APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1987, p. 2363–2367
0099-2240/87/102363-05$02.00/0

Vol. 53, No. 10

Effects of Potassium Ion Concentrations on the Antimicrobial Activities of Ionophores against Ruminal Anaerobes†

KARL A. DAWSON* AND JAMES A. BOLING

Department of Animal Sciences, University of Kentucky, Lexington, Kentucky 40546-0215

Received 10 April 1987/Accepted 10 July 1987

The antimicrobial activities of monensin and lasalocid against representative strains of ruminal bacteria were evaluated in medium containing three different concentrations of potassium (1.3, 7.9, or 23.3 mM). The growth of *Eubacterium ruminantium* was inhibited by low concentrations of ionophores (≤0.16 mg/liter), while the strain of *Streptococcus bovis* tested was resistant to high concentrations of ionophores (40 mg/liter) at all potassium concentrations tested. The MICs of the ionophores for strains of *Bacteroides succinogenes*, *Butyrivibrio fibrisolvens*, *Ruminococcus albus*, and *Ruminococcus flavefaciens* and for one strain of *Bacteroides ruminicola* increased with increasing potassium concentrations in the medium. High concentrations of ionophores (40 mg/liter) decreased the maximum cell yields or increased the lag times or both in cultures of one strain of *Bacteroides ruminicola* and two strains of *Selenomonas ruminantium* but did not completely inhibit the growth of these organisms. Increased potassium concentrations in the medium (from 7.9 to 23.3 mM) decreased the lag times or increased the cell yields or both when these three strains were grown in ionophore-containing medium, while the activities of lasalocid and monensin against these organisms were enhanced in the medium containing low potassium concentrations (1.3 mM). The data from this study suggest that extracellular potassium concentrations may influence the antimicrobial activities of ionophores in the rumen.

Monensin and lasalocid are widely known for their ability to alter ruminal fermentations and to improve the efficiency of feed utilization in ruminant animals (1, 12, 17). It is generally believed that the selective antimicrobial activity of ionophores may account for many of the changes in the ruminal fermentations associated with the use of these compounds as feed supplements (8). At low concentrations (≤2.5 mg/liter), these agents are believed to inhibit the growth and activity of methane-, formate-, hydrogen-, lactate-, and butyrate-producing bacteria while allowing the growth of propionate- and succinate-producing bacteria in the rumen (8). Studies of the antimicrobial activities of these compounds against a number of isolated ruminal bacteria support this hypothetical model for monensin and lasalocid activities in the rumen (8, 9, 11, 13).

Both monensin and lasalocid are classified as ionophores and are believed to alter microbial activities by dissipating the cation gradients which are normally established across bacterial cell membranes (1, 16, 17). Recent studies have shown decreased intracellular K^+ concentration associated with the flow of protons and Na^+ into *Streptococcus bovis* cells exposed to monensin (16). These changes in ion concentrations within the cell could account for the antimicrobial activities of the ionophores. Since this ionophore-mediated flow of ions through bacterial membranes is directly related to their relative concentration inside and outside the cells, factors which influence the internal and external cation concentration may be expected to influence the antimicrobial activity of ionophores in the rumen and alter the ability of ionophores to influence ruminal fermentations. Several investigators have suggested that cation concentrations may significantly influence the activities of ionophores in the rumen (1, 9, 14–16). However, there is currently little data describing the effects of external potassium ion concentrations on the antimicrobial activities of ionophores against a wide variety of ruminal bacteria. This study evaluated the effects of the potassium ion concentration on the antimicrobial activity of lasalocid and monensin against several representative ruminal anaerobes.

