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Effect of Ionophores and pH on Growth of *Streptococcus bovis* in Batch and Continuous Culture

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Batch cultures (pH 6.7) of *Streptococcus bovis* JB1 were severely inhibited by 1.25 and 5 μM lasalocid and monensin, respectively, even though large amounts of glucose remained in the medium. However, continuous cultures tolerated as much as 10 and 20 μM, respectively, and used virtually all of the glucose. Although continuous cultures grew with high concentrations of ionophore, the yield of bacterial protein decreased approximately 10-fold. When pH was decreased from 6.7 to 5.7, the potency of both ionophores increased, but lasalocid always caused a larger decrease in yield. The increased activity of lasalocid at pH 5.7 could largely be explained by an increased binding of the ionophore to the cell membrane. Because monensin did not show an increased binding at low pH, some other factor (e.g., ion turnover) must have been influencing its activity. There was a linear increase in lasalocid binding as the concentration increased, but monensin binding increased markedly at high concentrations. Based on the observations that (i) *S. bovis* cells bound significant amounts of ionophore (the ratio of ionophore to cell material was more important than the absolute concentration), (ii) batch cultures responded differently from continuous cultures, and (iii) pH can have a marked effect on ionophore activity, it appears that the term "minimum inhibitory concentration" may not provide an accurate assessment of microbial growth inhibition in vivo.

Monensin and lasalocid are polyether antibiotics that inhibit the growth of gram-positive bacteria (11, 23), and these ionophores are routinely fed to feedlot cattle which consume large quantities of cereal grain (24). The benefit of ionophores has usually been ascribed to reductions in methane formation, ammonia production, and coccidia, but they may also have a role in the prevention of ruminal acidosis (5). Ruminal acidosis causes depressions in food intake, tissue damage, founder, hemoconcentration, and, in severe cases, even death (27).

Streptococcus bovis is a rapidly growing, gram-positive, ruminal bacterium that can produce large amounts of lactate and is involved in the onset of ruminal acidosis (7). Acetate, formate, and ethanol are its primary fermentation products when growth rates are slow, and lactate only becomes a significant end product when carbohydrate availability and growth rate increase (20). Wolin (33) showed that lactate production was regulated by fructose 1,6-bisphosphate, and later studies showed that lactate dehydrogenase activity was also affected by changes in intracellular pH (21).

The effects of ionophores on pure cultures of ruminal bacteria have been examined in batch culture (2, 5, 6, 12), but few experiments have been performed in continuous culture (14). Although the ruminal pH of feedlot cattle is typically <6.0 (27), in vitro incubations have most often been performed at near-neutral pH. Because monensin and lasalocid are carboxylic ionophores (18), is seemed likely that pH would affect their antimicrobial activity (17). Results presented here indicated that (i) *S. bovis* persisted in continuous culture at concentrations of monensin and lasalocid that strongly inhibited growth in batch cultures, (ii) monensin and lasalocid caused a >10-fold decrease in the yield of continuous cultures, (iii) both ionophores were more potent growth inhibitors at pH 5.7 than at pH 6.7, (iv) the increased potency of lasalocid at low pH could be explained by an increased binding of ionophores to the cells, and (v) the activity of monensin was not directly related to the amount of ionophore present in the cell membrane.

MATERIALS AND METHODS

Organism and growth conditions. *S. bovis* JB1 (22) and S1 (4) were grown anaerobically at 39°C in media containing (per liter) 292 mg of K_2HPO_4, 292 mg of KH_2PO_4, 480 mg of $(NH_4)_2SO_4$, 480 mg of NaCl, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_2O$, 4 g of Na_2CO_3, 1.0 g of Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 0.5 g of yeast extract, and 0.6 g of cysteine hydrochloride. Batch cultures (pH 6.7) received 6.0 g of glucose per liter (final concentration) and 0 to 10 μM monensin or lasalocid. The ionophores were dissolved in ethanol and sterile filtered (0.2-μm membrane; final ethanol concentration, <1%). Batch cultures received the ionophore solution and a 3% inoculum (24-h culture) at time zero. Batch culture experiments were repeated on several days.

