Binding of Radiolabeled Monensin and Lasalocid to Ruminal Microorganisms and Feed \(^{1,2}\)

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ABSTRACT: Gram-negative, ionophore-resistant ruminal bacteria and Gram-positive, ionophore-sensitive species bound similar amounts of \(^{14}\)C]lasalocid, but neither group bound large amounts of \(^{14}\)C]monensin. Membrane vesicles also bound more lasalocid than monensin (\(P < .05\)). The binding was first-order at low cell or vesicle concentrations and saturable at high cell or vesicle densities. *Streptococcus bovis* was inhibited by both monensin and lasalocid (5 \(\mu\)M), but cells that were re-incubated in medium lacking ionophore grew rapidly. Lasalocid-treated cells grew very slowly when they were resuspended in fresh medium. Based on these results, it seemed that lasalocid had a higher affinity for bacterial membranes than monensin. Mixed bacteria, however, bound nearly equal amounts of \(^{14}\)C]monensin and \(^{14}\)C]lasalocid (\(P > .05\)). Monensin binding was greatly reduced when the mixed ruminal bacteria were pretreated with Tris+EDTA (\(P < .05\)), but Tris+EDTA did not affect the binding of lasalocid. Mixed ruminal protozoa always took up more lasalocid than monensin (\(P < .05\)), but feed particles bound equal amounts of \(^{14}\)C]lasalocid and \(^{14}\)C]monensin (\(P > .05\)). Based on the binding capacity of mixed ruminal bacteria, ruminal protozoa, and feed particles, there would be little free ionophore in ruminal fluid.

Key Words: Rumen Microorganisms, Ionophores, Monensin, Lasalocid, Binding

Introduction

Monensin has been used to increase the performance of beef cattle for more than two decades (Goodrich et al., 1984; Galyean and Owens, 1988), and lasalocid, a closely related ionophore, has also been approved for use as a ruminant feed additive (Russell and Strobel, 1989). Ionophores were originally marketed as enhancers of ruminal propionate production (Chalupa, 1977), but subsequent work showed that they could also inhibit lactate- and ammonia-producing ruminal bacteria (Dinius et al., 1976; Dennis et al., 1981; Russell et al., 1988; Chen and Russell, 1989). Because ionophores inhibited Gram-positive bacteria to a much greater extent than Gram-negative bacteria, it seemed that the Gram-negative outer membrane was able to protect the cell membrane from ionophores (Russell and Strobel, 1988, 1989).

Monensin is typically added to feed at 20 to 30 ppm (Galyean and Owens, 1988). Based on a feed intake of 10 kg of DM/d and a ruminal volume of 60 L, the ruminal ionophore concentration would be 5 to 7 \(\mu\)M. In vitro studies often used a similar concentration (Chalupa, 1977; Van Nevel and Demeyer, 1977; Russell and Martin, 1984), but these studies ignored the ability of ionophores to concentrate in bacterial membranes and the fact that the density of microorganisms is usually much greater in vivo than in vitro. Because little was known about the binding of radiolabeled ionophore to ruminal microorganisms and feed particles, we decided to study ionophore binding with radiolabeled \(^{14}\)C]monensin and \(^{14}\)C]lasalocid.

Materials and Methods

Mixed Cultures. Ruminal contents were removed from a 750-kg nonlactating, ruminally fistulated cow that was fed 2.5 kg of concentrate (16% CP) and 2.5 kg of chopped timothy hay twice a day. The contents

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\(^{2}\) Mention of tradenames, proprietary products, or specific equipment does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

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were strained through two layers of cheesecloth and incubated at 39°C until large feed particles had buoyed to the top and protozoa had settled to the bottom of the flask. Protozoa and bacteria were partitioned further by differential centrifugation (117 vs 10,000 × g, 5 min).