MATERIALS AND METHODS

Organisms and growth conditions. *Bacteroides ruminicola* GA33 and 23, *Bacteroides succinogenes* S85, *Butyrivibrio fibrisolvens* D1, *Eubacterium ruminantium* B4, *Ruminococcus albus* 7, *Ruminococcus flavefaciens* C94, and *Selenomonas ruminantium* D and GA192 were obtained from M. J. Allison, National Animal Disease Center, Ames, Iowa, and are maintained in the culture collection at the Department of Animal Sciences, University of Kentucky, Lexington. *Streptococcus bovis* S1 was an isolate obtained from the rumen of a Holstein steer maintained on a high-concentrate diet and not receiving an ionophore supplement. The identity of this organism was determined by the tests and characteristics outlined by Deibel and Seeley (10). All media used in this study were prepared and inoculated by the anaerobic culture techniques of Bryant (2). Stock cultures of these organisms were maintained in the anaerobic ruminal fluid-based agar medium described by Bryant and Robinson (3). All cultures were incubated at 37°C.

Sensitivity of isolates to ionophores. The sensitivity of ruminal isolates to monensin and lasalocid was measured in a basal medium containing (in grams per liter): glucose, 1.0; Trypticase peptones (BBL Microbiology Systems, Cockeysville, Md.), 5.0; hemin, 0.001; resazurin, 0.001; KH_2PO_4, 0.9; NaCl, 0.9; $CaCl_2$, 0.02; $MgCl_2 \cdot 6H_2O$, 0.02; $MnCl_2 \cdot 4H_2O$, 0.01 $CoCl_2 \cdot 6H_20$, 0.001; $FeSO_4$, 0.018; $(NH_4)_2SO_4$, 0.9; Na_2CO_3, 4.0; and cysteine hydrochloride, 0.5. The medium was supplemented with methionine, volatile fatty acids, and vitamins as described by Caldwell et al. (6). The pH of the medium was adjusted to 6.8 prior to sterilization. The potassium content of the medium was altered by exchanging KCl for NaCl and NaH_2PO_4 for

* Corresponding author.

† Published with the approval of the Director of the Kentucky Agricultural Experiment Station as journal paper no. 86-5-208.

TABLE 1. Mineral concentrations in test media

Potassium concn in medium	Content (mM)				Ratio, Na/K	
	Calculated[a]		Measured[b]		Calculated	Measured
	K	Na	K	Na		
Low	1.3	145.3	1.3	143.5	112	110
Medium	7.9	138.7	7.0	139.8	17.6	20.0
High	23.3	123.3	21.2	115.9	5.4	5.5

[a] Values were calculated from minerals added to each medium and values for individual components supplied by the manufacturer.
[b] Concentrations were measured by flame spectrophotometry after dilution in distilled water.

KH_2PO_4 on an equimolar basis to provide medium with similar ionic concentrations (Table 1). The concentrations of potassium and sodium in each medium were confirmed by flame spectrophotometry on a 560 atomic absorption spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn.). The intermediate potassium concentration was slightly less than those estimated in the ruminal fluid medium used for maintaining the stock cultures (8.8 to 15.6 mM). The ionophores were prepared in a doubling dilution sequence in 95% ethanol under a CO_2 gas phase. The solutions containing ionophores were added to the medium at a rate of 0.01 ml/5.0 ml of medium just prior to inoculation to give final concentrations ranging from 40 to 0.08 mg/liter. Five milliliters of medium in sealed culture tubes (13 by 150 mm) were inoculated with 0.1 ml of material from a 24-h culture grown in the basal medium without added antibiotic. The growth characteristics of all organisms except *R. flavefaciens* were determined by following the turbidity (OD_{660}) for at least 72 h after inoculation. The MIC was determined in two replicate sets of tubes and was defined as the lowest concentration of ionophore at which there was no measurable growth. All growth characteristics were measured relative to the initial turbidity measurement and to the growth in an antibiotic-free control (containing ethanol and no antibiotic). The purity of each culture was determined before and after each experiment by microscopic examination. Growth studies were repeated if microscopic examination suggested contamination or if the duplicate sets of cultures failed to give similar results. *R. flavefaciens* tended to grow in large clumps in the basal medium. This did not allow accurate turbidometric evaluation of growth. As a result, growth of these cultures was evaluated by visually comparing growth in tubes containing ionophores with that in the ionophore-free control medium.