Continuous cultures were grown with 5.9 mmol of glucose per liter at pH 6.7 or 5.7. After 3 days of continuous culture (dilution rate, 0.1 h^{-1}), 0.01 μM ionophore was added to the medium reservoir, and at least a 90% turnover was allowed before the ionophore concentration was increased. The final concentration of ethanol never exceeded 36.2 mM, and batch culture experiments indicated that this concentration did not affect the growth of *S. bovis*. Preliminary experiments (replicates having the same scheme of monensin addition) indicated that the continuous-culture studies were highly reproducible.

Analyses. Optical density was measured with a Gilford 260 spectrophotometer (600 nm; 18-mm tubes). Samples were centrifuged (7, 500 × *g*, 5 min, 5°C), and cell-free supernatants were stored at −15°C. Glucose was analyzed by an enzymatic procedure using hexokinase and glucose 6-phosphate dehydrogenase (1). Lactate, formate, and acetate were quantified by high-pressure liquid chromatography (Beck-

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man model 334 liquid chromatograph, model 156 refractive index detector, model 421 CRT data controller, CR1A integrator, Bio-Rad HPX-87H organic acid column, 50-μl loop; 0.013 N H_2SO_4, 0.5 ml/min, 50°C). Cell protein was measured by the method of Lowry et al. (8) after the cell pellets were boiled for 15 min in 0.2 N NaOH.

Binding assays. Washed cells (160 μg of protein per ml) were incubated in 50 mM Na_2HPO_4 containing 10 mM KCl (pH 6.5 or 5.5) and 0 to 10 μM ^{14}C-labeled monensin (0.62 μCi/mg; Eli Lilly and Co., Indianapolis, Ind.) or lasalocid (1.74 μCi/mg; Hoffmann-La Roche, Inc., Nutley, N.J.) for 4 h at 39°C. Preliminary studies indicated that there was little increase in lasalocid binding after 15 min, but monensin binding did not reach its maximum until 4 h. Cells were harvested by centrifugation (7,500 × *g*, 5 min, 5°C), and the supernatant was removed. Pellets were suspended in 1 ml of H_2O and tranferred to 10 ml of scintillation cocktail (aqueous counting scintillant; Amersham Corp., Arlington Heights, Ill.). Cells and water caused little, if any, decline in the efficiency of scintillation counting.

RESULTS

Batch cultures. When *S. bovis* JB1 was grown in batch culture with 30 mM glucose at pH 6.7, the doubling time was 26 min (k = 1.60 h^{-1}) and the final optical density was 2.4 (Fig. 1a). When an unadapted inoculum was added to medium containing 1.25 μM monensin, there was no effect on the initial rate of growth, but the doubling time eventually increased to 190 min and the final optical density was 1.7. The inhibition of growth was even more pronounced with 2.5 μM monensin, and little growth was observed with either 5 or 10 μM. Cultures treated with 5 or 10 μM monensin showed a small amount of growth during the first 12 h of incubation, but the final optical density was never >0.4 (see Fig. 4) even though large amounts of glucose remained in the medium (data not shown). Cultures treated with lasalocid also showed some growth during the first 3 h of incubation, but as little as 1.25 μM caused a 85% reduction in optical density (Fig. 1b). Even after 32 h of incubation, the lasalocid-treated cultures had not consumed all of the available glucose (data not shown). *S. bovis* S1, a strain previously reported to be highly monensin resistant (4), showed a similar sensitivity to monensin and lasalocid.

Continuous cultures. When *S. bovis* JB1 was grown in a glucose-limited chemostat at pH 6.7, monensin concentrations of 0.1 μM had little effect on cell yield (Fig. 2a). As monensin was increased in a logarithmic fashion from 0.3 to 20 μM, there was a linear decrease in cell yield (Fig. 2a). Declines in yield were accompanied by an increase in the specific rate of glucose consumption (Fig. 2b). When the glucose consumption rate was >0.01 mmol/mg of protein per h, the fermentation shifted from acetate, formate, and ethanol to lactate production, and lactate accounted for as much as 40% of the glucose consumption (Fig. 2c). Glucose never accumulated (data not shown).

