Continuous Culture of Some Anaerobic and Facultatively Anaerobic Rumen Bacteria

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SUMMARY

Strains of *Streptococcus bovis*, *Selenomonas ruminantium*, and an anaerobic lipolytic bacterium (5s) have been grown under carbohydrate-limiting conditions in continuous culture for long periods. With *S. ruminantium* and bacterium 5s the fermentation products varied with growth rate. Yield of organism in continuous culture of all three bacteria showed a maximum at a particular growth rate. The yield of *S. ruminantium* was much higher in continuous culture at optimum growth rate than in batch culture, and was also higher than can be explained on present concepts of the energy from fermentation available for growth. The various results are discussed in the light of results obtained with other bacteria and the conditions obtaining in the rumen.

INTRODUCTION

During the development of the culture apparatus described in the previous paper (Hobson, 1965) a strain of *Selenomonas ruminantium* was used because it is an anaerobic rumen bacterium and the strain had been well characterized in previous work (Hobson & Mann, 1961). It has been suggested that selenomonads are not bacteria, but should be classed as protozoa (e.g. Bisset & Davis, 1960). However, apart from other considerations, our own unpublished results show that the cell-wall composition of *S. ruminantium* strain 6 (nitrogen 10.0%; carbohydrate 4.7%; lipid 28%; hexosamine 3.6%; ash 0.7%; phosphorus 0.7%; with twelve amino acids present) is similar to that of other Gram-negative bacterial cell walls. This seems to be an additional reason for regarding the selenomonads as bacteria. When the unusual nature of the results with *S. ruminantium* in the present work became apparent some runs were done with a rumen strain of *Streptococcus bovis*, to examine the behaviour of a facultatively anaerobic organism which ferments the same carbohydrate. Finally, the lipolytic bacterium 5s was used in an extended series of tests; the reasons for this were twofold. First, bacterium 5s is also a strict anaerobe, but it ferments a simpler carbohydrate than does the selenomonad *S. ruminantium*. (This selenomonad lost the ability to ferment glycerol on prolonged growth on glucose.) Secondly, large amounts of organisms were needed for experiments on the lipolytic activity of bacterium 5s. The strains of anaerobic bacteria used would grow only in media containing amino acid mixtures, and not on simpler media containing only ammonia as nitrogen source as do some rumen bacteria. While a simpler medium might have been desirable for some aspects of the work the yields of bacteria have generally been determined for media in which were present most of the monomers of cell constituents.

The symbols used in this paper to describe growth in continuous culture are those of Herbert, Elsworth & Telling (1956). The yield constant, Y_{gluc} or Y_{glyc} is g. dry weight organism/mole substrate utilized, as introduced by Bauchop & Elsdon (1960).

METHODS

Organisms used

Streptococcus bovis. This was the strain 18c6 isolated from the sheep rumen described by Hobson & Mann (1955). Stock cultures were kept in Robertson's cooked meat medium or were freeze-dried.

Selenomonas ruminantium var. *lactyliticas*. This was the strain 6 isolated from the rumen and described by Hobson & Mann (1961). On original isolation and for some time after it rapidly fermented glycerol. At the time of the present experiments various cultures had been kept as stocks and continually subcultured on glucose slopes, and other cultures had been kept on glucose and then freeze-dried. On retesting, after the experiments on continuous culture on glucose had been done (some 3 years after isolation of the organism), it was found that neither stock cultures on glucose slopes nor the revived freeze-dried cultures metabolized glycerol to any extent. A little growth was obtained on the basal constituents of the medium, but continued subculture on liquid or solid media containing glycerol did not increase the growth or glycerol utilization.

Lipolytic bacterium. This organism, bacterium 5s, was isolated from a sheep on a hay and concentrate diet. It was similar in all respects, except that it fermented the additional carbohydrate cellobiose, to the lipolytic bacteria previously isolated from sheep (Hobson & Mann, 1961). These bacteria do not ferment glucose. Stock cultures were kept on glycerol slopes at 2°, and were subcultured every 2 weeks. No change in the characteristics of the strain were noted during the period of the experiments.

