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Substrate Preferences in Rumen Bacteria: Evidence of Catabolite Regulatory Mechanisms

JAMES B. RUSSELL AND R. L. BALDWIN*

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

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Five species of rumen bacteria with overlapping substrate fermentative capabilities were tested for substrate preferences and evidence of catabolite regulatory mechanisms. All five bacteria showed evidence of some type of catabolite regulatory mechanism. In the six-substrate test system that was used, utilization of every substrate was inhibited by another substrate in at least one of the bacteria. Inhibited versus noninhibited substrate data suggest that the five bacteria have different strategies of substrate utilization and thus occupy separate niches in the rumen. The significance of these observations to understanding the rumen ecosystem is discussed.

The knowledge that bacteria can preferentially use one substrate to the exclusion of others is not new. Epps and Gale first reported this effect in 1942 (8). Monod (22) further studied this phenomenon in the late 1940s and referred to it as the "glucose effect." Magasanik (20) coined the term "catabolite repression" to describe the phenomenon when it was discovered that substrates other than glucose could produce the same effect. After the discovery of cyclic AMP in bacteria in 1964 by Makman and Sutherland (21), a cyclic AMP-dependent mechanism of catabolite repression was gradually uncovered (23, 35).

In spite of elucidation of cyclic AMP-dependent catabolite repression, the complete mechanism by which bacteria preferentially use one substrate to the exclusion of others is not well understood. The correlation between inducibility and cyclic AMP level has been shown in a number of experiments to be poor (19, 24, 33, 34). The absence of cyclic AMP in *Bacillus licheniformis* (3) and *Lactobacillus plantarum* (11) despite sequential utilization of substrates suggests that other mechanisms and possibly other nucleotides are involved.

Two additional mechanisms have been proposed to explain sequential utilization not explained by catabolite repression. The first of these proposed mechanisms, "catabolite inhibition," suggests that sequential utilization is mediated by an interference of preferred substrate with the enzymatic utilization of other substrates (18, 19). The site of this interference was found to be at the point of transport of the secondary substrate. The second proposed mechanism, "phosphotransferase system-mediated repression," suggests that sequential utilization is mediated by exclusion of inducer at the cell membrane. The independence of these two mechanisms is not clear (25-27). "Inducer exclusion" and catabolite inhibition appear to be much faster mechanisms than catabolite repression. In this presentation, catabolite repression, catabolite inhibition, and inducer exclusion (phosphotransferase system-mediated repression) will be referred to collectively as "catabolite regulatory mechanisms."

There are several bacterial species present in the rumen which ferment a wide, overlapping range of substrates. If some of these bacteria have catabolite regulatory mechanisms, the diversity of bacterial species in the rumen might be better understood. The niche described as "soluble carbohydrate-fermenting bacteria" might actually be an aggregate of several smaller niches. Two bacteria capable of fermenting the same set of substrates might not be in direct competition if catabolite regulatory mechanisms cause them to ferment different substrates during critical periods of the feeding cycle.

The purpose of this study was to determine whether rumen bacteria exhibit sequential patterns of substrate utilization characteristic of catabolite regulatory mechanisms. Five rumen bacteria were incubated separately with multiple substrates to determine whether these bacteria preferentially use one or more substrates to the exclusion of others. Preferential and sequential substrate utilization would be considered evidence of catabolite regulatory mechanisms in rumen bacteria. The results might also indicate whether bacteria in the soluble carbohydrate-fermenting group actually occupy separate niches.

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MATERIALS AND METHODS

Organisms. *Megasphaera elsdenii* B159, *Butyrivibrio fibrisolvens* A38, *Selenomonas ruminatium* HD4, *Bacteroides ruminicola* GA33, and a newly isolated *Streptococcus bovis* were used. The *S. bovis* was isolated from the rumen of a cow fed alfalfa. A bright orange colony was isolated from a rumen fluid roll tube after 7 h of incubation at a 10^7 dilution. The bright orange colonies were not seen at higher dilutions (J. M. Macy). The isolated cells were gram positive, ovoid in shape, and occurred in pairs or in moderate chains (as many as eight cells). The culture fermented glucose, sucrose, maltose, cellobiose, lactose, and starch. The culture did not ferment xylose, arabinose, or ribose. Large amounts of L-lactic acid were produced, and a doubling time of 24 min was observed when glucose was the substrate. Growth occurred at 45°C but not at 50°C. When NaCl was added to the medium, growth was seen at 2% but not at 6.5%. A medium composed of salts and 0.8 g of glucose, 300 mg of ammonia, and 5 mg of yeast extract per liter gave good growth.

Media. Media used were similar to those described by Caldwell and Bryant (6). Cultures were maintained in tubes reduced with 0.044% cysteine·HCl·H_2O. Actual "test incubations" were carried out in media containing cysteine sulfide (6). Both carbon source and amino nitrogen source were different from the media described by Caldwell and Bryant. The incubations shown in Fig. 1 through 18 contained 0.5 g of Trypticase and 20 mg of DL-methionine per liter; the incubation shown in Fig. 19 contained 0.75 g of Trypticase and 30 mg of DL-methionine per liter; and the incubations shown in Fig. 20 through 23 contained 1.0 g of Trypticase and 40 mg of DL-methionine per liter. The level of carbon source (xylose, glucose, maltose, sucrose, cellobiose, or lactate) used in each incubation was as indicated in each of the figures. The cultures were maintained in media that were the same as the incubation media except that the carbon source was varied. The maintenance media used for the incubations shown in Fig. 1, 7, 12, 18, and 20 contained 0.180 g of glucose per liter. All other maintenance media contained the level of carbon source present in that particular incubation medium at the start of that incubation. Resazurin was used in the maintenance tubes but not in the incubation media.

Cell growth. Incubations were performed at 39°C in a 500-ml flask continuously gassed with O_2-free CO_2. The CO_2 was first passed through a large-capacity oxygen-removing furnace. The copper column of the oxygen-removing furnace was constantly observed for oxidation and was never oxidized for more than one-quarter of its length. The pH of all media remained between 6.75 and 6.9. Samples (10 ml) of incubation media were removed every 30 min through a butyl rubber septum in the bottom of the incubation flask. A portion of this sample was checked for optical density in a Gilford spectrophotometer (model 240) at 600 nm. The remainder of the sample was immediately centrifuged at 31,000 × *g* for 20 min at 0°C. *S. bovis* cultures were put thorugh a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.) being centrifuged. Supernatants were quickly frozen and stored at −15°C until carbon sources were analyzed. When substrates were added during the incubation, they were injected through the butyl rubber septum with a syringe from a sterile, anaerobic, 0.9% solution of that substrate. An appropriate amount was added to reach the desired concentration.