When pH of continuous cultures was decreased from 6.7 to 5.7 (no monensin), there was only a small decline in yield (Fig. 2a). However, monensin was a more potent inhibitor at pH 5.7 (Fig. 2a), and there was a nearly linear decrease in yield as monensin concentration was increased logarithmically from 0.01 to 20 μM. Once again, lactate accumulated when the specific rate of glucose consumption was >0.01 mmol/mg of protein per h (Fig. 2c), and lactate eventually represented >50% of the fermentation product. Even with

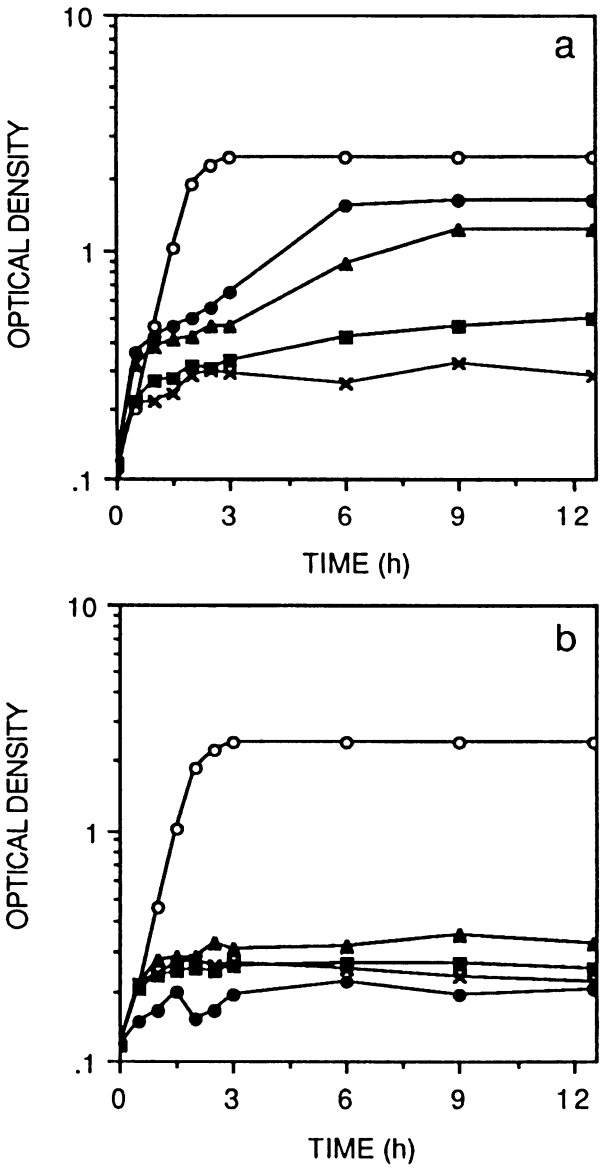

FIG. 1. Effect of monensin (a) or lasalocid (b) on growth of *S. bovis* in batch culture at pH 6.7. Symbols: o, 0; ●, 1.25; ▲, 2.5; ■, 5; ×, 10 μM.

20 μM monensin, >90% of the glucose was fermented (data not shown).

S. bovis JB1 was more sensitive to low concentrations of lasalocid than monensin, and even 0.1 μM caused a decrease in the yield (Fig. 3a) and an increase in the specific rate of glucose consumption (Fig. 3b) at pH 6.7. Lactate increased markedly when lasalocid was >1 μM (Fig. 3c). Lasalocid was a more effective inhibitor of growth at pH 5.7 than at pH 6.7 (Fig. 3b), and there was a faster rise in the specific glucose consumption rate (Fig. 3b) and lactate production (Fig. 3c). When lasalocid was at 10 μM, the cultures left approximately 50% of the glucose (data not shown).

