the turbidity of cultures (measured in an EEL colorimeter with a 640 nm filter; Evans Electroselenium Ltd, Harlow, Essex) after the construction of calibration curves using media 1, 3 and 8. The relationship between turbidity and dry weight did not vary significantly between these media.

Cultures were harvested after 16 h incubation, which for all but *S. bovis* corresponded to the beginning of the stationary phase. *Streptococcus bovis* cultures showed maximum growth within about 10 h, and so were in the stationary phase for up to 6 h before harvesting. There was, however, no evidence of significant lysis of *S. bovis* or any of the other bacteria when incubated for up to 24 h, nor was there evidence of uptake of glucose during the period after cessation of growth.

Cultures were centrifuged at 2 °C in a L2-50 preparative ultracentrifuge (Beckman Instruments, Glenrothes, Fife) at 100000g for 60 min. Then chemical analyses were made on the supernatants as follows: glucose by the glucose-oxidase method of Morley, Dawson & Marks (1968); lactate and alcohol by the methods of Conway (1957); formate by the method of Barker & Somers (1966); succinate by the method of Clark & Porteous (1964); C₂ to C₄ VFA by the g.l.c. method of Whitelaw *et al.* (1970). The total phosphorus content of the bacteria was estimated by the method of Roach (1966), by using an acidified extract prepared as follows: about 50 mg bacteria, which had been centrifuged at 3500 g for 20 min, washed twice in distilled water and dried for 16 h at 105 °C, were boiled for about 30 min in a mixture of 0.5 ml H₂SO₄, 2 ml HNO₃ and 1 ml HClO₄ (all BDH Aristar grade) for about 30 min.

Unless otherwise stated chemicals were BDH Analar grade except for K₂HPO₄ which was laboratory grade.

RESULTS

The changes in pH on addition of HCl to the eight types of media studied are shown in Fig. 1. Since the three media containing 20 mM-VFA salt (media 4, 6 and 7) had almost identical buffer capacities, only medium 4, 20 mM-acetate, is shown in Fig. 4. Medium 8, with 17.4 mM-phosphate and 90 mM-VFA, had a buffer capacity similar to a selection of 17 rumen fluid samples taken from five different sheep on various feeds. Medium 8 required the addition of 8 mmol HCl/100 ml to lower the pH from 6.7 to 4.7, while the rumen fluids required from 7.1 to 10 mmol. The glucose concentrations of the media had no effect on buffering capacity.

The concentrations of glucose used were chosen so that the lower (0.38 %, w/v) would provide glucose-limited cultures, and the higher (1.25 %, w/v) provide cultures with excess glucose. None of the bacteria showed detectable growth in the basal medium without glucose.

Fig. 1. pH changes on addition of HCl to nutrient media. ○, ●, △, ▲, □, ■, Media 1, 2, 3, 4, 5 and 8, respectively. Media 6 and 7 gave results almost identical to those of medium 4.

Streptococcus bovis, *L. plantarum* and *Bact. rumenicola* utilized all the 0.38 % (w/v) glucose during growth. With *Bu. fibrisolvens*, however, about half of the 0.38 % (w/v) glucose remained after growth in media without VFA salts. Only when propionate was present was all of the glucose used. When the glucose concentration of the medium with propionate was increased above 0.38 % (w/v) no increase in growth of *Bu. fibrisolvens* was observed.

The effects of the various phosphate concentrations and of the 90 mM-VFA salt mixtures on the dry weight yields, molar growth yields (calculated from the amount of glucose apparently utilized during growth) and final pH of the bacterial cultures are shown in Table 2. In representative media (1, 3 and 8) with 0.38 and 1.25 % (w/v) glucose, *S. bovis* and *L. plantarum* gave average carbon recoveries of around 93 %, *Bact. rumenicola* around 80 % and *Bu. fibrisolvens* around 75 %. Differences in carbon recoveries for any one bacterium in different media were small.

Table 2 shows that increasing the phosphate concentration of the medium generally lowered the molar growth yield. In media with excess glucose this was combined with increases in the dry weight yield. Control experiments using media containing K^+ as KCl, showed that the range of K^+ concentrations in these media had no significant effect on any of the bacteria. In media with 5.8 and 29 mM-phosphate the phosphorus levels in the bacteria ($\mu\text{g}/\text{mg}$ dried bacteria) were 20 and 21 (*S. bovis*), 27 and 28 (*L. plantarum*), and 22 and 28 (*Bact. rumenicola*), respectively.

The bacteria showed more individual patterns of response to VFA salts than to phosphate. Thus whilst *S. bovis* and *L. plantarum* showed a similar response to all three acids, *Bact. rumenicola* seemed to be more adversely affected by acetate than by the other acids and in the media with excess glucose 50 mM-acetate gave a depression of the molar growth yield equal to 76 % of the effect of the 90 mM-VFA mixture (of which acetate comprised about 55 %). Propionate (20 mM) was as effective in stimulating growth of *Bu. fibrisolvens* as was the 90 mM mixture, and both acetate and butyrate slightly stimulated growth. Control

Table 2. Effects of phosphate and VFA salts on the growth of *S. bovis*, *L. plantarum* and *Bact. ruminicola*, and of VFA salts on *Bu. fibrisolvens*

Cultures were incubated for 16 h at 38 °C.