Maximum growth rate. Table 3 shows the maximum growth rate of each bacterium when grown on a single substrate. Both nitrogen (1.0 g of Trypticase per liter) and substrate concentrations (4 g/liter) were kept high. All other media ingredients were the same as described above. Growth curves were constructed by measuring increase in optical density versus time with a Bausch & Lomb Spectronic 20 spectrophotometer at 600 nm. Whole tubes were inserted in the Spectronic 20 spectrophotometer every 10 min (*S. bovis*) or every 30 min (*B. ruminicola, S. ruminantium, M. elsdenii,* and *B. fibrisolvens*). The growth constant K was computed by taking the slope of the exponential phase of a logarithm of an optical density-versus-time curve. At least seven points were used in the computation of each K.

Substrate analyses. There are several methods available for the discrimination and assay of the carbohydrates used. Spectrophotometric and enzymatic methods were selected because they provided the sensitivity and discrimination needed and enabled analysis of the numbers of samples required in the study.

Xylose was assayed by the method of Schneider (29). Pentoses (xylose) were differentiated from hexoses by taking the difference of readings at 660 and 600 nm (7). The extinction coefficient for hexoses is the same at both wavelengths. Glucose was assayed by the hexokinase–glucose-6-phosphate dehydrogenase (ATP:D-hexose 6-phosphotransferase [EC 2.7.1.1.] – D-glucose-6-phosphate : $NADP^+$ 1-oxidoreductase [EC 1.1.1.49]) method of Bergmeyer and Klotsch (2). Lactate was assayed by the method of Hohorst (14). Disaccharides were assayed by hydrolysis and measurement of the resulting monosaccharides.

β-D-Glucoside glucohydrolase (EC 3.2.1.21) and β-D-fructoside fructohydrolase (EC 3.2.1.26) were specific for the hydrolysis of cellobiose and sucrose, respectively. α-D-Glucoside glucohydrolase (EC 3.2.1.20), however, was not specific within the mixture of carbohydrates used. This lack of specificity was due to the presence of two enzymes within the enzyme preparation. One of the enzymes attacked sucrose and maltose, and the other attacked sucrose but not maltose. The two enzymes have very similar properties and have proven impossible to separate by standard separation techniques (10, 17). Because of this lack of specificity and kinetic complexity, sucrose in samples was first hydrolyzed with β-D-fructoside fructohydrolase and assayed before maltose was analyzed by using α-D-glucoside glucohydrolase. Glucose resulting from hydrolysis of the three disaccharides was assayed by the hexokinase–glucose-6-phosphate dehydrogenase method (2). Fructose resulting from the hydrolysis of sucrose was measured by this method after conversion to glucose by D-glucose-6-phosphate isomerase (EC 5.3.1.9).

Sucrose was assayed by first letting the sample react with hexokinase-glucose-6-phosphate dehydrogenase system to remove glucose. Then, 50 IU of β-D-fructofuranoside fructohydrolase and 6 IU of D-glucose-6-phosphate ketol-isomerase were added to each cuvette containing 2.5 ml of buffer and 0.15 ml of sample. Optical density was recorded until the reaction reached completion. Cellobiose was assayed by placing 0.015 ml of sample in 0.04 ml of acetate buffer (0.1 M; pH 4.0) in a microcuvette. A 2-IU quantity of β-D-glucoside glucohydrolase was then added, and the mixture was allowed to react for exactly 45 min. The enzymatic reaction was stopped by raising the pH to approximately 10 with the addition of 0.003 ml of 1 N NaOH. The resulting glucose (from cellobiose) and the original glucose in the sample were assayed by the hexokinase-glucose-6-phosphate dehydrogenase procedure. Absorbance due to glucose present in the original sample before enzymatic hydrolysis of cellobiose was subtracted so that the resulting absorbance was indicative of the amount of cellobiose. Maltose was assayed by adding 50 IU of β-D-fructofuranoside fructohydrolase and 6 IU of D-glucose-6-phosphate ketol-isomerase to 1 ml of the hexokinase-glucose-6-phosphate dehydrogenase buffer system. Quantities of 0.35 ml of buffer and 0.015 ml of sample were added to each cuvette and allowed to react until completion. A 2-IU quantity of α-D-glucoside glucohydrolase was then added to each cuvette, and increase in optical density was recorded for exactly 30 min. All assays were compared with standard curves. None of the components of the incubation media interfered with the linearity of the assays. Interference of one carbon source upon assays of other carbon sources was found to be less than 2% in all cases.

β-D-Fructofuranoside fructohydrolase (150 IU/mg of protein), α-D-glucoside glucohydrolase (50 IU/mg of protein), and β-D-glucoside glucohydrolase (50 IU/mg of protein) were purchased from Boehringer Mannheim Corp., Indianapolis, Ind. Glucose-6-phosphate dehydrogenase (220 IU/mg of protein), D-glucose-6-phosphate ketol-isomerase (550 IU/mg of protein), and NADP were purchased from Sigma Chemical Co., St. Louis, Mo. Hexokinase (lyophilized, salt-free) was purchased from Calbiochem, La Jolla, Calif. Other chemicals were purchased from standard commercial sources.

RESULTS

Approach. Each bacterium was first grown on a mixture of all substrates that it could use (Table 1). In these incubations, growth curves were monitored for lags characteristic of catabolite repression. Substrate disappearances were monitored to determine whether substrates were used simultaneously or sequentially. When sequential utilization was observed, further incubations were performed to determine which substrate(s) was responsible for apparent repressive effects. In these later incubations, bacteria were first grown on the substrate(s) utilized last in the earlier study (nonpreferred substrate). After moderate growth was attained, a preferred substrate was injected into the medium. Disappearance of both substrates was monitored to determine what effect the preferred substrate had on utilization of the nonpreferred substrate(s). Simultaneous and accelerating utilization of both substrates was taken as indicating no repression of one substrate by the other. If addition of the preferred substrate completely halted the disappearance of the nonpreferred substrate, it was presumed that a catabolite regulatory mechanism was operating. If a linear or decelerating utilization of the nonpreferred substrate was accompanied by accelerating use of the preferred substrate and large increases in microbial numbers, the presence of a catabolite regulatory mechanism was assumed. It was felt that increasing cell numbers (optical density) should always result in increased rates of substrate disappearance unless cells were becoming less fit to use that particular substrate. Any such decrease in fitness was attributed to the presence of a catabolite regulatory mechanism. Even at slow growth rates, substrate utilization should accelerate due to a combination of growth and maintenance energy expenditures. It has been noted that maintenance expenditures are quite high in slow-growing cultures and that total expenditure is proportional to cell number (32).

If a repressive interaction were simply the result of competition for the same transport system, one would not expect to see a lag in the growth curve (protein synthesis) when the preferred substrate is depleted. Similarly, one would expect to see a fixed ratio of preferred versus nonpreferred substrate utilization. Accelerating rates of use of preferred substrates coupled with linear or decelerating rates of use of nonpreferred substrates are not consistent with simple competition for transport.