Media

For selenomonad strain 6. The medium used (CY medium) initially was one containing mineral solutions (a) and (b), 15 ml. each; Bacto Casitone, 1.5 g.; Bacto Yeast Extract, 0.25 g.; NaHCO₃, 0.4 g.; cysteine HCl, 0.05 g.; resazurin, 0.1 ml. of 0.1 % (w/v) solution; water to 100 ml. and glucose in the correct concentration. The constituents of mineral solutions (a) and (b) and the methods of preparing the media under oxygen-free carbon dioxide were described by Hobson & Mann (1961). This medium was used solidified with agar for stock cultures, and as liquid for batch cultures of the selenomonad. The medium for continuous cultures was originally the CY medium + glucose, but in later experiments this was changed by addition of 0.522 g. K₂HPO₄ and 0.272 g. KH₂PO₄/100 ml. (CYP medium; pH 6.7).

For lipolytic bacterium 5s. Initial experiments were made with medium CY with glycerol added. Later experiments used a medium (AHI medium) of composition: mineral solution (a, above), without NaCl, 15 ml.; solution (b, above), 15 ml.; acid-hydrolysed casein (Allen and Hanbury, London), 1 g.; Bacto Yeast Extract, 1 g.; NaHCO₃, 0.4 g.; cysteine HCl, 0.05 g.; resazurin solution, 0.1 ml. of 0.1 % solution; glycerol; water to 100 ml. The casein hydrolysate gave an acid solution, so the minerals, casein hydrolysate, yeast extract and water were mixed and the solution

adjusted to pH 6.8 with a little 10N-NaOH. At this pH value a faint opalescence developed; increasing the pH value caused a precipitate to form. The resazurin (and the agar when slopes were being made) was added, and the solution then autoclaved and a filter-sterilized solution of bicarbonate + cysteine + glycerol added in the usual way (Hobson & Mann, 1961). A heavy opalescence formed in the autoclaved medium, but this cleared as the medium cooled; the slight sediment remaining was allowed to settle before the medium, prepared in bulk, was dispensed into smaller portions for batch cultures. In the continuous cultures this sediment settled to the bottom of the medium flasks, leaving a clear supernatant fluid; this sediment was not disturbed as medium flowed between the flasks or into the culture vessel. For the later continuous culture work the sodium bicarbonate concentration was increased to 0.6%, and the cysteine concentration to 0.1%. The medium was of pH 6.8, and this is referred to as AH2 medium. The NaCl was omitted from these media as other experiments suggested that a better production of lipase occurred in the absence of NaCl.

Streptococcus bovis. This was grown in the CYP medium.

All cultures were incubated at 39°.

Analytical methods

Glucose was determined by an anthrone method, as was bacterial carbohydrate. Glycerol was determined by the method of Hanahan & Olley (1958). Volatile fatty acids were steam-distilled from the culture media, and the constituent acids identified by paper chromatography. These methods and that for lactic acid were previously described (Hobson & Mann, 1961). The fermentation acids were separated quantitatively on Celite columns by the method of Wiseman & Irwin (1957). This method did not separate lactic and succinic acids completely, so the total lactic + succinic fraction was determined by the column separation and succinic acid determined by subtracting from this a value for lactic acid determined on another portion of the culture.

Organism dry weights were determined by centrifuging 10 ml. of culture in a small conical tube, washing the sediment once with 10 ml. distilled water, again centrifuging, and finally drying the sediment to constant weight at 110° after removing most of the water.

The 'turbidity' of cultures was measured in 1.5 ml. tubes in an EEL colorimeter (Evans Electroselenium Ltd., Harlow, Essex) with an OR1 (640 m μ) filter. Experiments with bacterial suspensions showed that the red filter gave better linearity with amount of organisms, and covered a wider range, than did blue or yellow filters. The turbidity values were read in the EEL colorimeter on a scale 0-10-100- ∞ . Measurements of organism dry weight/unit volume and turbidity as EEL colorimeter readings were made during batch and continuous cultures. The measurements for each organism fell on the same straight line (Fig. 1). Deviations were found only in cultures where lysis of organisms was taking place or during granular growth. The turbidity was thus routinely used as a measure of the behaviour of the continuous cultures and in some cases to estimate yields of organisms.

The sizes of organisms were measured in wet preparations examined with a calibrated micrometer eyepiece in phase-contrast illumination. The sizes of ten

bacteria taken at random in a field were measured and an average length found; this was repeated at each sampling time.

A Pye pH meter was used to measure pH value as soon as possible after removal of a sample from the culture vessel.