Bacterium	Addition (mm)		0.38 % (w/v) glucose			1.25 % (w/v) glucose		
	Phos- phate	VFA salt	Dry wt (mg/ml)	Y_{glc} *	Final pH	Dry wt (mg/ml)	Y_{glc} *	Final pH
<i>S. bovis</i>	5.8		0.75	36.0	6.0	1.46	35.1	4.0
	17.4		0.71	34.0	6.2	1.56	33.7	4.0
	29.0		0.54	25.9	6.4	1.66	31.9	4.0
	17.4	90†	0.66	31.3	6.0	1.81	23.9	4.4
<i>L. plantarum</i>	5.8		0.47	22.5	6.0	0.63	16.4	4.2
	17.4		0.43	20.7	6.1	0.69	16.4	4.2
	29.0		0.37	17.8	6.2	0.74	16.4	4.2
	17.4	90†	0.45	21.6	6.0	0.82	13.5	4.6
<i>Bact. ruminicola</i>	5.8		0.94	45.2	5.9	1.37	40.7	4.4
	17.4		0.90	43.2	6.0	1.48	38.2	4.4
	29.0		0.84	40.4	6.2	1.52	36.6	4.5
	17.4	90†	0.76	36.6	6.0	1.32	28.3	4.8
<i>Bu. fibrisolvens</i>	17.4		0.44	49.5	6.1	—	—	—
	17.4	90†	0.89	56.5	5.7	—	—	—

—, Not studied.

* Molar growth yield, calculated from the amount of glucose utilized.

† Sodium acetate (50 mm) + propionate (20 mm) + butyrate (20 mm).

experiments with media containing Na^+ (as NaCl) showed that *S. bovis* was the only bacterium to show a response to changes in Na^+ concentration, growth being slightly inhibited by Na^+ in media containing 0.38 % (w/v) glucose. The results in Table 2 have been corrected for this effect of sodium, which was not shown in media with isosmotic solutions of other electrolytes or 1.25 % (w/v) glucose.

The effect of the 90 mM-VFA salt mixture on the rate of growth of *S. bovis* and *Bact. ruminicola* in media containing 1.25 % (w/v) glucose was determined by measuring the dry weight of cultures at intervals after inoculation. Volatile fatty acid salts had no detectable effect on the growth rate of *S. bovis* during the exponential phase (mean generation time 21 min), the higher cell yield in cultures with VFA salts being the result of an extension of the exponential phase. The difference in molar growth yield of *S. bovis* cultures grown in media with and without the VFA salts mixture was roughly constant from the mid-exponential to the early stationary phase, although in both cases the molar growth yield tended to fall during this period.

The growth rate of *Bact. ruminicola* was clearly depressed by the presence of the VFA salt mixture. During the exponential phase, *Bact. ruminicola* grew with a mean generation time of 86 min in the medium without added VFA salt; with the VFA salt present this increased to 121 min.

The effects of phosphate and the 90 mM-VFA salt mixture on the growth of the bacteria in media containing 1.25 % (w/v) glucose were tested under conditions of constant pH (Table 3). Only *Bu. fibrisolvens* showed increased growth in VFA-containing media when the pH was kept constant. Most of the cultures maintained at pH 6.3 grew more rapidly than in previous experiments in which the pH was allowed to fall, and the nature of the effects was clear after 8 h incubation.

Table 3. *Dry weight yields of bacteria grown at pH 6.3 in media with 1.25% (w/v) glucose*

Cultures were incubated for 8 h at 38 °C.

Addition (mm)		Dry wt (mg/ml)			
Phosphate	VFA salts	<i>S. bovis</i>	<i>L. plantarum</i>	<i>Bact. ruminicola</i>	<i>Bu. fibrisolvens</i>
5.8		2.58	0.80	2.37	—
29.0		2.34	0.59	1.95	—
17.4		2.54	0.70	2.16	0.16
17.4	90*	2.34	0.66	2.00	0.29

—, Not studied.

* Acetate (50 mm) + propionate (20 mm) + butyrate (20 mm).

Neither the concentration of phosphate nor the presence of VFA salts had a marked effect on the proportions of fermentation products of the bacteria. *Streptococcus bovis* and *L. plantarum* produced between 1.80 and 1.92 mol lactate/mol glucose utilized; acetate and propionate were occasionally found in very small amounts. From each mole of glucose utilized, *Bact. ruminicola* produced around 1.3 mol lactate, 0.2 mol succinate and 0.2 mol acetate. *Butyrivibrio fibrisolvens* produced about 0.8 mol formate, 0.35 mol acetate and 0.6 mol butyrate/mol glucose. In no case was there evidence of a net uptake of any of the acids by the bacteria.