RESULTS AND DISCUSSION

Strains of *B. ruminicola*, *B. succinogenes*, *E. ruminantium*, and *R. albus* were unable to grow within 24 h in the basal medium at the lowest (1.3 mM) potassium concentrations but did grow in medium containing the greater potassium concentration (Table 2). All of these organisms except *B. succinogenes* and *R. albus* grew in medium containing 1.3 mM potassium after an extended incubation period (>48 h). The growth rate and maximum absorbance of these cultures were similar to those of cultures grown in the medium containing greater (7.9 mM) potassium concentrations. Visual observations indicated that *R. flavefaciens* also failed to grow in basal medium containing low concentrations of potassium within 24 h but did grow in all of the antibiotic-free test media within 48 h. These data suggest that the lowest potassium concentrations provided in these test media were not sufficient to support optimal growth of several of the organisms tested. This was unexpected, since the potassium concentrations provided were relatively high compared with the range of potassium concentrations which have been shown to support the growth of *Bacteroides* species (4–7). These observations may reflect a greater need for potassium in the test media that we developed for use in this study.

Measurements of the MICs of monensin were dependent on the incubation time and the concentration of potassium in the medium (Table 3). In general, strains of *B. ruminicola* and *S. ruminantium* and *S. bovis* S1 were resistant to high concentrations of monensin, while strains of *B. fibrisolvens*, *R. albus*, *R. flavefaciens*, and *E. ruminantium* were sensitive to relatively low concentrations of monensin. These observations are similar to those of other investigators who have suggested that the growth of butyrate-, formate-, and hydrogen-producing bacteria is inhibited by ionophores while the growth of propionate- and succinate-producing bacteria is not greatly altered by ionophores (8, 11, 13).

The notable exception to these similarities was *S. bovis* S1, which was resistant to high concentrations of monensin. In general, ionophores inhibit the growth of gram-positive organisms (11, 13). However, some gram-positive organisms resist the antimicrobial activity of monensin. Dennis et al. (11) reported that one strain (strain 124) of *S. bovis* grew in medium containing 48 mg of monensin per liter, while other strains were inhibited by monensin concentrations above 12 mg/liter. In another study, Russell (16) demonstrated that the growth rate of *S. bovis* JB1 was decreased but fermentation activity continued in cells after growth had ceased in me-

TABLE 2. Effects of potassium concentration in basal medium on growth of representative ruminal anaerobes

Organism	Strain	Growth[a] at potassium concn:					
		1.3 mM		7.9 mM		23.3 mM	
		A_{660}	Time (h)	A_{660}	Time (h)	A_{660}	Time (h)
Bacteroides ruminicola	GA33	70 (0)	12	65 (3)	12	67 (4)	12
	23	54 (7)	72	62 (3)	12	64 (2)	12
Bacteroides succinogenes	S85	2 (1)	NG[b]	42 (11)	12	43 (16)	12
Butyrivibrio fibrisolvens	D1	25 (2)	24	29 (1)	24	29 (2)	24
Eubacterium ruminantium	B4	17 (8)	72	18 (1)	72	20 (2)	72
Ruminococcus albus	7	1 (1)	NG	15 (9)	72	30 (4)	24
Selenomonas ruminantium	D	9 (1)	12	11 (1)	12	9 (0)	12
	GA192	25 (2)	12	29 (5)	12	28 (6)	12
Streptococcus bovis	S1	33 (1)	12	33 (2)	12	33 (1)	12

[a] A_{660}, Mean maximum absorbance ($OD_{660} \times 100$) measured in two cultures. Values in parentheses are standard deviations. Time, Time required for the culture to reach maximum absorbance.
[b] NG, No growth during the 72-h incubation period.

