APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1979, p. 531-536
0099-2240/79/03-0531/06$02.00/0

Vol. 37, No. 3

Comparison of Substrate Affinities Among Several Rumen Bacteria: a Possible Determinant of Rumen Bacterial Competition

JAMES B. RUSSELL† * AND R. L. BALDWIN

Department of Animal Science, University of California, Davis, California 95616

Received for publication 4 January 1979

Five rumen bacteria, *Selenomonas ruminantium, Bacteroides ruminicola, Megasphaera elsdenii, Streptococcus bovis,* and *Butyrivibrio fibrisolvens* were grown in continuous culture. Estimates of substrate affinities were derived from Lineweaver-Burk plots of dilution rate versus substrate concentration. Each bacterium was grown on at least four of the six substrates: glucose, maltose, sucrose, cellobiose, xylose, and lactate. Wide variations in substrate affinities were seen among the substrates utilized by a species and among species for the same substrate. These wide differences indicate that substrate affinity may be a significant determinant of bacterial competition in the rumen where soluble substrate concentrations are often low. Growth of these bacteria in continuous culture did not always follow typical Michaelis-Menten kinetics. Inflated theoretical maximum growth rates and non-linear Lineweaver-Burk plots were sometimes seen. Maintenance energy expenditures and limitation of growth rate by factors other than substrate concentration (i.e., protein synthesis) are discussed as possible determinants of these deviations.

In the 1940's Monod observed that bacterial growth rate was dependent on substrate concentration and that the two were related according to saturation kinetics typical of enzyme systems (8). Since this time it has been generally accepted that bacterial growth rate and substrate concentration follow Michaelis kinetics. However, kinetic differences do exist between enzymes and bacteria. Pirt (11), in describing maintenance energy, corrected the Michaelis derivation of bacterial growth in continuous culture proposed by Herbert et al. (3) by adding a maintenance term. Because affinity is usually measured at high growth rates when substrate is easy to measure and maintenance as a percentage of total energy consumption is low, this maintenance term is generally neglected. Another important difference between bacterial growth rate and enzyme velocity lies in the fact that growth rate is limited by factors other than substrate concentration (13). This difference has been neglected.

Since substrate concentrations in natural environments are usually too low to permit maximum growth rate (5), it is reasonable to assume that relative substrate affinities can be a significant determinant of bacterial competitions in nature (10). Indeed, several mixed culture experiments in chemostats have shown that at a low dilution rate a bacterium with a low maximum growth rate can dominate a bacterium with a higher maximum growth rate (2, 7, 15, 16).

In the rumen there are often several species present that are capable of fermenting the same substrate. Because soluble substrate concentrations are usually low in the rumen (4), competition must exist among rumen bacteria for available substrates. It was hypothesized that if affinities differed significantly among rumen bacteria, relative substrate affinity could be a major determinant of bacterial growth and competition in the rumen. The experiments reported herein were undertaken to test this hypothesis.

MATERIALS AND METHODS

Media. The media used were similar to the medium described by Caldwell and Bryant (1), except that resazurin was not used, the type of reducing agent was changed, and concentrations of energy sources, yeast extract, and Trypticase were modified. Yeast extract and Trypticase were added at 0.5 and 1.0 g/liter, respectively. Cysteine-hydrochloride (0.06%) was used as the reducing agent instead of cysteine-sulfide. Each energy source was added at a level that would yield a maximum optical density (OD) of approximately 0.4. Preliminary experiments indicated that a linear plot of log OD versus time could be obtained by using this level of energy source with each of the five bacteria.

† Present address: Department of Animal Science, University of Illinois, Urbana, IL 61801.

Salt solution A, salt solution B, Na_2CO_3 (8%, wt/vol, in water), yeast extract (3.25%, wt/vol, in water), Trypticase (4.33%, wt/vol, in water), and volatile fatty acid mixture (adjusted to pH 9.4 with 10% NaOH) were prepared as separate solutions in round-bottom flasks. Each solution was brought to a boil under O_2-free CO_2, wired shut with a rubber stopper, and autoclaved for 30 min at 121°C. Glucose, maltose, sucrose, xylose (each 20%) cellobiose (15%), and L-lactate (10%, neutralized with 10% NaOH) were similarly prepared, except that they were brought to a boil under O_2-free N_2. Eleven liters of cysteine-hydrochloride and water were autoclaved for 2 h at 121°C and then immediately bubbled with O_2-free CO_2 and cooled to room temperature, and the other seven solutions were then added anaerobically. The medium reservoir was bubbled with O_2-free CO_2 throughout the incubation.