TABLE 1. *Possible substrates for each of the five bacteria*

Species	Substrates					
	Glucose	Xylose	Maltose	Sucrose	Cellobiose	Lactate
B. ruminicola	+	?[a]	+	+	+	−
B. fibrisolvens	+	+	+	+	+	−
M. elsdenii	+	−	+	+	−	+
S. bovis	+	−	+	+	+	−
S. ruminantium	+	+	+	+	+	+

[a] See text for explanation.

S. ruminantium. In Fig. 1, results from a six-substrate incubation performed with *S. ruminantium* are shown. In this incubation, *S. ruminantium* did not use maltose or cellobiose until xylose, sucrose, and glucose were depleted, implying sequential substrate utilization or the presence of a catabolite regulatory mechanism. When *S. ruminantium* was incubated initially with maltose, xylose, and cellobiose (Fig. 2), addition of glucose at 3 h caused almost complete cessation of maltose utilization, but did not alter the rate of xylose utilization. A lag in growth at 6 to 7 h, corresponding to depletion of glucose, is also evident in Fig. 2. After depletion of glucose, rapid maltose utilization resumed, and cellobiose and lactate were used. The simultaneous utilization of xylose and maltose (1 to 3 h) and maltose and cellobiose (5.5 to 6.5 h) indicates no inhibition among these pairs of substrates. Addition of sucrose after 3 h of growth on maltose caused complete cessation of maltose utilization (Fig. 3). Depletion of sucrose was followed by resumption of maltose utilization. Lactate accumulated during the entire incubation, but the rate of lactate production decreased between 6.5 and 7 h despite rapid utilization of maltose. Cellobiose utilization was completely inhibited by the addition of sucrose at 3.5 h (Fig. 4). Depletion of sucrose was accompanied by a lag in the growth curve, resumption of cellobiose utilization, and cessation of lactate accumulation. A slight decrease in lactate was noted between 9 and 10 h. The data in Fig. 5 show that glucose addition produced essentially the same result as sucrose (Fig. 4). Simultaneous and accelerating utilization of xylose and cellobiose (Fig. 6) indicated no inhibition between these two substrates. Depletion of xylose was associated with a decrease in lactate and cell numbers. The data shown in Fig. 1 through 6 indicate that sucrose, xylose, and glucose utilizations were not

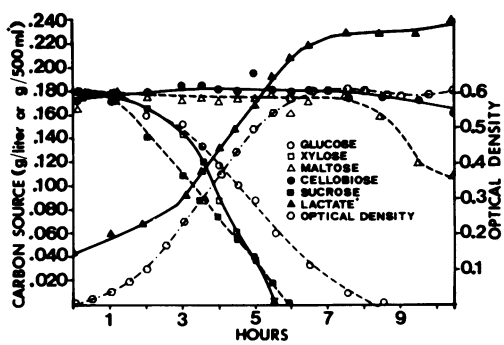

FIG. 1. *S. ruminantium incubated with glucose, xylose, maltose, cellobiose, sucrose, and lactate. Lactate is expressed as grams per 500 ml.*

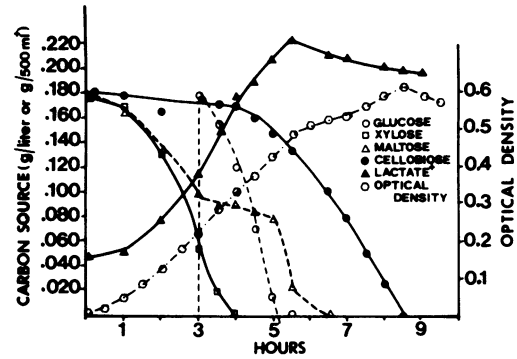

FIG. 2. *S. ruminantium incubated with xylose, maltose, cellobiose, and lactate. Glucose was added after 3 h. Lactate is expressed as grams per 500 ml.*

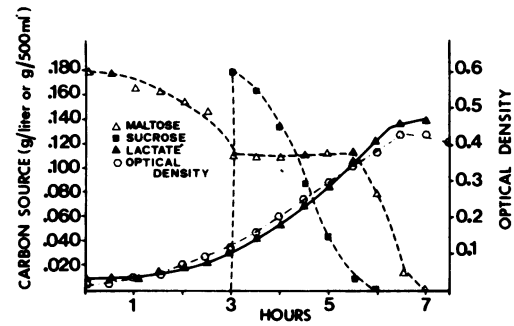

FIG. 3. *S. ruminantium incubated with maltose and lactate (lactate derived from inoculum). Sucrose was added after 3 h. Lactate is expressed as grams per 500 ml.*

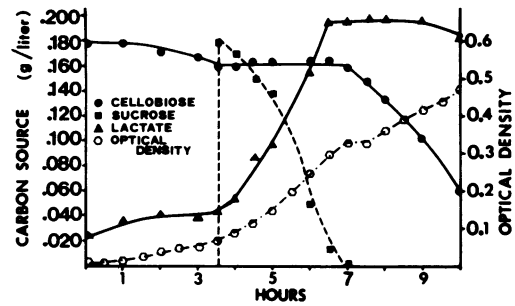

FIG. 4. *S. ruminantium incubated with cellobiose and lactate. Sucrose was added after 3.5 h.*

inhibited by any of the five other substrates. Maltose, cellobiose, and lactate utilizations were repressed by glucose and sucrose (Table 2). The growth of *S. ruminatium* on single substrates is shown in Table 3. Maximum growth rates were lower for lactate, cellobiose, and maltose than for the preferred substrates glucose, sucrose, and xylose. When a cellobiose medium was inoculated from a glucose-grown culture, *S. ruminatium* used cellobiose, and a growth rate of 0.19

h^{-1} is obtained. However, if this same cellobiose culture was used to inoculate another cellobiose medium, a growth rate of 0.06 h^{-1} was seen. It appears that cellobiose alone was poorly utilized and that lactate, derived in this case from the inoculum, was needed for rapid cellobiose utilization. In Fig. 2, 4, and 5, cellobiose was used when lactate concentration was high. The rather poor utilization of cellobiose at the beginning of the incubations shown in Fig. 4 and 5 might be explained by the fact that lactate concentrations were low.