TABLE 3. MICs of monensin for representative ruminal anaerobes as influenced by potassium concentration in a basal medium

Organism	Strain	MIC (mg/liter) at the following K concn in medium at:					
		24 h			72 h		
		1.3 mM	7.9 mM	23.3 mM	1.3 mM	7.9 mM	23.3 mM
Bacteroides ruminicola	GA33	>40.00	>40.00	>40.00	>40.00	>40.00	>40.00
	23	NG[a]	0.62	2.50	0.31	1.25	10.00
Bacteroides succinogenes	S85	NG	0.62	0.62	NG	2.50	1.25
Butyrivibrio fibrisolvens	D1	≤0.08	0.31	0.31	0.31	0.31	0.62
Eubacterium ruminantium	B4	NG	NG	≤0.08	≤0.08	0.16	≤0.08
Ruminococcus albus	7	NG	NG	0.16	NG	≤0.08	0.31
Ruminococcus flavefaciens	C94	NG	NG	≤0.08	0.62	2.50	5.00
Selenomonas ruminantium	D	>40.00	>40.00	>40.00	>40.00	>40.00	>40.00
	GA192	>40.00	>40.00	>40.00	>40.00	>40.00	>40.00
Streptococcus bovis	S1	20.00	20.00	20.00	>40.00	>40.00	>40.00

[a] NG, Organism failed to grow in the antibiotic-free control medium at the potassium concentration provided.

dium containing as little as 0.5 mg of monensin per liter. The growth of strain S1 was not significantly influenced by monensin concentrations of less than 20 mg/liter. These studies suggest considerable variation in the responses of different strains to monensin. Although several studies have evaluated the mechanisms by which ionophores influence growth, activities, and the intracellular ion concentrations of some ruminal bacteria (16), the mechanism of monensin resistance in these organisms is not understood.

The concentrations of monensin required to inhibit the growth of strains of *B. ruminicola* 23 in each of the test media were greater at 72 h than at 24 h of incubation. Although *B. ruminicola* GA33 was not completely inhibited by any of the monensin concentrations tested, a time-dependent adaptation was observed at intermediate potassium concentrations (7.9 mM) in this organism (Fig. 1). Maximum abosrbance (measured relative to the antibiotic-free control) in cultures of strain GA33 exposed to 20 mg of monensin per liter were observed after 60 h of incubation, while the maximum absorbance was observed within 15 h in cultures containing 0.62 mg of monensin per liter. After the initial exposure of these cultures to a high concentration of monensin (>10 mg/liter), the growth of these organisms was consistently characterized by an extended lag phase (>24 h) and then a period of rapid growth. Similar extended lag phases were not noted when cultures of strain GA33 were successively transferred in monensin-containing medium. This type of adaptation to monensin is consistent with the observation of other investigators, who have shown that strains of *B. ruminicola* will develop resistance to monensin and will grow in media with increased monensin concentrations after prolonged incubation periods (8).

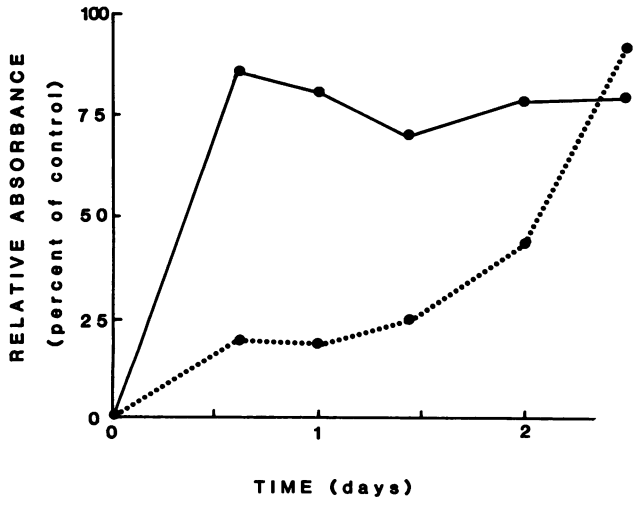

FIG. 1. Growth of *B. ruminicola* GA33 in medium containing 20 mg (·····) or 0.625 mg (——) of monensin per liter measured relative to a monesin-free control.