Organisms. *Megasphaera elsdenii* B159, *Butyrivibrio fibrisolvens* A38, *Selenomonas ruminantium* HD4, *Bacteroides ruminicola* GA33, and a recently isolated *Streptococcus bovis* were used (12).

Cell growth. Each bacterium was grown in a New Brunswick model C30 chemostat that was modified to exclude oxygen. The actual culture vessel was bubbled with O_2-free CO_2 to create a positive pressure. The culture vessel volume was 350 ml for *M. elsdenii, B. fibrisolvens, S. ruminantium,* and *B. ruminicola,* and a 170-ml vessel was used for *S. bovis.* All incubations were performed at 39°C.

Sampling and substrate analysis. An approximate dilution rate was set with a flow meter, but dilution rates were calculated from the total volume accumulated per unit time. At least a 98% turnover was allowed before a sample was taken at a new dilution rate. This 98% turnover time was calculated by dividing the natural logarithm of 0.02 by the dilution rate. Dilution rates were performed in decreasing order. Substrate samples were removed from the culture through a 6-inch (152.4 mm) 16-gauge needle and were immediately passed through a 0.45-μm membrane filter (Millipore Corp.). Separation of cells from the medium was always accomplished in less than 30 s. This substrate sample was stored at −15°C until time of analysis. Substrates were analyzed by methods described previously (13). OD was monitored at 600 nm using a Gilford spectrophotometer model 240. pH was also monitored and remained between 6.6 and 6.8 in all incubations. Actual plots used to estimate affinity were computed by using linear regression (14).

RESULTS

Approach. Bacterial substrate affinities can be so high that substrates are sometimes difficult, if not impossible, to detect in samples from the continuous culture vessel by using colorimetric or enzymatic methods. It was found that substrates could only be assayed when dilution rates were high enough to cause a decrease in OD and an increase in substrate concentration. The affinity constants listed in Tables 1–5 were derived over dilution rates that were high enough to allow measurable substrate concentrations. When affinity was low and K_S was high, the ranges of these dilution rates (range of regression line) were wide. When affinities were high, however, affinities could only be determined at very high dilution rates. In interpreting data obtained in the latter situation, the assumption was made that substrate affinities estimated at high dilution rates are similar to those expressed at low dilution rates where substrate measurements could not be performed. This assumption is supported by the fact that when affinities were determined over wide ranges of dilution rate (K_S high), Lineweaver-Burk plots were linear indicating constancy of K_S.

Problems of wall growth in continuous culture were discussed by Munson (9). The walls of culture vessels were examined during and after each incubation, and it was found that *M. elsdenii* and *S. bovis* cultures sometimes adhered to the walls. To minimize effects of wall growth, the highest dilution rates were performed first. By achieving these steady states first, the ratio of bacteria attached to the wall to bacteria suspended in solution was kept low. Linearity of the Lineweaver-Burk plot can also be used as an indicator of wall growth. When wall growth was a problem, nonlinear Lineweaver-Burk plots were seen.

B. fibrisolvens. Lineweaver-Burk plots were constructed for each *B. fibrisolvens* incubation, and the results are summarized in Table 1. The K_S values were determined by taking the negative reciprocal of the intercept of the abscissa, while the theoretical maximum growth rate was derived from the reciprocal of the intercept of the ordinate. A typical plot showing growth on glucose is shown in Fig. 1. Comparison of K_S values show that *B. fibrisolvens* has a very high affinity (low K_S) for glucose, maltose, less so cellobiose and considerably lower affinities for sucrose and xylose. Maximum theoretical growth rates from each plot are also given in Table 1. The values approximate closely the probable "washout rate" of each culture. At the upper limit of the regression line (Table 1), ODs