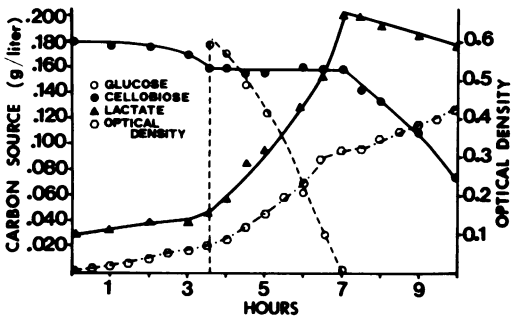

FIG. 5. *S. ruminantium incubated with cellobiose and lactate. Glucose was added after 3.5 h.*

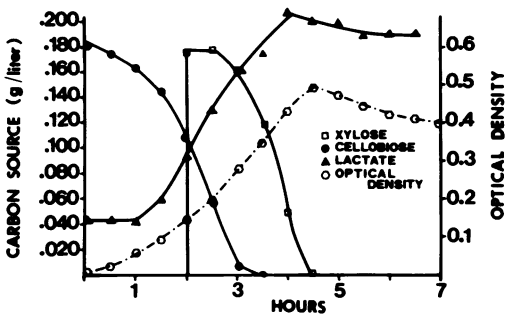

FIG. 6. *S. ruminantium incubated with cellobiose and lactate. Xylose was added after 2 h.*

S. bovis. The multiple-substrate incubation with *S. bovis* (Fig. 7) indicated that cellobiose and maltose were not utilized until glucose and sucrose were depleted. Addition of glucose at 1 h (Fig. 8) caused complete cessation of maltose utilization. After glucose was depleted, rapid maltose utilization resumed. Addition of sucrose also caused inhibition of maltose utilization (Fig. 9). However, inhibition was not as immediate as the inhibition caused by glucose (Fig. 8). This lag (0.25 h) before cessation of maltose utilization was also associated with a lag in sucrose uptake by the culture. Addition of sucrose to the cellobiose incubation (Fig. 10) resulted in a decrease in the rate of cellobiose utilization. This decreased utilization rate occurred despite rapidly increasing cell numbers and an increasing rate of sucrose disappearance. Both cellobiose and glucose were rapidly utilized after addition of glucose at 1.375 h (Fig. 11). Rates of cellobiose and glucose disappearance were approximately equal at the end of the incubation, indicating no repressive interaction between these two substrates. Taken in aggregate, the five *S. bovis* incubations indicate that glucose and sucrose are non-catabolite-regulated substrates in this microbe. Maltose was inhibited by both glucose and sucrose. Cellobiose utilization was repressed by sucrose but not by glucose (Table 2).

The highest maximum growth rate was seen in *S. bovis* when it was grown on sucrose (Table 3), and lesser growth rates were seen when it was grown on either maltose or cellobiose, the catabolite-regulated substrates. A relatively low growth rate was seen with glucose, and it is somewhat surprising that glucose was seen to inhibit maltose, a substrate which yields a higher growth rate.

TABLE 2. *Summary of substrate interactions for each bacterium*

Species	Non-catabolite-regulated substrates	Catabolite-regulated substrates	Inhibitors
B. ruminicola	Glucose		
S. bovis	Glucose	Maltose	Glucose, sucrose
	Sucrose	Cellobiose	Sucrose
M. elsdenii	Glucose, maltose, lactate	Sucrose	Glucose, sucrose
B. fibrisolvens	Maltose, sucrose	Cellobiose	Maltose, sucrose
		Glucose, xylose	Maltose, sucrose
S. ruminantium	Sucrose, xylose, glucose	Maltose	Glucose, sucrose
		Cellobiose	Glucose, sucrose
		Lactate	Glucose, sucrose

TABLE 3. *Maximum growth rates on single substrates*

Species	Growth rate (h^{-1}) in:					
	Glucose	Maltose	Sucrose	Cellobiose	Xylose	Lactate
S. ruminantium	0.72	0.35	0.67	0.06[a]	0.64	0.15
B. ruminicola	0.56	0.52	0.62	0.20	0.04[a]	
B. fibrisolvens	0.39	0.54	0.52	0.53	0.45	
S. bovis	2.04	1.85	2.1	1.83		
M. elsdenii	0.45	0.55	0.14			0.21

[a] See text for explanation.

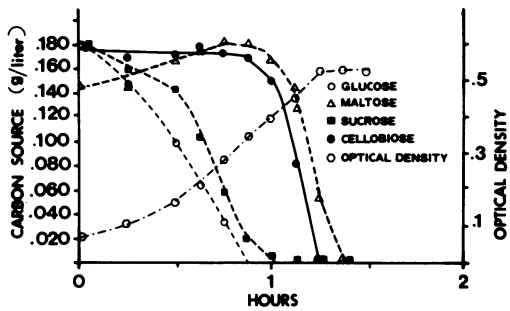

FIG. 7. *S. bovis incubated with glucose, sucrose, maltose, and cellobiose.*

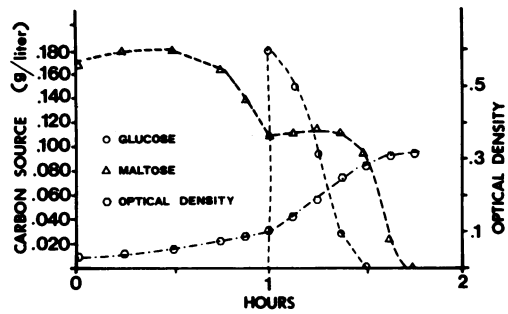

FIG. 8. *S. bovis incubated with maltose. Glucose was added after 1 h.*

B. fibrisolvens. *B. fibrisolvens* (Fig. 12) utilized maltose and sucrose in a rapid, accelerating rate during the first 5.5 h of incubation with glucose, xylose, maltose, cellobiose, and sucrose. After depletion of maltose and sucrose at 5.5 h, a pronounced lag in microbial growth occurred. The lag in growth was followed by sharp increases in rates of cellobiose, glucose, and xylose utilization. Addition of maltose to incubations containing glucose, xylose, and cellobiose (Fig. 13) after 5.5 h resulted in a decrease in cellobiose utilization. Depletion of maltose at 7.5 h was followed by marked increases in rates of glucose and xylose utilization and a growth lag. The data in Fig. 14 show accelerating utilization of cellobiose before addition of maltose at 2.5 h and a decelerating rate of cellobiose utilization thereafter. This deceleration occurred while bacterial