RESULTS

General. Batch cultures were inoculated from overnight cultures, the volume of inoculum usually being about 2% of the culture volume. The continuous culture apparatus was assembled with medium as previously described, inoculated and allowed to grow as a batch culture overnight. This did not usually give the maximum growth attainable in a small batch culture. The culture was then sampled and

Fig. 1

Fig. 2

Fig. 1. The relationship of bacterial dry weight and turbidity in batch and continuous cultures of selenomonad 6 (○) and lipolytic bacterium 5s (●). Turbidity of basal medium = 1–1.5. A turbidity of 80 EEL units = 1.49 mg. dry wt./ml. for selenomonad 6.

Fig. 2. Growth of lipolytic bacterium strain 5s in an insufficiently buffered medium. Glycerol concentration 65.6 μ mole/ml. Turbidity measured on an EEL colorimeter, +, culture pH, ○.

the inlet pump started at a suitable rate. The turbidity reading on the EEL colorimeter always decreased in the first few hours after starting the medium flow and then returned to a value corresponding to the dilution rate. Measurements of turbidity were made at intervals and a further sample taken for analysis when the culture had been stable for about 30 hr in most cases at high dilution rates, or about 72 hr or longer at low dilution rates. Since there was a slight variation in turbidity with time the value shown in the figures for turbidity at any dilution rate is an

average of the values found over the steady-state period. As the volume of culture needed to obtain turbidity and pH measurements was about 1.5 ml., this was a little over 1% of the culture volume and so did not affect the system. When about 10 ml. was taken for analysis the culture was not sampled again for a few hours, depending on the dilution rate, to allow return to steady-state conditions. No difficulties have been experienced with 'wall growth' with the three types of bacterium tested.

Stability of the apparatus. The cultures ran for long periods with a minimum of attention beyond an occasional adjustment of the CO₂ flow, and the apparatus was routinely left to run overnight, and occasionally over a weekend, without attention. The stability of the medium flow has been shown previously. Because of the great dependence of turbidity of the selenomonad culture on dilution rate a rather greater variation in turbidity readings during running at one nominal dilution rate was found than for other organisms, because of slight variations in pumping speed. With the bacterium 5s in a test run of 200 hr at one dilution rate the turbidity readings, measured at about equal intervals, were 23.2, 22.5, 22.4, 21.1, 21.1, 21.1, 21.1, 20.0, 22.6, 22.6, 23.3, 23.6. This period included a change of medium in the 5 l. reservoir.

pH stability. In initial experiments with the bacterium 5s the medium AH1 was not sufficiently buffered. Fig. 2 shows two sections from a graph of the results obtained. A number of dilution rates were tested and in each case the cycle was repeated, the time scale being slightly shorter at higher dilution rates. All the glycerol in the medium had been utilized at the times of maximum turbidity and minimum pH value. It is evident that a comparatively small change in pH value markedly decreased the growth rate of the bacteria and they were washed out until the entry of fresh medium restored the pH value, when growth and fermentation started, and the cycle was repeated. Small changes in pH value may have been partly responsible for the slight variations in turbidity found in other experiments during steady states. Finn & Wilson (1954) suggested that cycling of a yeast culture was due to pH variations. With the medium AH2 the pH value was steadier, the fluctuations over 70 hr being, for example, pH: 5.9, 6.0, 5.9, 5.9, 6.1, 6.0. The selenomonad cultures with medium CYP fluctuated between pH 6.1 and 6.2.

As a further example of the effect of an insufficiently buffered medium the *Streptococcus bovis* strain 18c6 was grown in a yeast medium with glucose (Hobson & Mann, 1955). In batch cultures the pH value fell rapidly and there was little correspondence between turbidity and glucose concentration except at low glucose concentrations. In continuous culture the pH value fell to about 4.9 (from 6.0) and the turbidity (at three dilution rates) to less than half that initially obtained in batch culture. All the glucose was not utilized.