When the yield data were plotted by the method of Eadie and Scatchard (J. B. Russell and H. J. Strobel, Arch. Microbiol. in press) (percent decline in yield/ionophore concentration versus percent decline in yield), the amount of monensin required to inhibit growth by 50% (−1/slope) at pH

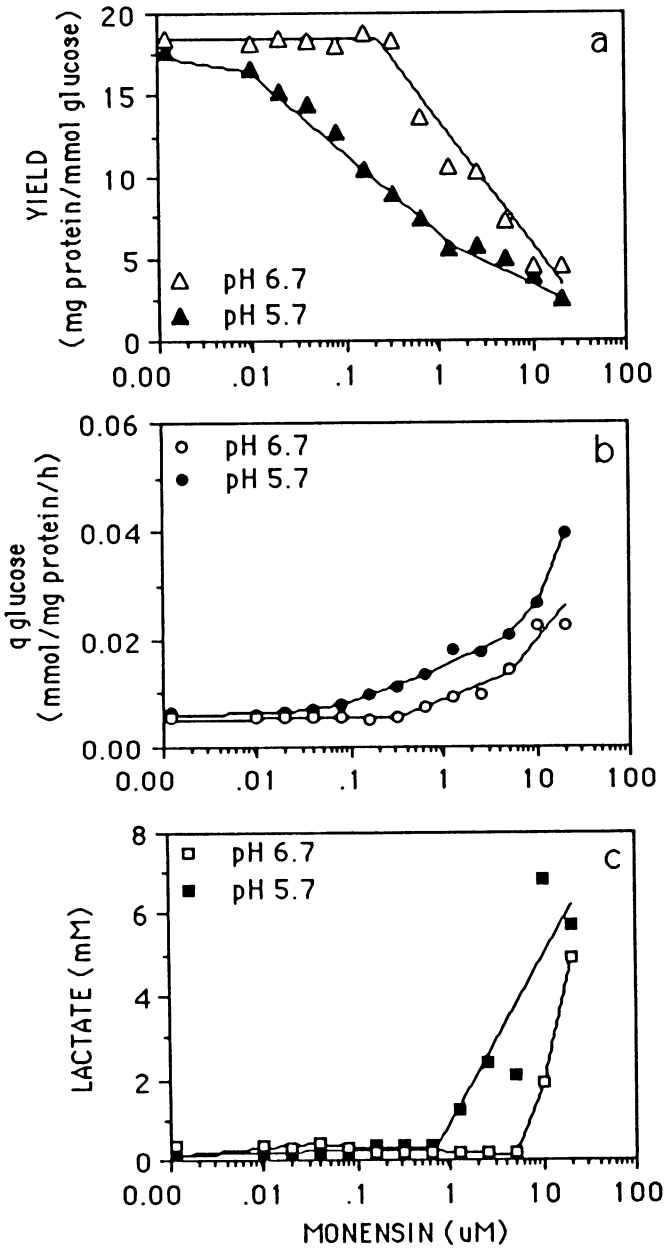

FIG. 2. Effect of monensin (log scale on yield (a), specific rate of glucose consumption (b), and lactate production (c) of *S. bovis* grown in continuous culture (5.9 mM glucose; dilution rate, 0.1 h^{-1}) at pH 6.7 (open symbols) or 5.7 (closed symbols).