Veillonella alcalescens and *Megasphaera elsdenii* were grown in media containing VFA in concentrations similar to those previously described, but with 0.38 % (w/v) lactate as energy source. The media contained 17.4 mm-phosphate. In the control medium without VFA, dry weight yields of 0.5 mg/ml (*Megasphaera*) and 0.4 mg/ml (*Veillonella*) were obtained. In media with the 90 mm-VFA mixture, 0.69 mg *Megasphaera*/ml, and 0.32 mg *Veillonella*/ml were obtained. Neither organism reduced the medium pH markedly, so effects relating to buffer capacity were evidently not involved. The stimulating effect of VFA salts on the growth of *Megasphaera* was apparently due to the acetate; in fact 50 mm-sodium acetate produced rather greater stimulation of growth than the 90 mm-VFA salt mixture, butyrate and propionate being slightly inhibitory. Propionate seemed to account for the major part of the inhibitory effect of the 90 mm-VFA salt mixture on *Veillonella*.

DISCUSSION

It has been shown that under certain conditions the dry-weight yield of some rumen bacteria can be increased by the addition of buffer compounds to the medium. The finding that this increase was accompanied by a decrease in the molar growth yield was less predictable and is a point apparently not considered by others (Sargeant, 1968) who have stressed the importance of pH as a factor limiting the yield of anaerobic fermentations.

The deleterious effects of phosphate on the molar growth yield of *S. bovis*, *L. plantarum* and *Bact. ruminicola* tended to be most marked in cultures with a high final pH. This suggests that HPO_4^{2-} may be responsible for much of the inhibitory effect, though the bacteria so far studied are generally thought to take up H_2PO_4^- rather than HPO_4^{2-} (Mitchell, 1954). The values for the phosphorus concentration of the bacteria are similar to those of *Escherichia coli* (Roberts *et al.* 1955) and are within the range quoted by Hungate (1966) for rumen bacteria.

Unlike phosphate, VFA salts showed the most marked depressions of molar growth yields of *S. bovis*, *L. plantarum* and *Bact. ruminicola* in media with excess glucose and low

final pH. This is consistent with the view that much of the inhibitory activity of VFA is possessed by the undissociated acid (Meynell, 1963; Hentges, 1967).

Bacteroides rumenicola appears to be more strongly inhibited by acetate than by the other acids tested, and since acetate is the only one of these acids produced by *Bact. rumenicola*, it seems possible that much of the effect of acetate may be due to end-product inhibition. The presence of acetate does not markedly alter the proportions of fermentation products of *Bact. rumenicola*, but presumably slows down the rate of fermentation of glucose, possibly as a consequence of accumulation of pyruvate. Both propionate and butyrate also slightly decrease the molar growth yields of *Bact. rumenicola*, so other mechanisms must account for their effects, and for the effects of all three VFA salts on the exclusively lactate-producing *S. bovis* and *L. plantarum*. Possibly a mechanism is involved similar to that proposed by Sheu & Freese (1972) and Sheu, Konings & Freese (1972), who suggested that VFA react with a component of the cell membrane of *Bacillus subtilis* to inhibit the active transport of important compounds into the cell. End-product effects presumably could explain the inhibitory action of propionate on *Veillonella*, and of propionate and butyrate on *Megasphaera*. Stimulation of the growth of *Megasphaera (Peptostreptococcus) elsdenii* by acetate has previously been reported by Hungate (1966).

The increase in dry-weight yield of *L. plantarum* in media with excess glucose and VFA salts is interesting in view of the studies of Rogosa, Mitchell & Wiseman (1951) who developed a medium containing acetate for the selective isolation of Lactobacilli. The present study suggests that C₂ to C₄ VFA might generally increase the growth of lactic acid bacteria by increasing the buffer capacity of the medium.

Shane, Gouws & Kistner (1969) divided their strains of *Butyrivibrio* into two groups on the basis of fermentation product formation and acetate utilization, and Roche *et al.* (1973) found that only group 2 isolates were stimulated by VFA, particularly propionate. The present isolate seemed to fit into Group 2 of Shane *et al.* (1969) in that it did not utilize acetate, produced 'high formate' and 'low lactate', and was stimulated by propionate.

Experiments on the microbiology of gnotobiotic lambs containing a limited rumen flora have shown that when the bacteria studied here were inoculated into lambs fed on various diets, *Bu. fibrosolvens* tended to dominate the rumen bacterial flora (Lysons *et al.* 1971; Mann & Stewart, 1974). Part of the success may result from the known ability of *Bu. fibrisolvens* to grown on a wide range of substrates, but it does seem that the favourable response of *Bu. fibrisolvens* to the presence of VFA is likely to be an important factor in its success, particularly under conditions of energy-limitation.

Other effects found in the experiments with gnotobiotic lambs, particularly the occasional profuse growth of *Veillonella*, cannot be explained simply in terms of the present results, emphasizing that other factors, not considered here, must play a part in determining the full balance of the bacterial flora of the rumen.

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