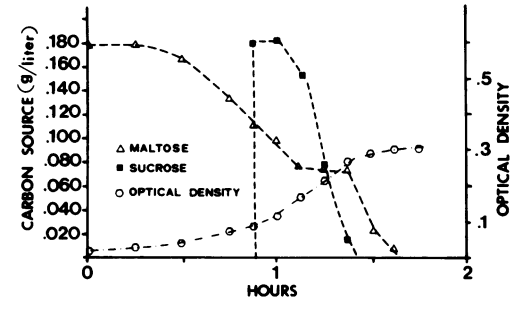

FIG. 9. *S. bovis incubated with maltose. Sucrose was added after 0.875 h.*

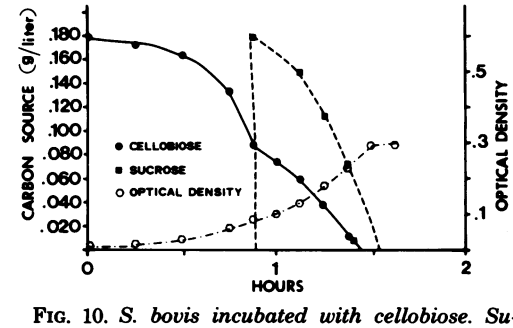

FIG. 10. *S. bovis incubated with cellobiose. Sucrose was added after 0.875 h.*

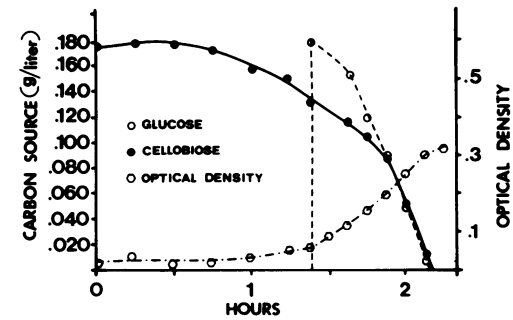

FIG. 11. *S. bovis incubated with cellobiose. Glucose was added after 1.375 h.*

mass increased and utilization of maltose accelerated. Similarly (Fig. 15), addition of sucrose after 2 h of incubation of *B. fibrisolvens* with

cellobiose was accompanied by a linear decrease in cellobiose. When sucrose was nearly depleted at 4.5 h, the rate of cellobiose disappearance increased once again. Cellobiose utilization decelerated after 6 h, but there was little bacterial growth after this time. Addition of sucrose after 6.5 h of incubation with glucose and xylose caused a non-accelerating rate of glucose and xylose utilization (Fig. 16). This non-accelerating rate occurred during exponential bacterial growth and accelerating rates of sucrose use. Figure 17 shows essentially the same phenomenon as Fig. 16 except that maltose was added instead of sucrose. In the *B. fibrisolvens* incubations, maltose and sucrose utilizations were not repressed. Cellobiose, glucose, and xylose utilizations were all inhibited by both maltose and sucrose (Table 2). Glucose and xylose (Table 3) gave lower maximal growth rates than the disaccharides sucrose, maltose, and cellobiose.

B. ruminicola. *B. ruminicola* showed no obvious lags in growth during a five-substrate incubation (Fig. 18). Rapid utilization of sucrose, cellobiose, and xylose did not occur until maltose was depleted. Addition of maltose after 5 h of incubation with sucrose, xylose, and cellobiose caused a sharp increase in the growth rate. Maltose was used at an accelerating rate until microbial growth began to decrease. During this same time (5 to 6.5 h) sucrose, cellobiose, and

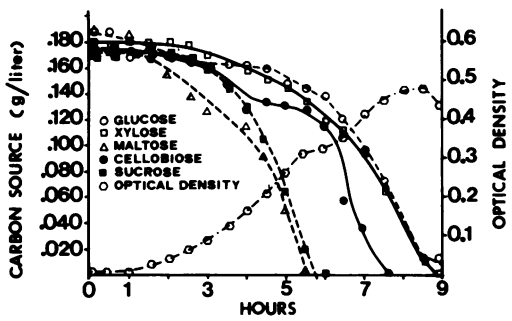

FIG. 12. *B. fibrisolvens incubated with glucose, xylose, maltose, cellobiose, and sucrose.*

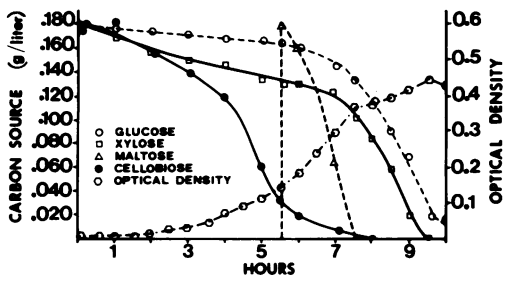

FIG. 13. *B. fibrisolvens incubated with glucose, xylose, and cellobiose. Maltose was added after 5.5 h.*

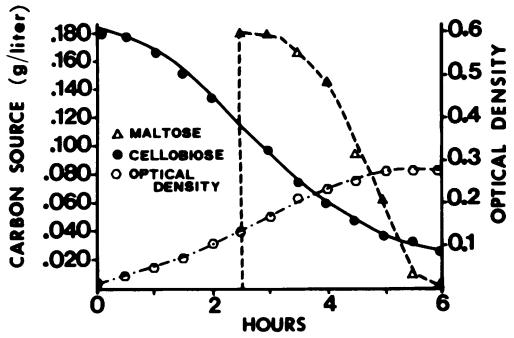

FIG. 14. *B. fibrisolvens incubated with cellobiose. Maltose was added after 2.5 h.*

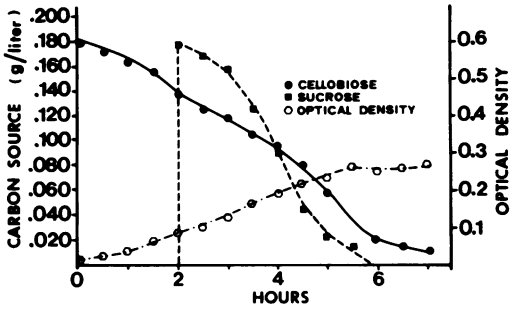

FIG. 15. *B. fibrisolvens incubated with cellobiose. Sucrose was added after 2 h.*

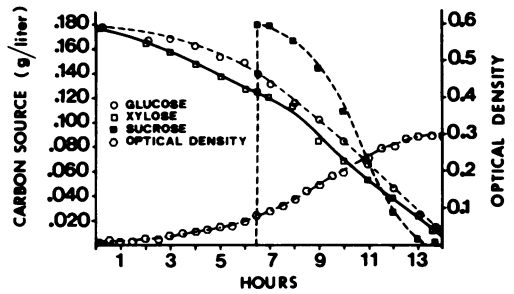

FIG. 16. *B. fibrisolvens incubated with glucose and xylose. Sucrose was added after 6.5 h.*

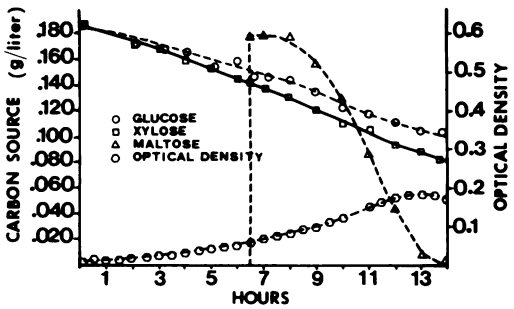

FIG. 17. *B. fibrisolvens incubated with glucose and xylose. Maltose was added after 6.5 h.*