Growth of Streptococcus bovis 18c6

In batch cultures grown in CYP medium with glucose 11.1, 22.2, 33.3, 44.4 and 55.5 μ mole/ml. growth was proportional to glucose added between 22.2 and 44.4 μ mole/ml. and virtually all the glucose was used; at 55.5 μ mole/ml. a divergence of growth and glucose concentration was beginning. At glucose 11.1 μ mole/ml., although all the glucose was utilized, growth was higher than expected from the other results because of the contribution of growth on the basal medium. This effect was similar to that found with the selenomonad (Fig. 4). Fig. 3 shows the

effect of dilution rate on growth taken from a run of 450 hr with a medium with glucose $27.7 \mu\text{mole/ml}$. The minimum doubling time ($t_d = (\log_e 2)/D$) was about 0.5 hr. No intracellular 'starch' was observed in the organisms from continuous culture. This might be expected from previous experiments on starch formation in this organism (Hobson & Mann, 1955).

Growth of Selenomonas ruminantium strain 6

Batch culture experiments were done to find the range over which concentration of organism varied with glucose concentration so that a glucose-limiting system might be used in continuous culture. The results of a number of experiments are shown in Fig. 3 and Table 1. These experiments were not always reproducible for

Fig. 3

Fig. 4

Fig. 3. Continuous culture of *Streptococcus bovis* 18c6. Steady-state turbidity and dilution rate (D). Glucose-limiting conditions, glucose concentration $27.7 \mu\text{mole/ml}$. Maximum turbidity of batch cultures at same substrate concentration c. 22.

Fig. 4. Growth of selenomonad 6 in batch cultures at various glucose concentrations.

two reasons. After maximum growth was attained the selenomonads tended to lyse fairly rapidly and the turbidity readings decreased. This rapid decrease in turbidity after maximum growth has been noted with other rumen bacteria in our own laboratories and by others (e.g. Bryant & Robinson, 1961*a*). Also, when growth was slow the cultures often did not attain the maximum yield of a rapidly growing culture, although the glucose was utilized. The values for Y_{gluc} given in Table 1 are uncorrected for growth on the basal medium as it does not seem possible to decide, either from Fig. 4 or a similar graph in which concentration of organism (mg. dry wt./ml.) was plotted against glucose added, whether growth on the basal medium was taking place or was suppressed in the presence of glucose. The results shown in Fig. 4 indicated that a glucose-limiting culture was possible with this basal medium at glucose concentrations about $27.7 \mu\text{mole/ml}$. At the higher glucose concentrations another nutrient, or possibly the pH value, was becoming limiting in the batch cultures. Volatile fatty acids (VFA), identified as acetic and propionic by paper chromatography, and lactic acid, were produced in the glucose fermentation in the batch cultures. At glucose $27.7 \mu\text{mole/ml}$. added, $27.4 \mu\text{mole}$ VFA and $186.6 \mu\text{mole}$ lactic acid were produced per $100 \mu\text{mole}$ glucose utilized. Assuming that the VFA are acetic and propionic acids with CO_2 equivalent to the acetic acid,

this gives a carbon recovery of 107%. At other concentrations of added glucose up to 83.3 $\mu\text{mole/ml}$, the proportions of VFA and lactic acid were similar, and carbon recovery on the above basis was somewhat over, or somewhat under, 100%. At higher concentrations of added glucose carbon recovery was low, being only about 60% at the highest concentration. The reason for this is not known, unless a large amount of glucose was converted to intracellular polysaccharide. The 'carbohydrate' content of the dried organisms at glucose 27.7 and 33.3 $\mu\text{mole/ml}$, added was 22 and 26% measured as apparent glucose by anthrone reagent, but no other determinations were made. If the glucose utilized but not recovered as fermentation products were assimilated then this would increase the low value of Y_{gluc} found at the highest glucose concentration.

Table 1. *Batch growth of selenomonad 6 on glucose*

Glucose added ($\mu\text{mole/ml}$.)	Glucose used ($\mu\text{mole/ml}$.)	Cell dry wt. (mg./ml.)	Y_{gluc} *
0	0	0.15	—
11.1	11.1	0.20	18.0
16.6	16.6	0.25	15.0
22.2	22.2	0.35	15.8
27.7	27.7	0.45	16.2
33.3	29.4	0.58	19.7
44.5	37.8	0.81	21.4
83.3	51.1	0.94	18.4
100.0	64.4	0.98	15.2
138.9	78.9	0.94	11.9

Medium as in text. Final pH in cultures containing glucose 5.6–5.7. *Not corrected for growth in basal medium, see text.