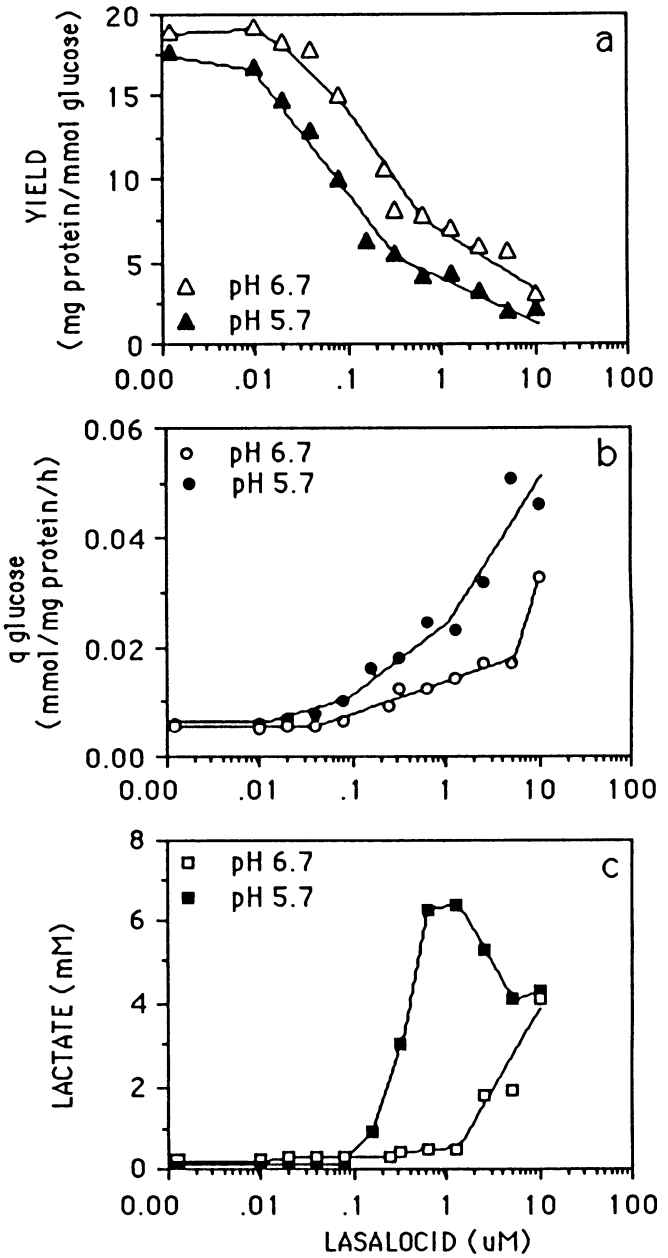

FIG. 3. Effect of lasalocid (log scale) on yield (a), specific rate of glucose consumption (b), and lactate production (c) of *S. bovis* grown in continuous culture (5.9 mM glucose; dilution rate, 0.1 h^{-1}) at pH 6.7 (open symbols) or 5.7 (closed symbols).

6.7 was 1.43 μM (Fig. 4a). Monensin was a considerably more potent growth inhibitor at pH 5.7 than at pH 6.7, but the plot was not linear. This nonlinearity indicated that monensin activity did not follow typical saturation kinetics and was proportionally more effective at low concentrations. Lasalocid was 6.5-fold more potent than monensin at pH 6.7, but this difference was not as great at pH 5.7 (particularly at low ionophore concentrations). Lasalocid was 2.5-fold more effective at pH 5.7 than at pH 6.7 (Fig. 4b).

Binding studies. When washed cells were incubated with ^{14}C-labeled monensin, there was little binding until the concentration was >4 μM (Fig. 5a). As monensin was increased further, there was a dramatic increase in binding, but pH had little effect on the amount bound. Lasalocid binding increased linearly with the ionophore concentration, and there was a threefold increase at pH 5.5 (Fig. 5b). Based on a intracelluar volume of 4.3 μl/mg of protein (29), there was 137- and 407-fold enrichment of ^{14}C-labeled lasalocid and monensin, respectively, when the cells were incubated with 10 μM ionophore. Since the enrichment was very high, extracellular ionophore which was trapped in the cell pellet had little effect on the estimate of binding. At higher concentrations (>30 μM), the ionophores precipitated and it was impossible to estimate the amount which was bound to cells.