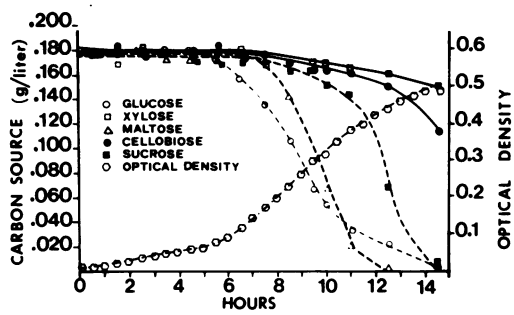

FIG. 18. *B. ruminicola incubated with glucose, xylose, maltose, cellobiose, and sucrose.*

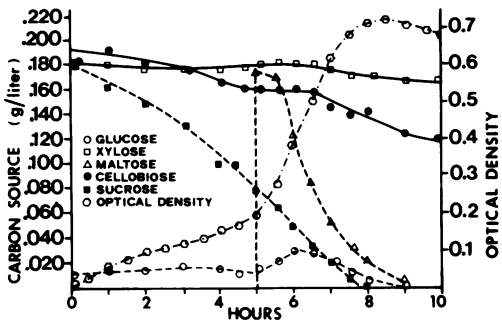

FIG. 19. *B. ruminicola incubated with glucose, xylose, cellobiose, and sucrose. Maltose was added after 5 h.*

xylose were used at linear or decelerating rates. This linear decrease in sucrose utilization (Fig. 19) is markedly different from the rapid and accelerating utilization of sucrose shown in Fig. 18. This contrast in sucrose utilization (Fig. 18 and 19) was not due to differences in cell number because optical densities during these latter stages of sucrose use were similar. A peculiar feature of this incubation was that glucose was detected during the entire incubation, even though glucose was not added to either the maintenance or incubation medium. The level of glucose increased at 1 h after addition of maltose. Maximum growth (Table 3) was very low with xylose alone and was substantially lower for cellobiose than for glucose, maltose, and sucrose. (See Table 2.) Bryant et al. (5) found that this particular strain of *B. ruminicola* could not grow on xylose alone. The growth rate shown in Table 3 for xylose (0.04 h^{-1}) could have resulted from yeast extract and Trypticase because the growth rate is very low. Slight utilization of xylose was seen in Fig. 18 and 19, but this utilization could have been for synthesis of cell material rather than for energy derivation.

M. elsdenii. *M. elsdenii* used glucose, maltose, and lactate simultaneously during the latter stages of a multisubstrate incubation (Fig. 20). No lags in growth were evident. Addition of glucose after 4 h of growth on sucrose produced complete cessation of sucrose used (Fig. 21). Addition of glucose also resulted in a greater rate of growth. Sucrose utilization, although not great, was completely halted by addition of maltose at 9 h (Fig. 22). Addition of maltose was also accompanied by a greater growth rate. Further sucrose utilization occurred after the depletion of maltose. Addition of lactate at 7 h did not result in an abrupt change in sucrose utilization (Fig. 23). Although sucrose utilization was linear after addition of lactate, microbial growth was limited, and lactate utilization did not show acceleration either. Thus, glucose and maltose, but not lactate, are inhibitors of sucrose utilization (Table 2). No interactions were seen among glucose, maltose, and lactate. Of the four substrates, sucrose gave the lowest growth rate (Table 3), one which was substantially lower than those with non-catabolite-regulated substrates. Growth on lactate was not as rapid as growth on glucose or maltose.

Carbon sources in most of the incubations with *M. elsdenii* were not exhausted when growth ceased. This residual substrate was due to a lack of aminonitrogen in the growth media. Aminonitrogen was limited because previous workers have found that the degree of catabolite

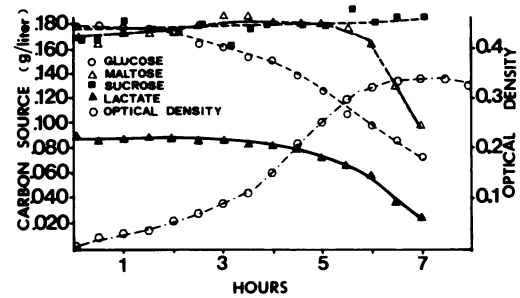

FIG. 20. *M. elsdenii incubated with glucose, maltose, lactate, and sucrose.*

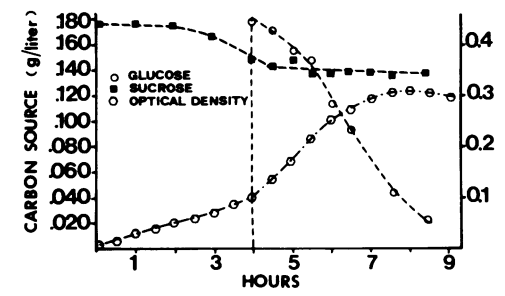

FIG. 21. *M. elsdenii incubated with sucrose. Glucose was added after 4 h.*

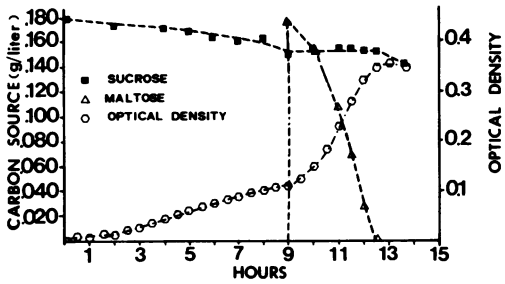

FIG. 22. *M. elsdenii incubated with sucrose. Maltose was added after 9 h.*

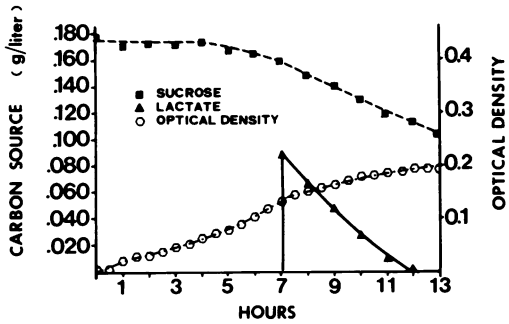

FIG. 23. *M. elsdenii incubated with sucrose. Lactate was added after 7 h.*

repression is dependent on the ratio of carbon to nitrogen (33).

DISCUSSION

The *B. ruminicola* incubations shown in Fig. 18 and 19 were perhaps the most surprising. Glucose was detected during the entire incubation, even though glucose was not added to either the maintenance medium or the incubation medium (Fig. 19). The sustained presence of glucose and the increase in glucose at 1 h after addition of maltose indicate that glucose was a result of the incubation rather than just a contaminant. Evidence of glucose production can also be seen in Fig. 18. At 11 h there was a pronounced shift in the slope of the glucose disappearance curve (Fig. 18). This shift corresponds to an increasing rate of sucrose utilization. These data might be explained by extramembranal (periplasmic space) cleavage of disaccharides or, alternatively, by hypothesizing that the cells are leaky to intracellular glucose.