The amount of monensin required to inhibit the growth of *B. fibrisolvens* in all of the test media was greater at 72 h than at 24 h (Table 4). Similarly, higher MICs were also observed for *B. succinogenes*, *R. albus*, *R. flavefaciens*, and *S. bovis* after prolonged incubation, but these higher MICs were only observed at high or intermediate potassium concentrations. Growth in these cultures did not appear to be the result of an adaptation process like that observed in strains of *B. ruminicola*, because decreased growth rates and increased lag times were observed even after successive transfer in monensin-containing medium. The selection of ionophore-resistant organisms in cultures of *B. ruminicola* and *B. succinogenes* after exposure to monensin has been observed by other investigators and appears to result in a stable population of resistant bacteria, but similar adaptation to monensin has not been observed in other species of ruminal bacteria (8, 13).

The amount of monensin required to inhibit the growth of all organisms except *S. ruminantium*, *B. succinogenes*, and *E. ruminantium* increased as the potassium concentration in the medium increased. The greatest effects of potassium on the antimicrobial activity of monensin was observed in strains of *B. ruminicola*, in which there was a 32-fold difference in the amount of monensin required to bring about inhibition of growth in medium containing 1.3 mM potassium and medium containing 23.3 mM potassium at 72 h.

The antimicrobial activity of lasalocid was similar to that observed for monensin; however, lasalocid tended to inhibit

TABLE 4. MICs of lasalocid for representative ruminal anaerobes as influenced by potassium concentration in a basal medium

Organism	Strain	MIC (mg/liter) at the following K concn in medium at:					
		24 h			72 h		
		1.3 mM	7.9 mM	23.3 mM	1.3 mM	7.9 mM	23.3 mM
Bacteroides ruminicola	GA33	>40.00	>40.00	>40.00	>40.00	>40.00	>40.00
	23	NG[a]	0.31	0.62	0.62	10.00	40.00
Bacteroides succinogenes	S85	NG	0.62	2.5	NG	1.25	2.5
Butyrivibrio fibrisolvens	D1	≤0.08	0.31	0.62	0.16	0.62	1.25
Eubacterium ruminantium	B4	NG	NG	NG	≤0.08	≤0.08	≤0.08
Ruminococcus albus	7	NG	NG	≤0.08	NG	0.16	0.62
Ruminococcus flavefaciens	C94	NG	NG	0.16	1.25	5.00	20.00
Selenomonas ruminantium	D	40.00	>40.00	>40.00	>40.00	>40.00	>40.00
	GA192	>40.00	>40.00	>40.00	>40.00	>40.00	>40.00
Streptococcus bovis	S1	5.00	5.00	5.00	>40.00	>40.00	>40.00

[a] NG, Organism failed to grow in the antibiotic-free control medium at the potassium concentration provided.

the growth of sensitive organisms at a lower concentration than did monensin (Table 4). *B. ruminicola* GA33 and strains of *S. ruminantium* were resistant to the high concentrations of lasalocid in all of the test media, while *E. ruminantium* was sensitive to all lasalocid concentrations tested. Strains of *B. ruminicola*, *B. fibrisolvens*, *R. albus*, and *R. flavefaciens* tended to show growth at much higher concentrations of lasalocid after extended incubation periods. The activity of lasalocid against most of the species examined in this study was dependent on the potassium concentration in the medium. MICs for these strains grown in the medium with a high potassium concentration were from 2 to 64 times greater than those observed in medium containing low and intermediate potassium concentrations. These data indicate that extracellular potassium concentrations can significantly influence the antimicrobial activity of both monensin and lasalocid against certain strains of ruminal bacteria and may influence the ability of ionophores to alter the composition of the microflora in the rumen.

Even though the growth of *B. ruminicola* was not completely inhibited by high concentrations of ionophores, the maximum cell yields obtained in the presence of the ionophores were found to be a function of both the ionophore concentration and the potassium concentration in the medium (Fig. 2). Cell yields at 40 mg of monensin per liter were smallest in medium containing potassium concentrations of 1.3 mM and tended to increase as the potassium concentration increased and the ionophore concentration decreased. Similarly, growth of strains of *S. ruminantium* in the presence of lasalocid was influenced by the potassium concentration in the medium. Cell yields increased and lag times decreased as the potassium concentration increased in medium containing 40 mg of lasalocid per liter (Fig. 3).