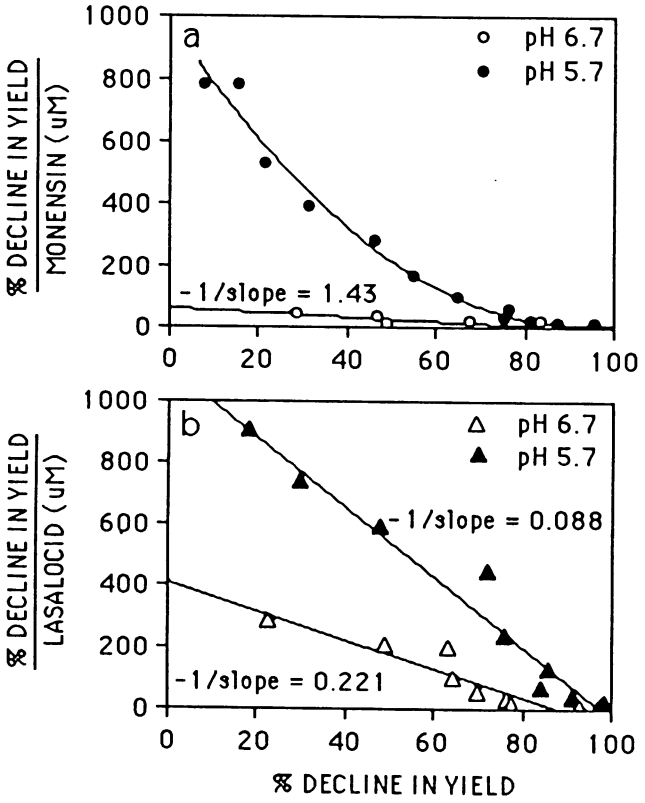

FIG. 4. Eadie-Scatchard plot of percent decline in yield/ionophore concentration versus percent decline in yield for (a) monensin or (b) lasalocid at pH 6.7 (open symbols) and 5.7 (closed symbols). −1/slope is the amount of ionophore needed to decrease yield 50%.

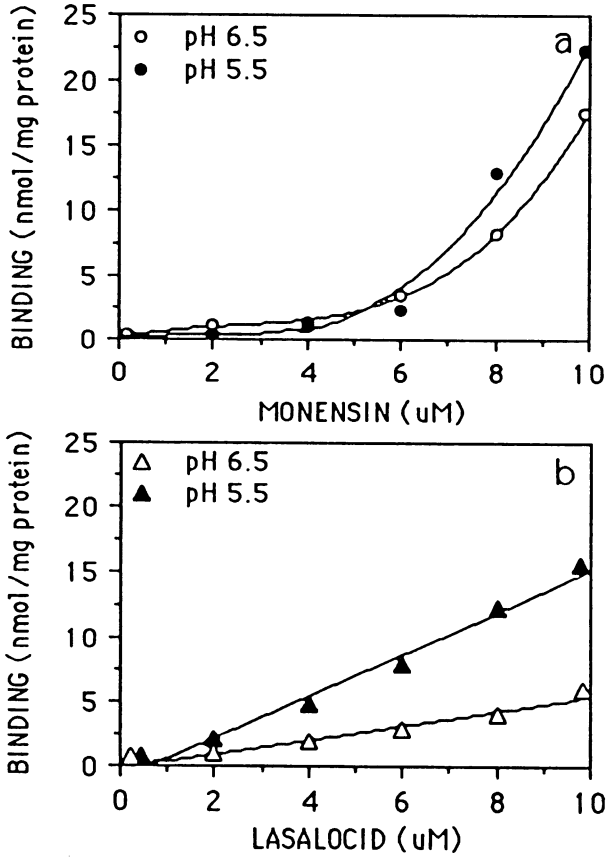

FIG. 5. Binding of ^{14}C-labeled monensin (a) or lasalocid (b) to *S. bovis* at pH 6.5 (open symbols) or 5.5 (closed symbols). The binding assays contained 160 mg of protein per liter and were conducted for 4 h at 39°C.

DISCUSSION

When *S. bovis* JB1 was grown in batch culture, 5 μM monensin and 1.25 μM lasalocid caused a marked decrease in final optical density (Fig. 1) even though large amounts of glucose remained in the medium (data not shown). Previous work indicated that monensin-treated batch cultures metabolized glucose at a rate which was 95% lower than exponentially growing cells (19). However, continuous cultures which were treated with 10 and 5 μM monensin and lasalocid, respectively (pH 6.7), fermented virtually all of the glucose and specific rates of glucose consumption approached those of untreated batch cultures (Fig. 2b and 3b). Since *S. bovis* can use either a phosphotransferase (10) or facilitated diffusion (unpublished data) to take up glucose and neither of these mechanisms was sensitive to monensin (Russell and Strobel, in press), it appeared that some other adaptation was responsible for the high glucose consumption of continuous cultures (e.g., ATPase activity; see below).