Outer membrane lipopolysaccharides were disrupted by washing mixed ruminal bacteria twice with 120 mM Tris HCl plus 10 mM EDTA (pH 8.0) and once with 120 mM Tris HCl (pH 6.5). Otherwise, the cells were washed in phosphate buffer (45 mM Na₂HPO₄, 5 mM KH₂PO₄, pH 6.5). The final cell concentration of bacteria or protozoa was varied from 0 to 4 mg of protein/mL.

Feed Particles. Ground alfalfa hay (< 300 μm particle size, 90% DM) was hydrated in phosphate buffer (pH 6.5), and the final concentration was varied from 0 to 60 mg/mL.

Pure Cultures. Butyrivibrio fibrisolvens 49, Clostridium aminophilum F, Clostridium sticklandii SR, Megasphaera elsdenii B159, Prevotella ruminicola (strains B14, 23, M384, and M20-78), Ruminococcus flavefaciens B146, Selenomonas ruminantium HD₄, and Streptococcus bovis (strains JB1, 26, and K277FFA) were grown anaerobically in medium containing (per liter) the following: 292 mg of K₂HPO₄, 240 mg of KH₂PO₄, 480 mg of (NH₄)₂SO₄, 480 mg of NaCl, 100 mg of MgSO₄·7H₂O, 64 mg of CaCl₂·2H₂O, 4 g of Na₂CO₃, 1 g of Trypticase (BBL Microbiology Systems, Cockeysville, MD), .5 g of yeast extract, .6 g of cysteine hydrochloride, .1 mg resazurin, and a VFA mixture (Caldwell and Bryant, 1966). Glucose (4 g/L; strains 49, B159, B14, 23, M384, M20-78, HD₄ JB1, 26, and K277FFA), cellobiose (3 g/L; strain B146), Trypticase (15 g/L; strain SR; BBL Microbiology Systems), or Casamino Acids (15 g/L; strain F; Difco Laboratories, Detroit, MI) were provided as energy sources. The cultures were incubated at 39°C in serum bottles (Wheaton, Millville, NJ) that were sealed with butyl rubber stoppers. Stationary cultures were harvested (11,700 × g, 5°C, 10 min) and resuspended in phosphate buffer (pH 6.5), and the final cell concentration was varied from 0 to 4 mg/mL of protein.

Membrane Vesicles. P. ruminicola B14 and S. bovis JB1 were harvested during log phase. P. ruminicola cells were treated with EDTA and lysozyme (Kaback, 1971), and S. bovis was treated with lysozyme and mutanolysin (Russell et al., 1988). Vesicles were then prepared by osmotic lysis and treatment with RNase and DNase (Russell et al., 1988). The vesicles were resuspended in phosphate buffer (pH 6.5) and frozen until use (−5°C).

Binding Assays. Mixed bacteria, mixed protozoa, feed, or vesicles were placed in 13-mm × 100-mm glass tubes containing [14C]-labeled lasalocid (1.74 μCi/mg; Hoffmann-La Roche, Nutley, NJ) or monensin (.62 μCi/mg; Eli Lilly, Indianapolis, IN). The final concentration of ionophore was 5 μM, and the ethanol concentration was less than 4%. The cells or feed were incubated with ionophore for 15 min at 39°C, and preliminary experiments indicated that greater than 90% of the binding occurred within the first 5 min of incubation. Cell suspensions (.9 mL) were placed in 1.5-mL polypropylene microcentrifuge tubes and centrifuged for 5 min (13,000 × g, 22°C). The binding of ionophore was estimated from the decrease in radioactivity (100 μL of supernatant; Beckman LS5000CE Liquid Scintillation System, Beckman Instruments, Fullerton, CA; Ecoscint scintillation cocktail, National Diagnostics, Manville, NJ). Incubations lacking cells or feed were used as a control, and the cpm were initially 300 and 1,000 for monensin and lasalocid, respectively.

