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Sodium-Dependent Transport of Neutral Amino Acids by Whole Cells and Membrane Vesicles of *Streptococcus bovis*, a Ruminal Bacterium

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***Streptococcus bovis* JB1 cells were able to transport serine, threonine, or alanine, but only when they were incubated in sodium buffers. If glucose-energized cells were washed in potassium phosphate and suspended in potassium phosphate buffer, there was no detectable uptake. Cells deenergized with 2-deoxyglucose and incubated in sodium phosphate buffer were still able to transport serine, and this result indicated that the chemical sodium gradient was capable of driving transport. However, when the deenergized cells were treated with valinomycin and diluted into sodium phosphate to create both an artificial membrane potential and a chemical sodium gradient, rates of serine uptake were fivefold greater than in cells having only a sodium gradient. If deenergized cells were preloaded with sodium (no membrane potential or sodium gradient), there was little serine transport. Nigericin and monensin, ionophores capable of reversing sodium gradients across membranes, strongly inhibited sodium-dependent uptake of the three amino acids. Membrane vesicles loaded with potassium and diluted into either lithium or choline chloride were unable to transport serine, but rapid uptake was evident if sodium chloride was added to the assay mixture. Serine transport had an extremely poor affinity for sodium, and more than 30 mM was needed for half-maximal rates of uptake. Serine transport was inhibited by an excess of threonine, but an excess of alanine had little effect. Results indicated that *S. bovis* had separate sodium symport systems for serine or threonine and alanine, and either the membrane potential or chemical sodium gradient could drive uptake.**

Streptococcus bovis is a very rapidly growing ruminal bacterium that flourishes in the rumen if diets contain an abundance of starch (14). *S. bovis* produces acetate, formate, and ethanol when carbohydrates and growth rate are restricted, but its fermentation is homolactic at rapid growth rates (25). Lactate is a stronger acid than the volatile fatty acids, and its accumulation can exceed the buffering capacity of ruminal fluid. As rumen pH declines, the growth rates of less acid-resistant ruminal bacteria are inhibited, and *S. bovis* soon dominates the population (14, 26). Rumen acidosis is a common problem in beef cattle, and it causes decreased food intake, rumen ulceration, founder, and even death (28).

S. bovis is able to use ammonia as a sole source of nitrogen (33), but growth rates are more than twice as fast when peptides and amino acids are provided (27). In bacteria, amino acid transport is often driven by the proton motive force (15), but sodium gradients may contribute to the driving force in *Escherichia coli* (4, 31), certain marine bacteria (10), and alkalophilic bacteria (16, 29). In gram-negative bacteria, amino acid transport often involves binding proteins, and uptake is thought to be driven by ATP hydrolysis (1). Gram-positive bacteria, which lack a periplasm, do not have binding proteins per se.

Recent studies showed that *S. bovis* used the phosphoenolpyruvate system to transport glucose and disaccharides (17), but amino acid transport has not been studied. Most of the ruminal bacteria have a requirement for sodium, and they have been compared to the marine bacteria (3). Because the concentration of sodium in the rumen is usually 90 mM, it seemed conceivable that sodium might be involved in active transport.

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MATERIALS AND METHODS

Cell growth. The JB1 strain of *S. bovis* was used, and previous work indicated that this strain was characteristic of the species (27). Cultures were grown anaerobically in medium 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 $CaCl_2 \cdot 2H_2O$, 600 mg of cysteine hydrochloride, 8 g of Trypticase (BBL Microbiology Systems, Cockeysville, Md.), and 0.5 g of yeast extract; the medium also contained 20 mM glucose. When strain JB1 was grown in sodium-deficient medium, sodium salts were replaced by potassium salts, and purified amino acids (12 g/liter), micro-minerals (22), and vitamins (22) were substituted for Trypticase and yeast extract. The medium was adjusted to pH 6.7, and the final pH was never lower than 6.2. The incubation temperature was 37°C.

Glucose-energized cells. Batch cultures were harvested during logarithmic growth by centrifugation (1,200 × *g*, 5 min, 25°C) at an optical density of approximately 1.2 (660 nm). The cells were washed twice in either potassium phosphate or sodium phosphate (100 mM K^+ or Na^+, pH 6.5) and resuspended in 150 μl of the same buffer. Concentrated cell suspensions (4 μl, 8.8 μg of protein per μl) were added to 200 μl of potassium or sodium phosphate buffer and energized with glucose for 5 min (10 mM, 28°C). Transport was initiated by the addition of 100 nCi of ^{14}C-labeled amino acid (final concentrations, 2.9 μM serine or alanine and 2.2 μM threonine) and allowed to continue for 0 to 30 s. Transport was terminated by the addition of 2 ml of ice-cold

0.1 M LiCl to the reaction mixture and rapid filtration of the mixture through 0.45-μm-pore-size cellulose nitrate membrane filters. Filters were washed with 2 ml of 0.1 M LiCl and dried for 25 min at 120°C. When needed, 0 to 10 μM nigericin or monensin was added to the cells prior to energization with glucose.