Because the continuous cultures utilized virtually all of the glucose, the fractional rate (hour^{-1}) of glucose consumption (yield × *q* glucose) should have been proportional to the dilution rate (0.1 h^{-1}; Fig. 6) (31). Previous work indicated that *S. bovis* JB1 had a maintenance energy requirement of 0.83 mmol of glucose/g of bacteria per h and a theoretical maximum growth yield of 72 mg of bacteria per mmol of glucose (20). Based on these earlier determinations (20), 38% of the glucose consumption at a dilution rate of 0.1 h^{-1} would have been expended to maintain the cells (Fig. 6). However, when monensin and lasalocid were added to the continuous cultures, there was a decrease in yield and an increase in the specific rate of glucose consumption which was not influenced by the type of ionophore or pH (Fig. 6).

For many years it was assumed that *S. bovis* (and streptococci in general) was homofermentative, but continuous-

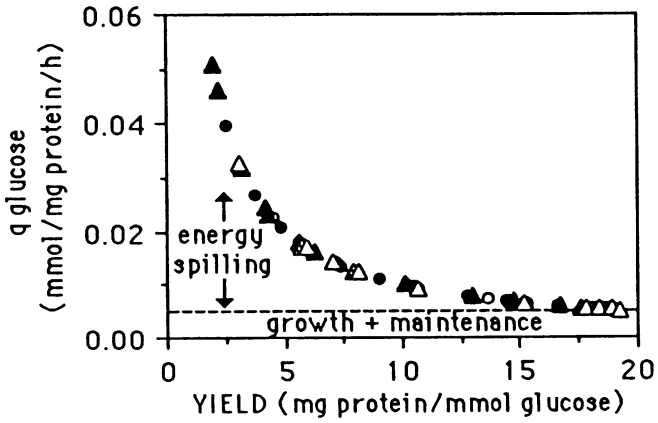

FIG. 6. Specific rate of glucose utilization (q) versus yield for continuous cultures (5.9 mM glucose; dilution rate, 0.1 h^{-1}) of *S. bovis* with monensin (○, ●) or lasalocid (△, ▲) at pH 6.7 (open symbols) or 5.7 (closed symbols). The area is the fractional rate of glucose utilization (hour^{-1}). The maintenance rate was taken from reference 20.

culture studies indicated that acetate, formate, and ethanol were the predominant end products at slow growth rates (20). This change in fermentation end products could be explained by a decrease in intracellular fructose 1,6-bisphosphate, an activator of the lactate dehydrogenase (21, 33). When *S. bovis* was grown in continuous culture with increasing concentrations of ionophores, there was a shift to lactate even though the growth rate was low (Fig. 2c and 3c). Since the specific rate of glucose consumption increased markedly (Fig. 2b and 3b), it is possible that a rise in intracelluar fructose 1.6-bisphosphate was responsible for lactate accumulation.

Previous studies also demonstated that lactate production was regulated by pH as well as growth rate. When *S. bovis* was grown in a slow-dilution-rate chemostat at pH 4.7, lactate was the predominant end product, intracellular pH decreased, and this decline in intracellular pH decreased the lactate dehydrogenase's requirement for fructose 1.6-bisphosphate (21). Because ionophores can translocate protons across the cell membrane (19), it is not surprising that the shift to lactate was more dramatic at pH 5.7 than at pH 6.7 (Fig. 2c and 3c).

When ionophores were added to continuous cultures of *S. bovis*, there was a 10-fold decrease in yield and the shift in fermentation end products (ATP availability) accounted for <20% of this decrease. The maintenance energy of bacteria was originally described as a time-dependent function which was directly proportional to cell mass (9, 15), but there has been considerable disagreement as to whether the maintenance energy of a bacterium is a biological constant (16, 28, 30). Because maintenance energy is described as the utilization of energy for non-growth-related functions (15), one might argue that the ionophores increased the maintenance energy requirement of *S. bovis*.

Because *S. bovis* changes its fermentation products as a function of growth rate (20) and ionophore concentration (Fig. 2c and 3c), it was not possible to estimate maintenance energy changes from traditional plots of 1/yield versus 1/growth rate. Tempest and Neijssel (32) and Stouthamer (28) have used the expression "energy spilling" to describe the growth-independent energy utilization of cultures that were limited by factors other than carbon source. Although "spill" suffers from the connotation that the phenomenon is accidental or unintentional, this term circumvents the question of a variable maintenance (Fig. 6). Neijssel (13) showed that the uncoupler 2,4-dinitrophenol (1 mM) had an effect on both the maintenance energy requirement and theoretical maximum growth yield of *Klebsiella aerogenes*.