Our data suggest that potassium ion concentration in the environment will significantly influence the effects of ionophores on the metabolic processes in anaerobic microbial cells. In many instances, the antimicrobial activity of the ionophores was reversed in the presence of a higher potassium concentration. The mechanism by which potassium influences the antimicrobial activity of ionophores is probably related to the ability of ionophores to alter the flow of cations across the cell membrane (1, 16). Russell (16) has reported that the potassium concentrations were 70-fold greater inside *S. bovis* cells than outside. This resulted in a potassium concentration gradient across the cell membrane which was 25 times greater than that established with sodium ions (greater inside) or hydrogen ions (greater outside) and suggests that the potassium gradient may be important to the action of the ionophores. Potassium ion concentrations decreased in cells exposed to ionophores as potassium ions flowed out of the cells in response to the concentration gradient (16). Since ionophores act as cation-transporting antiporters, the flow of potassium out of the cell was accompanied by an influx of sodium ions and protons, a decrease in the transmembrane potentials, and limitations on energy production in the bacterial cells. Data obtained with *S. bovis* suggest that potassium gradients are important to the action of the ionophores and that the magnitude of the potassium gradient may significantly influence the activity of the ionophores.

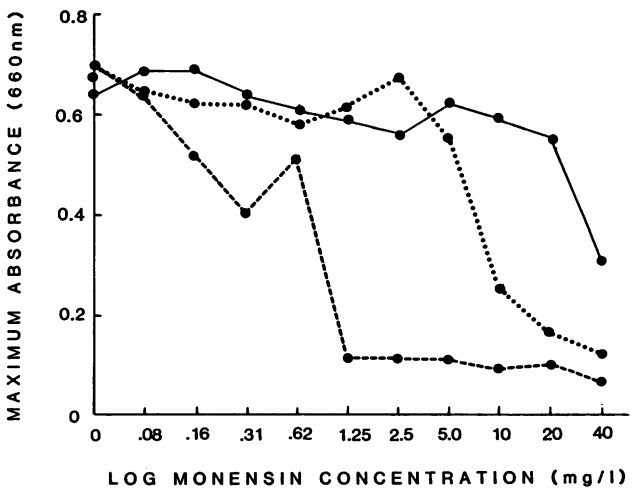

FIG. 2. Effects of monensin concentration on maximum cell yields in cultures of *B. ruminicola* GA33 in medium containing 1.3 mM K (-------), 7.9 mM K (·····), and 23.3 mM K (——).

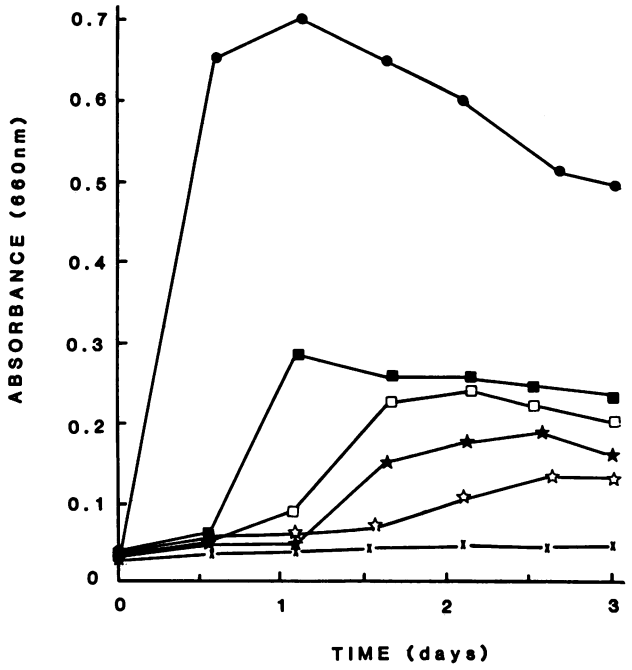

FIG. 3. Growth of *S. ruminantium* D in medium without lasalocid (●) and medium containing 40 mg of lasalocid per liter plus 1.3 (×), 4.6 (☆), 7.9 (★), 15.6 (□), and 23.3 (■) mM K.