In the first continuous culture runs CY medium was used. This was initially at about pH 6.5, but during growth this fell to between pH 5.7 and 6.0. This was felt to be rather low, so further runs were made with CYP medium initially at pH 6.7 and the culture remained at pH 6.1 to 6.2 with glucose 27.7 $\mu\text{mole/ml}$, and pH 6.4 with 13.9 $\mu\text{mole/ml}$. However, the results with CY medium and CYP medium all integrated. Figure 5, 6 and 7 show results taken from the steady-state values of five runs of 268 hr, 721 hr, 294 hr, 143 hr and 60 hr. The shortest run was to obtain two points at the higher dilution rates. In all runs the dilution rates examined overlapped with other runs. One run was with CY medium, the others with CYP medium. Points from one run (290 hr) on CY medium with glucose 13.9 $\mu\text{mole/ml}$, are included, as are points from a run of 130 hr on the basal CY medium. The glucose concentration in the culture vessel, corrected for blank values of the basal medium, averaged 4% of the incoming glucose at glucose 27.7 $\mu\text{mole/ml}$, in the medium, and was virtually nil at 13.9 $\mu\text{mole/ml}$, up to dilution rates (D) of 0.465 hr^{-1} ; no measurements were made above this dilution rate. At a number of times during cultivation the total volatile fatty acids were measured (Fig. 7). Only a few determinations of lactic acid were made. The value obtained at $D = 0.1 \text{ hr}^{-1}$ was 7 μmole lactic/100 μmole glucose utilized, at $D = 0.26$ the value was 87 μmole and at $D = 0.286$ the value was 73 μmole . These figures are obviously subject to errors; taking average values for the VFA formed at these dilution rates from Fig. 7 carbon recoveries of 96, 104 and 94% are obtained. These figures thus show a change

in the fermentation products with change in dilution rate and they also show that little of the glucose was incorporated into cellular material.

Measurement of the size of organisms during runs showed no variation in width, but the length varied somewhat from a minimum of 2.1μ to a maximum of 4.0μ , being generally about 3.1μ . No correlation between length of organism and dilution rate was observed. However, during some of the runs growth became very granular for a day or two. During the granular growth period very long organisms were seen which seemed to be undivided selenomonads. The granular growth returned to normal after a day or two and no reason for it could be found. The selenomonads varied in shape at times, some being almost straight rods, as compared

Fig. 5

Fig. 6 .

Fig. 5. Continuous culture of selenomonad 6; relationship between turbidity and dilution rate (D) at different steady states. Glucose-limiting conditions, glucose concentration $27.7 \mu\text{mole/ml.}$ $\circ$, $13.9 \mu\text{mole/ml.}$ $+$, basal medium $\bullet$. The values for $27.7 \mu\text{mole/ml.}$ glucose are from five runs, and with these and the ones for $13.9 \mu\text{mole/ml.}$ glucose the values are corrected for growth in the basal medium. At dilution rates up to 0.465 hr^{-1} an average of 96 % of the glucose was being utilized. No measurements were made above this dilution rate. Maximum turbidity of batch cultures at $27.7 \mu\text{mole/ml.}$ *c.* 11.

Fig. 6. Continuous culture of selenomonad 6; relationship between Y_{gluc} and dilution rate (D). Values are taken from five runs at $27.7 \mu\text{mole/ml.}$ glucose in the medium and one run at $13.9 \mu\text{mole/ml.}$ glucose.

with the normal curved shape. The percentage of motile organisms was generally much higher in the continuous cultures than in batch cultures. Tests were made at intervals during all runs, especially when the percentage of straight organisms was high, for the reaction of the organisms with antisera specific to the selenomonads (Hobson, Mann & Smith, 1962). In all cases good agglutination was observed.

Culture of lipolytic bacterium 5s

The bacterium 5s was originally grown in both batch and continuous culture in CY medium with various amounts of glycerol. In batch cultures the growth on the basal medium was high and at glycerol $65.2 \mu\text{mole/ml.}$ only 52 % of the glycerol was used; similar results were obtained in continuous culture. Further experiments showed that an amino acid mixture and yeast extract were needed for good growth

and 1% (w/v) casein hydrolysate+1% yeast extract medium (see Methods, medium AH1) gave optimum growth and glycerol utilization; but to prevent the pH value cycling in the continuous culture, and to get a more reduced medium, the medium was slightly altered (medium AH2). The results of experiments with batch cultures (AH1 medium) are shown in Table 2; the final pH value was 5.1 to

Fig. 7

Fig. 8

Fig. 7. Continuous culture of selenomonad 6; relationship between VFA produced and dilution rate (D). Values are taken from five runs at $27.7 \mu\text{mole/ml.}$ glucose in the medium and one run at $13.9 \mu\text{mole/ml.}$ glucose.