In Fig. 19 both sucrose and cellobiose showed substantial disappearances during the first 5 h of the incubation despite nondecreasing glucose levels. This glucose had to result from cleavage of either sucrose or cellobiose. It seems unlikely that a product of sucrose or cellobiose utilization, glucose, would repress sucrose or cellobiose utilization. The sequential nature of the substrate utilizations shown in Fig. 18 and the decrease in the rate of cellobiose utilization and the linearity of sucrose utilization after maltose addition shown in Fig. 19 suggest the presence of a catabolite regulatory mechanism in *B ruminicola.* Identification of the repressor is unclear because of the liberation of glucose by maltose, sucrose, and possibly cellobiose. *B. ruminicola* was shown to be a poor utilizer of xylose (Fig. 18 and 19 and Table 3).

The only species of *Bacteroides* previously tested for catabolite regulatory mechanisms was *Bacteroides fragilis* (16, 30). Hylemon and Phibbs (16) found no detectable cyclic AMP, phosphodiesterase, adenylate cyclase, or diauxic growth during growth on lactose and glucose. Such work, however, does not rule out the possibility of other mechanisms in *Bacteroides.*

B. fibrisolvens showed pronounced lags in growth after depletion of the preferred substrates sucrose and maltose (Fig. 12 and 13). These lags strongly suggest that catabolite repression is involved in its substrate utilizations. Lags are seen in catabolite repression because protein synthesis must be devoted to the synthesis of additional enzymes needed for catabolism of the nonpreferred substrate (20). It appears that the addition of maltose or sucrose caused a decrease in the rate of cellobiose utilization despite increasing cell numbers (Fig. 13, 14, and 15). *B. fibrisolvens* A38, although not an aggressive cellulose digester, does have some cellulolytic activity (4, 15). The effect of maltose and sucrose on cellulase production would be of interest. Catabolite repression of a cellulase has already been reported in an aerobic species, *Cellulomonas fimi* (31).

The HD4 strain of *S. ruminantium* is a lactate producer and a lactate utilizer. The procedure used in these experiments to assay lactate (13) is specific for L-(+)-lactate. The HD4 strain produces only small amounts of the D-(−)-isomer and uses only the L-(+)-isomer (28). Overall lactate production is slightly underestimated in these incubations, but lactate utilization is accurately monitored by this procedure. In batch culture, glucose was first fermented to lactate (13, 28). After glucose was depleted, lactate was then fermented. The pronounced lags shown in Fig. 2 and 5 before the utilization of lactate started suggest that lactate utilization is subject to catabolite repression. One possible hypothesis is that a lactate dehydrogenase favoring conversion of lactate to pyruvate is subject to catabolite repression. Hishinunuma et al. (12) also found a similar repression of lactate utilization by glucose in *S. ruminantium.*

The repressive effects shown in Fig. 1 through

22 are summarized in Table 2. The utilizations of all six substrates are repressed in at least one of the five bacteria. Although glucose and maltose appear to be the most preferred substrates, even their utilization is inhibited in *B. fibrisolvens* and *S. bovis*. Xylose is used by *S. ruminantium*, *B. ruminicola*, and *B. fibrisolvens*, but only in *S. ruminantium* is xylose a non-catabolite-regulated substrate. *S. ruminantium* is probably a more aggressive utilizer or fermenter of pentoses than either *B. fibrisolvens* or *B. ruminicola*. Both *M. elsdenii* and *S. ruminantium* can ferment lactate, but only in *M. elsdenii* is lactate utilization not inhibited. Because lactate is used by *S. ruminantium* only after depletion of sucrose, xylose, and glucose, *S. ruminantium* is probably not a very active lactate utilizer in the rumen. Sucrose utilization is inhibited in *M. elsdenii* and *B. ruminicola*. *B. fibrisolvens*, *S. ruminantium*, and *S. bovis* are probably more important sucrose utilizers. Cellobiose utilization is inhibited in all three bacteria that can use it. Cellobiose use is even inhibited in *B. fibrisolvens* A38, a cellulolytic strain (5). The competition of these bacteria for cellobiose released by active cellulolytic bacteria in the rumen is probably minor if other substrates are available.

A comparison between Tables 2 and 3 shows, generally, that catabolite-regulated substrates have lower maximal growth rates than do non-catabolite-regulated substrates. The only exceptions to this comparison appear to be glucose in *S. bovis*, and cellobiose in *B. fibrisolvens*. In no case did a catabolite-regulated substrate exhibit a higher maximal growth rate than all of the preferred substrates.

If these five bacteria did not exhibit substrate preferences and sequential substrate utilization, it is doubtful that they would all be present in the rumen. Indeed, the overall diversity of rumen microbial species is somewhat surprising. If several species used all soluble substrates simultaneously without preference, competition would yield one species, the victor. The data shown in Table 2 indicate that five rumen microbial species have different strategies of substrate utilization.

Definition of the exact mechanisms involved in the preferences and sequential substrate utilizations of these bacteria will require extensive biochemical elaboration. Generally, catabolite repression does not result in an immediate cessation of nonpreferred substrate utilization once a preferred substrate is introduced because enzymes synthesized before the introduction are still present. Interactions of this type were seen in *S. ruminantium* (Fig. 2), *S. bovis* (Fig. 10), *B. fibrisolvens* (Fig. 13, 14, 15, 16, and 17), and *B. ruminicola* (Fig. 19). Inducer exclusion and catabolite inhibition can result in immediate cessation of nonpreferred substrate utilization because the substrate is not transported. Interactions of this type were seen in *S. ruminantium* (Fig. 3, 4, and 5), *S. bovis* (Fig. 8 and 9), and *M. elsdenii* (Fig. 21 and 22). It is possible that both types of mechanisms may operate in the same bacterium. It is also realized that the bacterial strains used in these experiments might not necessarily be representative of all strains of a particular species or genus. Further work will be needed to validate such mechanisms in other strains.

Although preferences obviously play an important part in competition between bacteria, this is not the only factor involved. Affinities and capacities of substrate utilization are also quite important. Experiments are currently being conducted in this laboratory to measure the affinity and capacity of each of the five bacteria for each of the six substrates used in this experiment. It is hoped that such an analysis will give a more quantitative picture of rumen microbial interactions and competitions.