TABLE 1. *Affinity of B. fibrisolvens for different substrates*

Substrate	K_S (mM)	Theoretical K_{max} (h^{-1})	Correlation coefficient	Range of dilution[a] rates (h^{-1})
Glucose	0.009	0.50	0.99 (4)[b]	0.27–0.47
Maltose	0.006	0.50	0.86 (4)	0.35–0.49
Sucrose	0.262	0.83	0.95 (7)	0.42–0.66
Cellobiose	0.010	0.62	0.98 (4)	0.55–0.63
Xylose	0.367	0.71	0.94 (5)	0.49–0.60

[a] The range of where substrates were measured. Lactate is not utilized.

[b] Number of points in regression line.

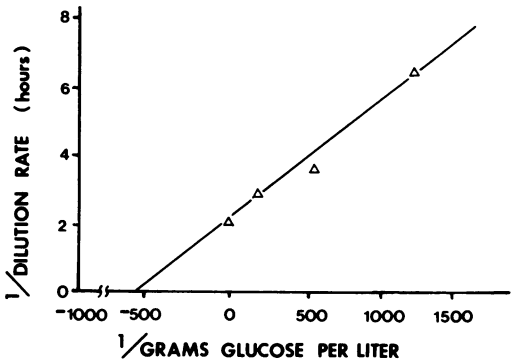

FIG. 1. *Lineweaver-Burk plot for the growth of B. fibrisolvens on glucose. The affinity constant, K_S, is 0.009 mM. The correlation coefficient of this plot is 0.99.*

were significantly reduced from maximum, and in each case it appeared that relatively small increases in dilution rate would wash out the culture.

B. ruminicola. The data for *B. ruminicola* are shown in Table 2. Comparisons of the K_S values among the substrates indicate that affinities for maltose, sucrose, and cellobiose are very low and are significantly lower than the affinity for glucose. The theoretical K_{max} for glucose appeared to correlate well with the probable "washout rate" of the culture, but the theoretical K_{max} values for maltose, sucrose, and cellobiose appeared to be very high. Because the upper limit of each regression line was near "washout" (OD was very low), it is unlikely that growth rates as high as these theoretical values could ever be achieved. A high degree of linearity in each of these plots is evidenced by the high correlation coefficients. The sucrose plot is shown in Fig. 2.

When *B. ruminicola* was grown on either sucrose or maltose, hexose was seen to accumulate in incubation media. The accumulation of hexose from sucrose was related to dilution rate (Table 6). Hexose accumulation from maltose was also higher at higher dilution rates, but showed greater variability in this regard (Table 7). Therefore, calculation of each affinity constant was based on total carbohydrate concentration in the medium rather than just disaccharide concentration. Hexose accumulation was not seen with cellobiose.

S. ruminantium. Glucose, maltose, sucrose, and xylose affinities were high in *S. ruminantium* (Table 3), and the theoretical K_{max} values appeared to approximate the probable washout rate of each culture. The lactate plot is shown in Fig. 3. Previous work (12) indicated that maximum growth rate on lactate was much lower than that for the other four substrates. Because maximum growth rate was so low, only low dilution rates could be used in the determination of K_S. At a dilution rate of 0.315 h^{-1}, OD was reduced to 0.021. This lactate plot shows a nonlinear relationship between 1/substrate concentration and 1/dilution rate that becomes nearly vertical at a low dilution rate. Wall growth was not observed during or at the end of the incubation, and it was concluded that maintenance energy expenditure was the probable cause of the deviation from linearity. Because the plot is nonlinear, it is impossible to give a precise esti-

TABLE 2. *Affinity of B. ruminicola for different substrates*

Substrate	K_S (mM)	Theoretical K_{max} (h^{-1})	Correlation coefficient	Range of dilution[a] rates (h^{-1})
Glucose	0.168	0.59	0.99 (4)[b]	0.49–0.54
Maltose	0.975	2.10	0.98 (7)	0.09–0.53
Sucrose	2.94	5.00	0.99 (7)	0.23–0.75
Cellobiose	11.76	4.00	0.97 (4)	0.18–0.49

[a] The range where substrates were measured. Xylose and lactate do not support growth.
[b] Number of points in regression line.