Deenergized cells. Glucose-limited cultures (4 mM glucose; final optical density, 0.4) were given 4 mM 2-deoxyglucose when growth ceased. After the cells were incubated for another 30 min, the cultures were centrifuged (1,200 × *g*, 5 min, 25°C), washed twice in potassium phosphate buffer (see above), and resuspended in 2 ml of the same buffer. Some deenergized cells were treated with valinomycin (2 μM, 60 min, on ice) to load them with potassium. Treated and untreated cells were centrifuged again (13,000 × *g*, 5 min, 25°C) and resuspended in 150 μl of potassium phosphate buffer. Concentrated cell suspensions (4 μl, 9 μg of protein per μl) were then added to 200 μl of either potassium or sodium phosphate buffer which contained ^{14}C-labeled amino acid. Transport was measured for 0 to 180 s as described above.

Membrane vesicles. The method of vesicle preparation (8, 19) was modified in several respects. Cells were grown in anaerobic medium (see above) supplemented with 0.75 g each of glycine and DL-threonine per liter. Cultures were harvested by centrifugation (11,000 × *g*, 10 min, 4°C) during logarithmic growth. Cell pellets were washed twice with 100 mM potassium phosphate (pH 7.0) and suspended in 20 mM sodium maleate (pH 6.5) containing 20 mM $MgCl_2$, 0.7 M lactose, 3 × 10^6 U of lysozyme (E. Merck AG, Darmstadt, Federal Republic of Germany), and 2,500 U of mutanolysin (Sigma Chemical Co., St. Louis, Mo.). After 45 min of incubation at 37°C, protoplasts were harvested by centrifugation (27,000 × *g*, 20 min, 4°C). Protoplasts were diluted into 1 liter of 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM $MgSO_4$, 30 × 10^6 U of DNase, and 2.4 × 10^6 U of RNase (Sigma). As estimated from the decrease in optical density, the formation of osmotically sensitive protoplasts was approximately 90%. The solution was incubated for 20 min at 37°C, and potassium EDTA (pH 7.0, 15 mmol/liter) was added. After 10 more min, $MgSO_4$ (20 mmol/liter) was added, and the solution was centrifuged (23,000 × *g*, 60 min, 4°C). The pellet was suspended in 50 mM potassium phosphate (pH 7.0) containing 10 mM $MgSO_4$ and centrifuged at low speed (750 × *g*, 60 min, 4°C). The supernatant was centrifuged at high speed (48,000 × *g*, 30 min, 4°C). The membrane vesicles were resuspended in 50 mM potassium phosphate (pH 7.0) containing 10 mM $MgSO_4$ (final protein concentration, 6.7 μg/μl). Vesicles were frozen in liquid nitrogen until use.

Membrane vesicles were treated with valinomycin (2 μM, 30 min, on ice) to load them with potassium or potassium and sodium. The vesicles were then diluted 50-fold into solutions containing 100 mM choline chloride, lithium chloride, or sodium phosphate (pH 6.5). Transport of L-[^{14}C]serine was assayed as described above.

Competition. L-[^{14}C]serine transport was assayed in the presence of a 70-fold excess of nonlabeled L-threonine or L-alanine. The experiments were performed with membrane vesicles which had been frozen in liquid nitrogen or with glucose-energized cells.

Materials. L-[U-^{14}C]serine (170 mCi/mmol), L-[U-^{14}C]threonine (226 mCi/mmol), and L-[U-^{14}C]alanine (171 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, United Kingdom. All other chemical were reagent grade and were obtained from commercial sources.

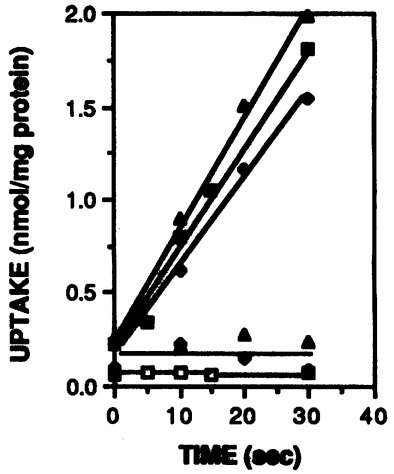


FIG. 1. Effect of sodium (closed symbols) or potassium (open symbols) on the uptake of threonine (▲ and △), serine (■ and □), and alanine (◆ and ◇) by *S. bovis* cells which were energized for 5 min with glucose. Cells were washed and incubated in either 100 mM sodium or potassium phosphate (pH 6.5).