Growth Inhibition and Desorption. S. bovis JB1 was grown anaerobically in 18-mm × 100-mm glass tubes (Belco Glass, Vineland, NJ) in media (described above) containing 6 g/L of glucose. Exponentially growing cultures (approximately .3 optical density, 600 nm, Gilford Stasar II, Gilford Instrument, Oberlin, OH) were treated with 10 μM monensin or lasalocid. The cells were harvested by centrifugation, resuspended in a similar volume of medium (6 g/L of glucose) that lacked ionophore, and incubated at 39°C.

Other Analyses. Washed cells were treated with 2 N NaOH (100°C, 15 min), and protein was determined by the method of Lowry et al. (1951).

Statistics. The experiments were performed on duplicate days. The data were regressed and analyzed for linear and quadratic effects (Neter et al., 1985). Predictive equations and 95% confidence intervals were calculated using the parameter estimates and the variances of the estimates.

Results

Pure Cultures. Gram-positive, monensin-sensitive ruminal bacteria (Butyrivibrio fibrisolvens 49, Clostridium aminophilum F, Clostridium sticklandii SR, Ruminococcus flavefaciens B146, Streptococcus bovis JB1) and Gram-negative, monensin-resistant ruminal bacteria (Megasphaera elsdenii B159, Prevotella ruminicola B14, Selenomonas ruminantium HD₄) were washed in phosphate buffer, adjusted to a cell density of 1.98 ± .26 mg of protein/mL, and incubated with 5 μM [14C]ionophore. The Gram-negative and -positive bacteria bound 1.5 ± .4 and 1.5 ± .7 nmol of lasalocid/mg of protein, respectively, and the binding was not different (P > .05; Table 1). Lasalocid binding to different strains of P. ruminicola (B14, 23, M384, M20-7) and S. bovis (JB1, 26 and K277FFA) was 1.1 ± .4 and 1.1 ± .2 nmol/mg of protein, respectively. Monensin binding to both Gram-positive and -negative bacteria was 10-fold lower than lasalocid binding (P < .05) and near the detection limit of our assay (approximately .1 nmol/mg of protein).
P. ruminicola B14 (Figure 1a) and S. bovis JB1 (Figure 1b) never bound large amounts of [14C]monensin (< 10%), even if the cell density was as great as 3.8 mg of protein/mL, but in each case there was a large increase in [14C]lasalocid binding as the cell density increased (P < .05). P. ruminicola B14 took up more [14C]lasalocid than S. bovis JB1 (P < .05), but the K_b (amount of cell protein needed to take up 50% of the ionophore as estimated from the x-intercept of a Lineweaver Burk plot) of P. ruminicola B14 cells was higher than the K_b of S. bovis cells (1.77 vs .48 mg of protein/mL, P < .05). Pretreatment of the cells with unlabeled monensin (8.3 μM had no effect (P > .05) on the binding of [14C]lasalocid to S. bovis or P. ruminicola. P. ruminicola B14 (Figure 2a) and S. bovis (Figure 2b) membrane vesicles bound less [14C]monensin than [14C]lasalocid (P < .05). Based on the relationship between percentage bound and cell protein, the K_b of P. ruminicola and S. bovis JB1 vesicles for lasalocid were similar (.67 vs .66 nmol/mg of protein, respectively; P > .05).

Mixed Ruminal Microorganisms. Mixed ruminal protozoa bound as much as 80% of the [14C]lasalocid (K_b of 1.02 mg of protein/mL), but these same cells took up very little monensin until the cell protein concentration was greater than 2 mg/mL (Figure 3). Mixed ruminal bacteria had the same capacity to bind [14C]monensin and [14C]lasalocid (P > .05), and the K_b values were similar (1.46 vs 1.69 mg of protein/mL, respectively, P > .05). When the mixed ruminal bacteria were washed with Tris+EDTA to disrupt outer membrane lipopolysaccharides, the binding of [14C]monensin was greatly reduced (P < .05), but this treatment had no effect (P > .05) on the binding of [14C]lasalocid (Figure 4).