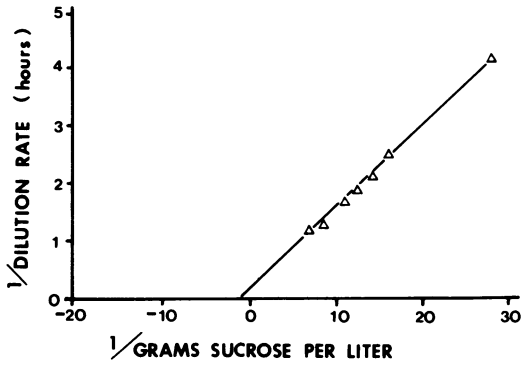

FIG. 2. *Lineweaver-Burk plot for the growth of B. ruminicola on sucrose. The affinity constant, K_S, is 2.94 mM. The correlation coefficient of this plot is 0.99.*

TABLE 3. *Affinity of S. ruminatium for different substrates*

Substrate	K_S (mM)	Theoretical K_{max} (h^{-1})	Correlation coefficient	Range of dilution[a] rates (h^{-1})
Glucose	0.046	0.95	1.00 (3)[b]	0.79–0.93
Maltose	0.058	0.83	0.95 (6)	0.26–0.74
Sucrose	0.004	1.25	0.98 (4)	0.83–1.19
Xylose	0.070	1.11	0.95 (8)	0.50–0.92
Lactate	See text			

[a] The range where substrates were measured. Cellobiose does not support growth (13).
[b] Number of points in regression line.

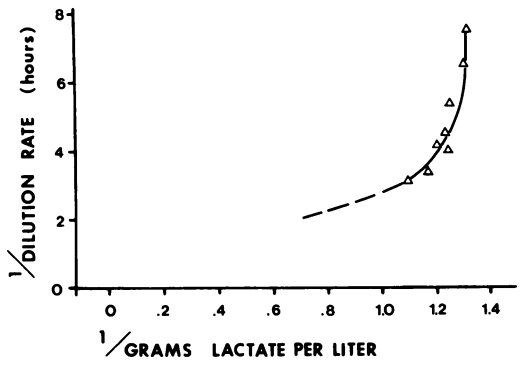

FIG. 3. *The Lineweaver-Burk plot for the growth of S. ruminantium on lactate.*

mate of K_S. K_S must be high (affinity low) because substrate levels never became low even at low dilution rates (Fig. 3).

M. elsdenii. Incubations with *M. elsdenii* indicated that affinity was lower for lactate than for glucose and that affinity was very low for maltose (Table 4). The theoretical K_{max} value for glucose appeared to be realistic, but the values for maltose and lactate appeared to be inflated because growth rates this high were not seen in batch cultures with very high substrate concentrations (12). Wall growth was not a problem in these incubations. Previous work (12) showed that maltose, glucose, and lactate gave much higher maximum growth rates than sucrose. The plot shown in Fig. 4 shows a nonlinear relationship similar to that described for lactate in *S. ruminantium*. Maintenance energy expenditures were again thought to be the cause of this non-linearity. Affinity for sucrose, although it cannot be predicted from the plot in Fig. 4, must be low because sucrose levels were high even at low dilution rates.

S. bovis. The data presented in Table 5 indicate that *S. bovis* has a high affinity for sucrose. A somewhat lower affinity was seen for maltose, and low affinities were found for cellobiose and glucose. Theoretical K_{max} values are realistic for the high affinity substrates, sucrose and maltose, but deviated from reality as the affinity became lower (glucose and cellobiose). The 3-min doubling time predicted for glucose clearly seems impossible. Wall growth was a particular problem with this bacterium (especially with growth on maltose and sucrose), but the bright orange color of this strain made identification of wall growth easy. By taking the high dilution rates first, linear Lineweaver-Burk plots could be obtained, but the speed at which wall growth became visually apparent limited the number of points that could be collected.