Fig. 8. Continuous culture of lipolytic bacterium 5s; relationship between turbidity and dilution rate (D) at different steady states. Glycerol-limiting conditions, glycerol concentration $65.6 \mu\text{mole/ml.}$ ○. At points marked ● a true steady state was not achieved; the density drifted downwards at about 1 unit per 12 hr. Basal medium growth +. At $D = 0.083$, 98% of the glycerol was used, at $D = 0.206$, 100%; at $D = 0.298$, 98.6%; at $D = 0.310$, 90.7%; at $D = 0.370$, 88.1%; at $D = 0.514$, 75.1%; at $D = 0.591$, 64.3%; at $D = 0.850$, 58.6%; at $D = 0.883$, 36.5%. The values are taken from two runs with glycerol and one run with the basal medium. Maximum turbidity of batch cultures at same substrate concentration c. 32. A turbidity of 30 EEL units = 1.36 mg. dry wt. bacteria/ml.

Table 2. Batch growth of lipolytic bacterium strain 5s on glycerol

Glycerol added ($\mu\text{mole/ml.}$)	Glycerol used ($\mu\text{mole/ml.}$)	Dry wt. bacteria (mg./ml.)	Y_{glyc}	VFA formed ($\mu\text{mole/100}$ μmole glycerol used)
0	0	*	—	—
65.2†	65.2	1.48	22.7	65.5
130.4	67.6	1.36	20.1	61.4
195.6	82.1	1.17	14.8	42.5
260.8	89.1	1.30	14.6	40.3

* Too small to measure accurately.

† Fermentation products ($\mu\text{mole/100} \mu\text{mole}$ glycerol used): acetic acid, 17.9; propionic acid, 47.4; succinic acid, 31.7; lactic acid, 7.1. Carbon recovery 109%.

5.3, depending on the glycerol concentration. As with the selenomonad cultures these results were not very reproducible because of the rapid decrease in turbidity after maximum growth, and this was accelerated by increasing the amount of glycerol in the medium. At glycerol $65.2 \mu\text{mole/ml.}$ all the glycerol was utilized, but

at higher concentrations only a fraction was used. This might have been partly due to the pH value attained in the cultures. The carbon recovery showed that the fermentation products were accounted for and little glycerol carbon was utilized to form cellular material. Except at low values, the turbidity readings were related to mg. dry-weight organism/ml. (Fig. 4) in batch and continuous culture. The results of two continuous cultures, of 320 and 550 hr, are shown in Figs. 8, 9, 10. The turbidity readings are not corrected for growth in the basal medium since Fig. 4 does not show the same relationship between turbidity and dry weight of organisms at low and high turbidities and any correction would be very small and

Fig. 9

Fig. 10

Fig. 9. Continuous culture of lipolytic bacterium 5s; relationship between Y_{glyc} and dilution rate (D). Values are taken from two runs at a glycerol concentration of $65.6 \mu mole/ml.$ in the medium.

Fig. 10. Continuous culture of lipolytic bacterium 5s; relationship between VFA produced and dilution rate (D). Values are taken from two runs at a glycerol concentration of $65.6 \mu mole/ml.$ in the medium. At points marked $\bullet$ a true steady state was not achieved (see Fig. 8).

uncertain in magnitude. Results from other cultures also gave graphs similar to Fig. 8, which is unlike the selenomonad results. The medium remained at about pH 6 at dilution rates up to about $D = 0.3$; above that it increased to pH 6.6 at the highest dilution rate. Also included in Fig. 8 is a graph showing growth on the basal medium from one run of 190 hr. As in the case of selenomonad 6, the turbidity in continuous culture of bacterium 5s was always less than in batch culture in the basal medium.

Bacterium 5s never showed granular growth like the selenomonad strain 6; growth was always uniformly turbid. Like the selenomonad the motility of bacterium 5s was much greater in the continuous culture than in batch culture. When the pH value was suitable for growth the 5s organisms were uniform in length and generally single, except at the highest dilution rates, when a few chains began to appear. However, in the cultures where the pH value was uncontrolled and the growth cycled, slowing down of growth as the pH value decreased was accompanied by the appearance of long coiled filaments of uniform diameter and of length up to about ten times the normal. These forms disappeared as the pH value increased

again and growth re-started. Misshapen and spherical forms were also noted at low culture pH values when growth had stopped; these unnatural forms were commonly seen in batch cultures which had passed maximum growth.