Feed Particles. Ground alfalfa hay (Figure 5) that had been hydrated in phosphate buffer bound similar amounts of [14C]lasalocid and [14C]monensin (P < .05), the K_b values were similar (8.5 and 7.9 mg of dry weight/mL, respectively, P > .05). Ground corn bound 40% less [14C]ionophore than alfalfa (P < .05).
Growth Inhibition and Desorption. *S. bovis* JB1 grew rapidly (1.6 h⁻¹) with glucose as an energy source, and its growth was inhibited by the addition of either 10 μM lasalocid (Figure 6a) or monensin (Figure 6b). When the lasalocid-treated cultures were centrifuged anaerobically and resuspended in media that did not contain lasalocid, growth resumed, but the maximum growth rate was much less than that of untreated cultures (0.24 vs 1.3 h⁻¹). Monensin-treated cultures (Figure 6b) that were resuspended in medium lacking monensin grew nearly half as quickly as the untreated control (0.67 vs 1.3 h⁻¹).

Discussion

Monensin and lasalocid mediate the exchange of cations for protons across cell membranes (Pressman, 1976). When these metal/proton antiporters bind a cation, the molecule assumes a torus-shape (doughnut), the cation is shielded, and the electroneutral ion/ionophore complex can transverse the membrane. After the cation dissociates, the carboxyl group of the ionophore can bind a proton, and this other electroneutral complex proceeds in the opposite direction. This disruption of ion balance across the cell membrane leads to a loss of intracellular K, an accumulation of intracellular Na, an acidification of the cytoplasm, a loss of transport activity, and a depletion of ATP (Russell and Strobel, 1989).

Both monensin and lasalocid are hydrophobic, but it seems that these ionophores differ in their lipid solubility. Lasalocid dissolved less readily in ethanol than does monensin, and membrane vesicles of ruminal bacteria took up at least 10-fold more lasalocid than monensin. Because membrane vesicles of *P. ruminicola*, a Gram-negative, ionophore-resistant species, and *S. bovis*, a Gram-positive, ionophore-sensitive species, took up similar amounts of lasalocid and monensin, it did not seem that ionophore resistance could be explained by differences in the cell membrane per se.
Figure 5. The binding of $^{14}$C]lasalocid (●) and $^{14}$C]monensin (▲) to ground alfalfa hay (< 300 μm particle size, 90% dry matter). Feed particles were suspended in phosphate buffer [pH 6.5] and incubated with ionophores [5 μM] at 39°C for 30 min. The shaded areas show the 95% confidence intervals.

Ionophores must pass through outer layers of the cell envelope before they can reach the cell membrane. Gram-negative bacteria are surrounded by an outer membrane, but Gram-positive bacteria lack this cell wall structure. Because P. ruminicola whole cells had a higher $K_b$ than the membrane vesicles ($P < .05$), it seemed that the outer membrane of P. ruminicola had a lower affinity for ionophore than the cell membrane. Whole cells and membrane vesicles of S. bovis JB1, a Gram-positive bacterium, had similar $K_b$ values ($P > .05$).

Pure cultures of ruminal bacteria bound at least fivefold more lasalocid than monensin ($P < .05$), but mixed ruminal bacteria that were taken directly from the rumen took up similar amounts of these two ionophores ($P > .05$). Costerton et al. (1981) noted

![Figure 5](image)

Figure 6. The effect of ionophores on the growth of Streptococcus bovis JB1 in batch cultures with 6 g/L of glucose. The cells were treated with 10 μM (a) lasalocid or (b) monensin at 1 h (open arrows). At 2 h (closed arrows), the cells were harvested by centrifugation and resuspended in media containing [closed symbols] or lacking [open symbols] 10 μM ionophore. A control that did not receive any ionophore is also shown [×].