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LITERATURE CITED

1. **Adhya, S., and H. Echols.** 1966. Glucose effect and the galactose enzymes of *Escherichia coli*: correlation between glucose inhibition of induction and inducer transport. J. Bacteriol. **92**:601–608.
2. **Bergmeyer, H. U., and H. Klotsch.** 1965. Sucrose, p. 99–102. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis. Academic Press Inc., New York.
3. **Bernlohv, R. W., M. K. Haddox, and N. D. Goldberg.** 1974. Cyclic quanosine 3′,5′ monophosphate in *E. coli* and *B. licheniformis*. J. Biol. Chem. **249**:4329–4331.
4. **Bryant, M. P., and N. Small.** 1956. The anaerobic monotrichous butyric acid-producing curved rod-shaped bacteria of the rumen. J. Bacteriol. **72**:16–21.
5. **Bryant, M. P., N. Small, C. Bouma, and H. Chu.** 1958. *Bacteroides ruminicola* n. sp. and *Succinimonas amylolytica* the new genus and species. Species of succinic acid-producing anaerobic bacteria of the bovine rumen. J. Bacteriol. **76**:15–23.
6. **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.
7. **Dische, Z.** 1962. Color reactions of pentoses, p. 483–488. *In* R. L. Whistler and M. L. Wolfrom (ed.), Methods in carbohydrate chemistry. Academic Press Inc., New York.
8. **Epps, H. M. R., and E. F. Gale.** 1942. The influence of glucose during growth on the enzymatic activities of *Escherichia coli*: comparison of the effect with that produced by fermentation acids. Biochem. J. **36**: 619–623.
9. **Haggerty, D. M., and R. F. Schleif.** 1975. Kinetics of the onset of catabolite repression in *Escherichia coli* as determined by *lac* messenger ribonucleic acid initia-

tions and intracellular cyclic adenosine 3',5'-monophosphate levels. J. Bacteriol. **123**:946–953.
10. **Halvorson, H. O., S. Winderman, and J. Gorman.** 1963. Comparison of the α-glucosidases of *Saccharomyces* produced in response to five non-allelic maltose genes. Biochim. Biophys. Acta **67**:42–53.
11. **Hasan, N., and I. F. Durr.** 1974. Induction of β-galactosidase in *Lactobacillus plantarum*. J. Bacteriol. **120**:66–73.
12. **Hishinunuma, F., S. Kanegasaki, and H. Takahaski.** 1968. Ruminal fermentation and sugar concentrations. Agric. Biol. Chem. **32**:1325–1330.
13. **Hobson, P. N., and R. Summers.** 1972. ATP pool and growth yield in *Selenomonas ruminantium*. J. Gen. Microbiol. **79**:351–360.
14. **Hohorst, H. J.** 1965. Lactate, p. 266–270. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis. Academic Press Inc., New York.
15. **Hungate, R. E.** 1966. The rumen and its microbes, p. 40. Academic Press Inc., New York.
16. **Hylemon, P. B., and P. V. Phibbs, Jr.** 1974. Evidence against the presence of cyclic AMP and related enzymes in selected strains of *Bacteroides fragilis*. Biochem. Biophys. Res. Commun. **60**:88–95.
17. **Khan, N. A., and N. R. Eston.** 1967. Purification and characterization of maltose and α-methyl glucosidase from yeast. Biochim. Biophys. Acta **146**:173–180.
18. **McGinnis, J. F., and K. Paigen.** 1969. Catabolite inhibition: a general phenomenon in the control of carbohydrate utilization. J. Bacteriol. **100**:902–913.
19. **McGinnis, J. F., and K. Paigen.** 1973. Site of catabolite inhibition of carbohydrate metabolism. J. Bacteriol. **114**:885–887.
20. **Magasanik, B.** 1961. Catabolite repression. Cold Spring Harbor Symp. Quant. Biol. **26**:249–256.
21. **Makman, R. S., and E. W. Sutherland.** 1965. Adenosine 3',5' phosphate in *Escherichia coli*. J. Biol. Chem. **240**:1309–1314.
22. **Monod, J.** 1947. The phenomenon of enzymatic adaptation. Growth **11**:223–289.
23. **Perlman, R., and I. Paston.** 1968. Regulation of β-galactosidase synthesis in *E. coli* by cyclic adenosine 3',5' phosphate. J. Biol. Chem. **243**:5420–5426.
24. **Peterkofsky, A., and C. Gazdar.** 1974. Glucose inhibition of adenylate cyclase in intact cells of *E. coli* B. Proc. Natl. Acad. Sci. U.S.A. **71**:2324–2328.
25. **Peterkofsky, A., and C. Gazdar.** 1975. Interaction of enzyme I of the phosphoenol pyruvate: sugar phosphotransferase system with adenylate cyclase of *E. coli*. Proc. Natl. Acad. Sci. U.S.A. **72**:2920–2924.
26. **Saier, M. H., Jr., and S. Roseman.** 1976. Sugar transport. The CRR mutation: its effect on repression of enzyme synthesis. J. Biol. Chem. **251**:6598–6605.
27. **Saier, M. H., Jr., and S. Roseman.** 1976. Sugar transport. Inducer exclusion and regulation of the melibrose, maltose, glycerol, and lactose transport systems by the phosphoenol pyruvate: sugar phosphotransferase system. J. Biol. Chem. **249**:6606–6615.
28. **Scheifinger, C. C., M. J. Latham, and M. J. Wolin.** 1975. Relationship of lactate dehydrogenase specificity and growth rate to lactate metabolism by *Selenomonas ruminantium*. Appl. Micribiol. **30**:916–921.
29. **Schneider, W. C.** 1945. Phosphorus compounds in animal tissues. I. Extraction and estimation of deoxypentose nucleic acid and pentose nucleic acid. J. Biol. Chem. **161**:293–303.
30. **Siegel, L. S., P. B. Hylemon, and P. V. Phibbs, Jr.** 1977. Cyclic adenosine 3',5'-monophosphate levels and activities of adenylate cyclase and cyclic adenosine 3',5'-monophosphate phosphodiesterase in *Pseudomonas* and *Bacteroides*. J. Bacteriol. **129**:87–96.
31. **Stewart, B. J., and J. M. Leatherwood.** 1976. Derepressed synthesis of cellulase by *Cellulomonas*. J. Bacteriol. **128**:609–615.
32. **Stouthamer, A. H., and C. Bettenhaussen.** 1973. Utilization of energy for growth and maintenance in continuous and batch cultures of microorganisms. Biochim. Biophys. Acta **301**:53–70.
33. **Ullmann, A., F. Tillier, and J. Monod.** 1976. Catabolite modulator factor: as possible mediator of catabolite repression in bacteria. Proc. Natl. Acad. Sci. U.S.A. **76**:3479–3482.
34. **Wayne, P. K., and O. M. Rosen.** 1974. Cyclic 3',5' adenosine monophosphate in *E. coli* during transient and catabolite repression. Proc. Natl. Acad. Sci. U.S.A. **71**:1436–1440.
35. **Zubay, G., D. Schwartz, and J. Beckwith.** 1970. Mechanism of activation of catabolite sensitive genes: a positive control system. Proc. Natl. Acad. Sci. U.S.A. **66**:104–110.