When carbohydrate concentration in the sucrose incubation of *S. bovis* was high due to high dilution rates, some hexose was measured. This level of hexose was rather constant and only made up a significant portion of total carbohydrate at high dilution rates (Table 8). Traces of hexose (<0.006 g/liter) were seen when maltose was used as a substrate, and no hexose could be detected in cellobiose incubations. The sucrose affinity constant, K_S, was based on total removal of carbohydrate, not just hydrolysis of sucrose.

DISCUSSION

The theoretical basis for estimation of K_S and K_{max} in a chemostat is that dilution rate (D) and

TABLE 4. *Affinity of M. elsdenii for different substrates*

Substrate	K_S (mM)	Theoretical K_{max} (h^{-1})	Correlation coefficient	Range of dilution[a] rates (h^{-1})
Glucose	0.111	0.53	0.90 (5)[b]	0.15–0.41
Maltose	1.34	1.66	0.97 (7)	0.11–0.39
Lactate	0.370	1.00	1.00 (5)	0.20–0.58
Sucrose	See text			

[a] The range where substrates were measured. Xylose and cellobiose are not utilized.
[b] Number of points in regression line.

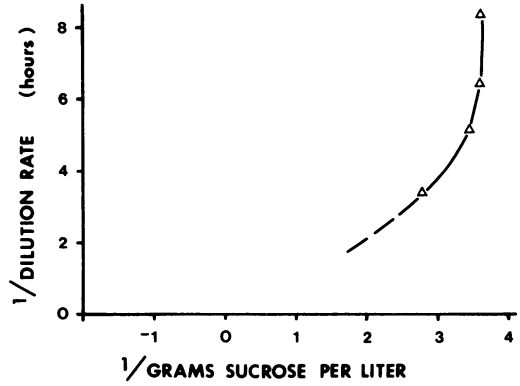

FIG. 4. *Lineweaver-Burk plot for the growth of M. elsdenii on sucrose.*

TABLE 5. *Affinity of S. bovis for different substrates*

Substrate	K_S (mM)	Theoretical K_{max} (h^{-1})	Correlation coefficient	Range of regression[a] line (h^{-1})
Glucose	5.56	20.0	0.99 (5)[b]	1.94–2.72
Sucrose	0.058	3.50	0.92 (4)	2.78–3.45
Maltose	0.155	2.94	0.96 (3)	2.25–2.63
Cellobiose	1.27	5.88	0.96 (4)	2.46–2.99

[a] The range where substrates were measured. Lactate and xylose are not utilized.
[b] Number of points in regression line.

hence growth rate (K) are related to substrate concentration (S) according to a Michaelis-Menten type function (18): $D = K = K_{max} S/K_S + S$. In the preceding equation, growth rate, K, approaches K_{max} when K_S is infinitely small compared to substrate concentration (S). If K were limited by factors other than substrate concentration (i.e., protein synthesis), and K_S is not very small compared to S, maximum observed growth rate would be less than the theoretical maximum growth rate, K_{max}. Magnitude of difference between theoretical and observed growth rates are thus related to the value of the affinity term, K_S. Large values of K_S (low affinity) lead to larger differences than do smaller values of K_S (high affinity).

The limitation of growth rate by factors other than substrate concentration is illustrated in Fig. 5. K_{max} is approached as substrate concentration becomes large, and the observed maximum growth rate $K_{max\text{-}0}$ is less than K_{max}. Increasing substrate levels above S' results in no further increase in growth rate because of the limitation. The deviation of $K_{max\text{-}0}$ from K_{max} is reflected in the ratio of K_S to S', namely, $K_{max\text{-}0}$ approaches K_{max} as K_S becomes small compared to S'.

Probable factors limiting maximum growth rate in bacteria have been discussed by Smith (13). His conclusion was that at maximum growth rate "... the cell is already so congested that there is no room for further reactants, or rather that the addition of further reactants would slow down the reaction." If Smith's conclusion is plausible, and it does seem so, addition of further substrate could not result in higher growth rates as would be predicted by a Michaelis-Menten function.

Deviations of theoretical maximum growth rates predicted by the Lineweaver-Burk plots from observed maximum growth rates were observed several times in this study, and each time the affinity constant, K_S, was high. The most notable example was the 3-min doubling time predicted for *S. bovis* on glucose. Other deviations were seen with *S. bovis* on cellobiose, *B. ruminicola* on cellobiose, sucrose, and maltose, and *M. elsdenii* on maltose.