DISCUSSION

The results of the present experiments show that it is possible to grow anaerobic rumen bacteria for prolonged periods in continuous culture. The cultivations reported here were done under carbohydrate-limiting conditions, but it would obviously be possible to use nitrogen-limiting conditions. The rumen is a kind of continuous culture, but apart from the fact that there is a wide diversity of microbial types present, it differs from the chemostat type of continuous culture described here in several ways, and, while the present results do give some pointers to the behaviour of rumen bacteria *in vivo*, it was not the purpose of these experiments to try to make an 'artificial rumen'. For instance, the rumen 'culture' has a very low dilution rate *in vivo*, only slightly more than one change of volume per day and, especially in the case of stall-fed animals, the micro-organisms do not attain a true steady state. Nutrient addition to the rumen, and to some extent saliva additions which form the main liquid flowing through the rumen, are intermittent and the concentrations of bacteria rise and fall in correspondence to these additions. Also, because of intermittent nutrient addition, it seems likely that different factors may limit bacterial growth at different times, and that the growth of all organisms will not be limited by the same nutrient. The change in amounts of fermentation products with growth rate, noted with the selenomonad strain 6 and to a lesser extent with the bacterium 5s in continuous culture, suggests that the results of *in vitro* batch cultures may not always be a quantitative guide to fermentation in the rumen. Some change in products with growth rate has been noted with other bacteria, for example *Streptococcus faecalis* (Rosenberger & Elsdén, 1960). If the results here are any guide to happenings *in vivo* then the maximum growth rates of the bacteria have some significance. If the maximum growth rate is taken as that at the beginning of the washout portion of the curves, then the selenomonad strain 6 and bacterium 5s have minimum doubling times ($\log_2 2/D$) of about 1.4 hr. This is very much longer than that of *S. bovis* and shows how it is possible in some cases of overfeeding of carbohydrate for *S. bovis* to increase so rapidly as to become the predominant rumen bacterium in a matter of hours. The effect of pH value on the growth of bacterium 5s is interesting (Fig. 2): at pH values below about 5.7 the growth rate markedly decreased, and growth appeared to cease at about pH 5.3. The pH value of rumen contents decreases after feeding and then increases and a value of pH 5.7 is above the minimum found on some rations. For instance, Purser & Moir (1959) and Bryant & Robinson (1961*b*) found minimum values of about pH 5.5 and 5.6 in animals fed on concentrates at 2 to 4 hr after feeding, compared with about pH 7.0 to 7.2 before feeding. Bryant & Robinson's results show that viable bacterial numbers rapidly increased till just before the time of minimum pH value, while the pH remained above 6, and then decreased slowly. If the results of Fig. 2 are typical of the main types of rumen bacteria then it would seem that pH value is probably the factor which controls growth rate of the bacteria, especially in the first few hours after feeding, and not the substrate concentration. The changes in morphology

of the selenomonad strain 6 and bacterium 5s point to a limitation in the techniques of counting morphological types of rumen bacteria at different times after feeding. As previously pointed out (Hobson & Mann, 1961) the selenomonads grown *in vitro* are much smaller than many seen *in vivo*, although there is a serological relationship between the organisms *in vitro* and *in vivo* (Hobson *et al.* 1962). One of the reasons for measuring the size of organisms in the selenomonad cultures was to see whether large forms were produced at high growth rates. Although the size of organism changed, no crescentic organisms of a size similar to that of those seen in the rumen were ever noted.