The present study supports this hypothesis and suggests that increased external potassium concentrations will decrease the magnitude of the potassium gradient and prevent the efflux of potassium from cells exposed to ionophores. Increased potassium concentrations in the medium will thus prevent dissipation of the transmembrane potential and moderate the activity of the ionophores against both ionophore-resistant and ionophore-sensitive ruminal bacteria.

ACKNOWLEDGMENT

The technical assistance of Wanda Cain is gratefully acknowledged.

LITERATURE CITED

1. **Bergen, W. G., and D. B. Bates.** 1984. Ionophores: their effect on production efficiency and mode of action. J. Anim. Sci. **58**:1465–1483.
2. **Bryant, M. P.** 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. Am. J. Clin. Nutr. **25**:1324–1328.
3. **Bryant, M. P., and I. M. Robinson.** 1961. An improved nonselective medium for ruminal bacteria and its use in determining diurnal variation in numbers of bacteria in the rumen. J. Dairy Sci. **44**:1446–1456.
4. **Bryant, M. P., I. M. Robinson, and H. Chu.** 1959. Observations on the nutrition of *Bacteroides succinogenes*—a ruminal cellulolytic bacterium. J. Dairy Sci. **42**:1831–1847.
5. **Caldwell, D. R., and C. Arcand.** 1974. Inorganic and metal-organic growth requirements of the genus *Bacteroides*. J. Bacteriol. **120**:322–333.
6. **Caldwell, D. R., M. Keeney, J. S. Barton, and J. F. Kelley.** 1973. Sodium and other inorganic growth requirements of *Bacteroides amylophilus*. J. Bacteriol. **114**:782–789.
7. **Caldwell, D. R., and R. F. Hudson.** 1974. Sodium, an obligate growth requirement for predominant rumen bacteria. Appl. Microbiol. **27**:549–552.
8. **Chen, M., and M. J. Wolin.** 1979. Effects of monensin and lasalocid-sodium on the growth of methanogenic and rumen saccharolytic bacteria. Appl. Environ. Microbiol. **38**:72–77.
9. **Dawson, K. A., and J. A. Boling.** 1984. Factors affecting resistance of monensin-resistant and sensitive strains of *Bacteroides ruminicola*. Can. J. Anim. Sci. **64**(Suppl.):132–133.
10. **Deibel, R. H., and H. W. Seeley.** 1974. Family II: *Streptococcaceae*, p. 490–509. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
11. **Dennis, S. M., T. G. Nagaraja, and E. E. Bartley.** 1981. Effects of lasalocid or monensin on lactate-producing or -using rumen bacteria. J. Anim. Sci. **52**:418–426.
12. **Goodrich, R. D., J. E. Garrett, D. R. Gast, M. A. Kirick, D. A. Larson, and J. C. Meiske.** 1984. Influence of monensin on the performance of cattle. J. Anim. Sci. **58**:1484–1498.
13. **Henderson, C., C. S. Stewart, and F. V. Nekrep.** 1981. The effect of monensin on pure and mixed cultures of rumen bacteria. J. Appl. Bacteriol. **51**:159–169.
14. **Perski, H. J., P. Schonheit, and R. K. Thauer.** 1982. Sodium dependence of methane formation in methanogenic bacteria. FEBS Lett. **143**:323–326.
15. **Rumpler, W. V., D. E. Johnson, and D. B. Bates.** 1986. The effect of high dietary cation concentrations on methanogenesis by steers fed diets with and without ionophores. J. Anim. Sci. **62**:1737–1741.
16. **Russell, J. B.** 1987. A proposed mechanism of monensin action in inhibiting bacterial growth: effects on ion flux and protonmotive force. J. Anim. Sci. **64**:1519–1525.
17. **Schelling, G. T.** 1984. Monensin mode of action in the rumen. J. Anim. Sci. **58**:1518–1527.