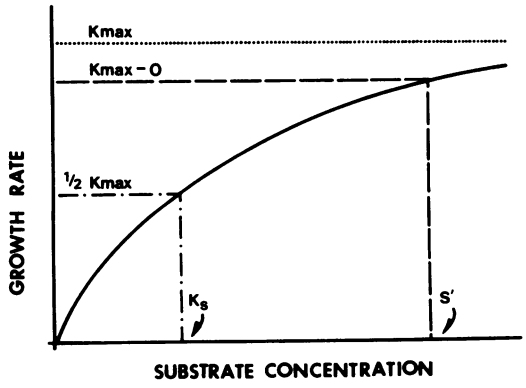

FIG. 5. *Relationship of substrate concentration and growth rate.*

The deviations from linearity of the Lineweaver-Burk plots shown in Fig. 3 and 4 can most easily be explained by maintenance energy expenditures. Maintenance energy expenditures make up a greater proportion of total substrate utilizations as growth rate decreases (11), and in each case (Fig. 3 and 4) the plot became nearly vertical at low growth rates. Such a plot indicates that as dilution rates become low, substrate levels become disproportionately larger. Because the ability to decrease substrate is based on growth rate and since maintenance energy has the effect of decreasing growth rate, a high maintenance energy expenditure could be expected to cause such a deviation. No obvious deviations from linearity were seen with *B. ruminicola* on sucrose and *M. elsdenii* on maltose even though the plots extended to as low as dilution rates, and it is suggested that maintenance energy expenditures may be abnormally high for the growth of *S. ruminantium* on lactate and *M. elsdenii* on sucrose.

The appearance of relatively large amounts of hexose in the sucrose incubation of *S. bovis* (Table 8) and the sucrose and maltose incubations of *B. ruminicola* (Tables 6 and 7) could be explained by either an extracellular (periplasmic space) hydrolysis of disaccharide or leakage from

TABLE 6. *B. ruminicola incubated in sucrose*

Dilution rate (h^{-1})	Sucrose concn (g/liter)	Hexose concn (g/liter)
0.228	0.023	0.014
0.373	0.038	0.026
0.463	0.051	0.025
0.516	0.056	0.025
0.583	0.062	0.030
0.713	0.085	0.042
0.754	0.105	0.041

TABLE 7. *B. ruminicola incubated in maltose*

Dilution rate (h^{-1})	Maltose concn (g/liter)	Hexose concn (g/liter)
0.090	0.015	0.001
0.119	0.024	0.000
0.135	0.032	0.000
0.213	0.042	0.004
0.316	0.040	0.016
0.418	0.066	0.006
0.533	0.095	0.010

TABLE 8. *S. bovis incubated in sucrose*

Dilution rate (h^{-1})	Sucrose concn (g/liter)	Hexose concn (g/liter)
2.78	0.069	0.027
3.12	0.308	0.058
3.40	0.559	0.032
3.45	0.605	0.028

cells of intracellular hexose. In *S. bovis* grown on sucrose, neither hexose nor sucrose could be detected at dilution rates below 2.78 h^{-1} (Table 8), but in *B. ruminicola* hexose and disaccharide could be detected at low dilution rates. Because sucrose affinity was high (Table 5) and because growth rate was very high (Table 8), a leakage of hexose seems possible. Intracellular hexose could accumulate under such a condition if rate of transport were to exceed rate of utilization for biosynthesis. Leakage of hexose from relatively starved cells (low dilution rate) does not seem likely. Because hexose accumulation occurred at low dilution rates (Tables 5 and 6), extracellular hydrolysis seems a more likely explanation than leakage of intracellular hexose. Extracellular hydrolysis and subsequent transport of hexose is consistent with the observation that glucose affinity is higher than maltose and sucrose affinities (Table 5). Further work is necessary to ascertain the exact origin of this hexose.