The theory of continuous culture proposed by Herbert *et al.* (1956) predicts that concentration of organism, equal to the maximum value found in batch culture, will not vary with growth rate until maximum growth rate (equated with dilution rate) is reached, but will then decrease rapidly. The limiting substrate concentration in the culture will generally remain very small until maximum growth rate is approached and will then increase rapidly. Some bacteria appear to behave in the theoretical manner, others diverge from the theoretical, but published data are scanty. A divergence at the higher growth rates, resulting in steady states at dilution rates apparently above the maximum growth rates and a slow decrease of concentration of organism as the dilution rate (D) increases (e.g. Fig. 5) was attributed by Herbert *et al.* (1956) to inadequate mixing in the culture vessel. This is a possibility, but it seems to be a phenomenon generally found and more probably indicates an inadequacy in the present theoretical treatments. Further divergences from the theoretical curve of concentration of organism against dilution rate have been found at low dilution rates. A decrease in organism dry weight/ml. at low dilution rates, found under carbohydrate-limiting conditions by Herbert (1958) with *Aerobacter aerogenes*, was ascribed to endogenous metabolism of cell substance. Herbert suggested a change in the equations for continuous culture which might account for this theoretically. It is also possible to account for this decrease in concentration of organism at low growth rates by supposing that some of the substrate is fermented to provide maintenance energy, thus decreasing the substrate available for cell multiplication. This will have the greatest effect when growth rate is low. All the bacteria grown in the present experiments showed a decrease in concentration of organism at low growth rates. All graphs (Figs. 3, 5, 8) also showed a maximum value for bacterial concentration. Since this peak in the graphs occurred with all the bacteria it seems unlikely to be an artifact. What is very unusual are the graphs for the selenomonad strain 6 (Figs. 5, 6). These show not only a pronounced decrease in bacterial concentration at low growth rates, but a very high yield of organism at optimum growth rate. The yield of organism during an anaerobic fermentation has been linked with the energy available in the 'high-energy' phosphate bonds of ATP. Bauchop & Elsdon (1960) found that for some bacteria of known fermentation pathways growing in a medium containing preformed monomers a yield of about 10 g. dry wt. organism/mole ATP (Y^{ATP}) produced during fermentation of the carbohydrate energy source was obtained. One of the bacteria tested by them, however, seemed to have a higher yield than could be accounted for by this method. Gunsalus & Schuster (1961) calculated that the energy available in a mole of ATP is sufficient to polymerize monomers into 33 g. of cell material, so that the experimental value of Y^{ATP} appears to be inefficient.

The theoretical yield of organism per mole of carbohydrate fermented can be calculated for simple fermentations, but calculation becomes difficult for more complex fermentations.

The yield of organism, Y_{glyc} , for bacterium 5s growing on glycerol approximates to 22 at the optimum growth rates in continuous culture and in batch culture where glycerol is limiting. At higher glycerol concentrations in batch culture Y_{glyc} decreases because fermentation of glycerol continues after growth is limited by a nitrogenous, or other, constituent of the medium, or by the decrease in pH value of the culture. The energy available in fermentation at this stage might be released by an ATPase type of enzyme, or might be used for maintenance of the organisms, although in this latter case the pH value (or other conditions in the medium) seems to be such that lysis of organisms takes place more rapidly than their structure can be maintained. The value of Y_{glyc} found for bacterium 5s approximates to formation of 2 ATP molecules during the fermentation (on the basis of $Y^{ATP} = 10$) and is in agreement with the results of Bauchop & Elsdén (1960) with a *Propionibacterium* fermenting glycerol.

The yield of selenomonads (Y_{gluc}), though, is about 62 at optimum growth rates (taking the average of values between the lines in Fig. 6). This would correspond to the formation of about 5–6 mole ATP/mole of glucose fermented. This seems improbable on present concepts of the pathways of fermentation. However, consideration of all the results suggests that the high yield is unlikely to be due to fermentation of the amino acids of the basal medium, or a secondary fermentation of lactate, and it must be concluded that this value is actually Y_{gluc} for this bacterium. There then remain the two possibilities that the selenomonads have sources of energy for growth other than those so far found in known modes of fermentation, or that Y^{ATP} is not universally 10, but is greater for some anaerobes. While this paper was being written Hungate (1963) reported that a strain of *Ruminococcus albus* growing in a continuous culture at a dilution rate of 0.1 hr^{-1} on cellobiose gave a value for Y_{gluc} of 55. This high yield was unexplained. The maximum value for Y_{gluc} calculated from the different batch cultures of the selenomonad approximates to 20, suggesting an ATP formation of 2 mole/mole glucose fermented on the basis of $Y^{ATP} = 10$. This appears reasonable, but since the yield of the selenomonad varies with growth rate in continuous culture it can be shown that the apparent yield of the batch cultures is probably entirely fortuitous, and bears no relationship to the actual yields under optimum growth conditions.

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