![Figure 6](image)

Table 1. The binding of $^{14}$C]lasalocid and $^{14}$C]monensin to pure cultures of ruminal bacteria

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gram</th>
<th>Lasalocid</th>
<th>Monensin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butyrivibrio fibrisolvens 49</td>
<td>+</td>
<td>1.1</td>
<td>&lt;.1</td>
</tr>
<tr>
<td>Clostridium aminophilum F</td>
<td>+</td>
<td>1.3</td>
<td>.3</td>
</tr>
<tr>
<td>Clostridium sticklandii SR</td>
<td>+</td>
<td>1.5</td>
<td>&lt;.1</td>
</tr>
<tr>
<td>Ruminococcus flavefaciens B146</td>
<td>+</td>
<td>1.3</td>
<td>&lt;.1</td>
</tr>
<tr>
<td>Streptococcus bovis JB1</td>
<td>+</td>
<td>1.2</td>
<td>&lt;.2</td>
</tr>
<tr>
<td>Fibrobacter succinogenes S85</td>
<td>-</td>
<td>2.3</td>
<td>.4</td>
</tr>
<tr>
<td>Megasphaera elsdenii B159</td>
<td>-</td>
<td>1.1</td>
<td>&lt;.1</td>
</tr>
<tr>
<td>Prevotella ruminicola B4</td>
<td>-</td>
<td>1.7</td>
<td>&lt;.1</td>
</tr>
<tr>
<td>Selenomonas ruminantium HD4</td>
<td>-</td>
<td>.7</td>
<td>&lt;.1</td>
</tr>
</tbody>
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$a$: Incubations contained 1.98 ± .26 mg/mL of protein.
that bacteria in natural environments such as the rumen often have a much thicker layer of glycocalyx than pure cultures. Based on these observations, it seemed that monensin might be binding to surfaces outside the cell membrane. This hypothesis was supported by the effect of Tris+EDTA on monensin binding. When mixed ruminal bacteria were treated with Tris+EDTA to destabilize the divalent cation bridges of lipopolysaccharides on the outer membrane, there was a marked decrease in monensin binding. Ruminal protozoa do not have an outer membrane, nor do they accumulate extracellular polysaccharide (Coleman, 1980), and untreated mixed ruminal protozoa never bound large amounts of monensin.

Czerkawski (1986) estimated that the dry matter content of feed in the rumen was 50 to 100 g/L and that microorganisms were present at 7 to 11 g/L. Protozoal numbers vary, but in some cases protozoa can account for half of the microbial mass in the rumen (Coleman, 1980). Because the K_b (the amount of cell protein needed to bind half of the ionophore) of mixed ruminal bacteria and protozoa were 1.5 and 1.0 g of protein/L, respectively, and the K_b of alfalfa hay 7.7 mg of DM/L, there would be little free lasalocid in the rumen when animals are fed a typical dose of 300 mg/d. Mixed ruminal protozoa did not bind as much monensin as lasalocid, but the K_b values of mixed ruminal bacteria and alfalfa hay for monensin were still much lower than the in vivo concentrations.

The potency of antibiotics has often been designated by the minimum inhibitory concentration, and this term has been used in the assessment of the effect of ruminal ionophores (Dennis et al., 1981; Nagaraja and Taylor, 1987). The extrapolation of in vitro experiments to in vivo conditions can, however, present problems in interpretation. The total count of bacteria in the rumen is usually 10^{10} cells/mL or greater, but the density of bacteria in vitro is usually 10-fold less (10^8 to 10^9/mL). Because the ratio of ionophore:bacterial mass seems to be a more important criterion of ionophore potency than the absolute concentration per se (Chow and Russell, 1990), much of the early in vitro work on ruminal ionophores was performed at high and unphysiological concentrations.

**Implications**

Because the ruminal ionophores monensin and lasalocid readily bind to a variety of materials within the rumen (bacteria, protozoa, feed particles), the concentration of free (unbound) ionophore that would be available to ionophore-sensitive bacteria is apt to be very low in vivo.

**Literature Cited**