The K_S values listed in Tables 1-5 show a great deal of variation within a species for different substrates and among species for the same substrate. These wide variations in K_S seem to indicate a great deal of specialization among these bacteria for this group of substrates. *B. fibrisolvens* had approximately a 500-fold greater affinity for glucose than did *S. bovis,* but sucrose affinity was approximately fivefold higher for *S. bovis* than *B. fibrisolvens.* Maltose and glucose affinities were both higher for *B. fibrisolvens* than for *S. ruminantium,* but the situation was reversed for xylose and sucrose. *M. elsdenii* had low affinities for maltose and sucrose, but relatively high affinities for lactate and glucose. Affinity for glucose was much higher in *B. ruminicola* than affinities for maltose, sucrose, and cellobiose, while sucrose and maltose affinities were substantially higher than glucose and cellobiose affinities in *S. bovis.*

The wide differences discussed above suggest that substrate affinity could be a significant determinant of bacterial competitions in the rumen where soluble substrates are often low. Other factors such as pH, maintenance energy expenditures, maximum growth rate, and substrate preference also play an obvious role in these competitions. Both substrate preferences and effects of pH on maximum growth rates have been described for the five bacteria six substrate test system used in this study (12; J. B. Russell, W. M. Sharp, and R. L. Baldwin, J. Anim. Sci., in press). Maintenance energy expenditures are currently being studied. It is hoped this test system will provide the basis of a more quantitative understanding of bacterial competition and feed utilization in the rumen.

LITERATURE CITED

1. **Caldwell, D. R., and M. P. Bryant.** 1966. Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria. Appl. Microbiol. **14**:794-801.
2. **Harder, W., J. G. Kuenen, and A. Maten.** 1977. Microbial selection in continuous culture. J. Appl. Bacteriol. **43**:1-24.
3. **Herbert, D. R., R. Elsworth, and R. C. Telling.** 1956. The continuous culture of bacteria: a theoretical and experimental study. J. Gen. Microbiol. **14**:601-622.
4. **Hungate, E.** 1966. The rumen and its microbes, p. 230. Academic Press Inc., New York.
5. **Koch, A. L.** 1971. The adaptive responses of *Escherichia coli* to a feast and famine existence. Adv. Microb. Physiol. **6**:147-218.
6. **Linton, D., D. Harrison, and A. T. Bull.** 1977. Molar growth yields, respiration and cytochrome profiles of *Beneckea natriegens* when grown under carbon limitation in a chemostat. Arch. Microbiol. **115**:135-142.
7. **Meers, J. L.** 1973. Growth of bacteria in mixed cultures. Crit. Rev. Microbiol. **2**:139-184.
8. **Monod, J.** 1949. The growth of bacterial cultures. Ann. Rev. Microbiol. **3**:371-394.
9. **Munson, R. J.** 1970. Turbidostats, p. 349-376. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 2. Academic Press Inc., New York.
10. **Neijssel, O. M., and D. W. Tempest.** 1975. The regulation of carbohydrate metabolism in *Kelbsiella aerogenes* NCTC 418 organisms, growing in chemostat culture. Arch. Microbiol. **106**:251-258.
11. **Pirt, S. J.** 1965. The maintenance energy of bacteria in growing cultures. Proc. R. Soc. London Ser. B **163**:224-231.
12. **Russell, J. B., and R. L. Baldwin.** 1978. Substrate preferences in rumen bacteria: evidence of catabolite regulatory mechanisms. Appl. Environ. Microbiol. **36**: 319-329.
13. **Smith, J. M.** 1969. Limitations on growth rate. Symp. Soc. Gen. Microbiol. **19**:1-13.
14. **Snedecor, G. W., and W. G. Cochran.** 1967. Statistical methods, 5th ed. Iowa State University Press, Ames.
15. **Veldkamp, H., and H. W. Jannasch.** 1972. Mixed culture studies with the chemostat. J. Appl. Chem. Biotechnol. **22**:105-123.
16. **Veldkamp, H., and J. G. Kuenen.** 1973. The chemostat as a model for ecological studies. Bull. Ecol. Res. Comm. (Stockholm) **17**:347-355.
17. **Tempest, D. W.** 1970. The continuous culture of microorganisms, p. 259-276. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 2. Academic Press Inc., New York.