Previous work with *S. bovis* JB1 showed that monensin caused an efflux of potassium and a net influx of sodium and protons (19). Since *S. bovis* has sodium and proton ATPases (29), ion influx could divert ATP from growth-related functions and decrease yield. Carboxylic ionophores such as monensin and lasalocid are lipid soluble, but only the uncharged species can transverse the cell membrane. According to the model of Pressman (18), these carboxylic ionophores can bind either protons or metal cations and the binding is both sequential and cyclic. If the free ionophore first binds a proton, the neutral complex crosses the membrane and a proton is released. The free ionophore can then bind a metal ion, and the complex moves across the membrane in the opposite direction. The metal ion is released and the cycle continues. Proton flux is always opposite to that of the metal ion, but the direction of movement is determined by the relative concentration of protons and metal ions across the cell membrane (24).

Since the carboxyl groups of monensin and lasalocid are responsible for proton translocation, it seems that pH might affect activity. Assuming that lasalocid has a pK_a of 5.8 (17), there should have been a fivefold increase in protonated species as pH was decreased from 6.7 to 5.7. Because a similar change in pH was associated with a threefold increase in binding (Fig. 5b), it appeared that the increased activity of lasalocid at low pH (Fig. 4b) could largely be explained by an increased number of lasalocid molecules in the cell membrane. Since monensin has a pK_a of 7.95 (17), nearly all of the monensin should have been protonated at either pH 6.7 or 5.7, and there was no difference in monensin binding at these pH values (Fig. 5a). Because pH did not affect binding, it appeared that some other factor (e.g., increased ion turnover) was increasing the activity of monensin at low pH.

The effect of lasalocid on yield showed typical saturation kinetics at pH 6.7 and 5.7 (Fig. 4b), but the binding assays did not show saturation over similar concentrations (Fig. 5b). Monensin binding was greater at high concentrations (Fig. 5a), but an Eadie-Scatchard plot of yield at pH 5.7 showed that monensin had proportionally less biological activity at high concentrations (percent decline in yield/micromolar concentration of monensin; Fig. 4a). Thus, the effect of ionophore concentration on yield cannot be explained solely by binding. Some carboxylic ionophores can form dimers in lipid membranes (25).

The effects of ionophores on ruminal bacteria in vitro have frequently been assessed at near-neutral pH and a concentration of 7 μM (5 ppm). Based on a daily intake of 350 mg of ionophore per day and a rumen volume of 70 liters, such a concentration seems realistic. However, in vitro incubations of ruminal bacteria usually contain much lower cell densities than those found in vivo. Assuming that bacterial protein concentrations in vivo are approximately 10 g/liter (3), a binding capacity of even 0.7 nmol/mg of protein could completely remove all of the available ionophore (7 μM). *S. bovis* (160 mg/liter) was able to bind as much 22 nmol of monensin and 15 nmol of lasalocid per mg of protein (Fig. 5). It is not known whether other ruminal bacteria (e.g., gram-negative bacteria) have as great a binding capacity as *S. bovis*, but it is likely that free ionophore concentrations in vivo are significantly <7 μM. Because pH had a marked influence on the effect of both monensin and lasalocid, the use of a near-neutral pH in vitro may not provide a true assessment of ionophore activity in vivo.

Previous workers have reported that some strains of *S. bovis* are highly resistant to monensin (4, 5). The S1 strain was originally resistant to 58 μM monensin (4), but in our experiments this strain was no more resistant than the JB1 strain (Fig. 1a). Continuous cultures tolerated as much as 20 μM monensin, but the yield was still depressed even after 13 days of continuous culture (45 doublings). These results are consistent with the supposition that ruminal bacterial resistance to ionophores is due to the outer membrane of gram-negative species and that monensin is more toxic to gram-positive bacteria (23, 24).

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