RESULTS

Glucose-energized cells. When cells of *S. bovis* JB1 were washed in potassium phosphate buffer, energized with glucose, and incubated in potassium phosphate buffer, there was little transport of threonine, serine, or alanine (Fig. 1). However, when the cells were washed and incubated in sodium phosphate buffer, rapid uptake was observed. Potassium phosphate-washed cells were able to take up the labeled amino acids at the same rate as sodium phosphate-washed cells as long as they were incubated in sodium phosphate buffer (data not shown). In separate experiments, initial rates of transport were determined at amino acid concentrations ranging from 1 to 50 μM. The V_{max} values for threonine, serine, and alanine were 10, 7, and 3 nmol/min per mg of protein, respectively, and the K_m values were 6.3, 8.6, and 8.8 μM, respectively.

When glucose-energized cells were treated with 0.2 μM nigericin and diluted into sodium phosphate buffer, the rate of serine transport was 4.4-fold lower than that in controls not receiving nigericin (Fig. 2). Nigericin was a somewhat more potent inhibitor of serine transport than monensin was, but transport decreased more than 85% when either ionophore was present at 5 μM or greater. Cells which were incubated in potassium phosphate once again showed no detectable transport, regardless of nigericin or monensin treatment (data not shown). Similar results were obtained for threonine and alanine.

Deenergized cells. When glucose-depleted cells were deenergized with 2-deoxyglucose, washed in potassium phosphate buffer, and incubated in potassium phosphate buffer, there was little uptake of serine (Fig. 3). When the same cells were incubated in sodium phosphate buffer, there was linear uptake of serine over the first 30 s of incubation. Since the cells were deenergized, it appeared that the chemical sodium gradient ($\Delta\tilde{\mu}_{Na}$) was driving uptake. When the deenergized cells were treated with valinomycin and diluted into sodium phosphate to establish a potassium diffusion potential ($\Delta\psi$) and $\Delta\tilde{\mu}_{Na}$, the rate of uptake was much greater than that in untreated cells (only $\Delta\tilde{\mu}_{Na}$). Based on the dilution (50-fold), the initial $\Delta\psi$ would have been approximately 100 mV. Valinomycin-treated cells which were diluted into potassium

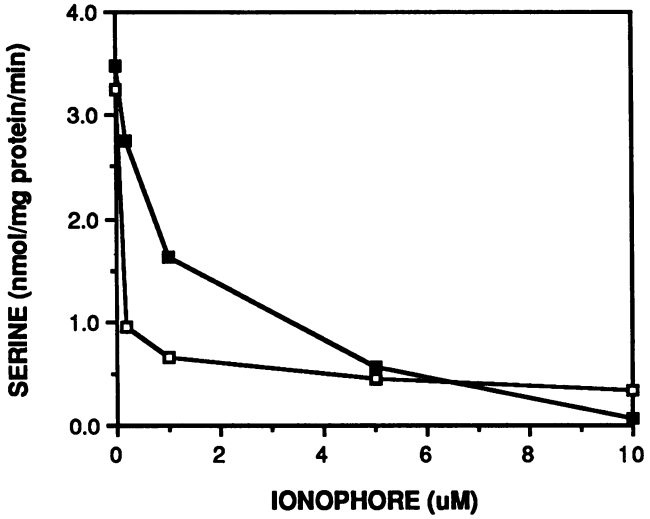


FIG. 2. Effect of nigericin (□) or monensin (■) on the rate of serine transport by glucose-energized cells which were preincubated with 0 to 10 μM ionophore. Cells were washed and incubated in 100 mM sodium phosphate (pH 6.5).

phosphate would not have had a $\Delta\psi$ or a $\Delta\tilde{\mu}_{Na}$, and there was no uptake of serine. Similar results were obtained for threonine and alanine (data not shown).

When valinomycin-treated cells were diluted into 100 mM choline chloride, there was virtually no transport of serine (Fig. 4a). As NaCl was added at concentrations ranging from 0 to 100 mM, the rate of serine transport increased dramatically. Concentrations of NaCl greater than 80 mM caused little further increase in the rate of serine transport. When the data were expressed as a Hill plot, the slope (n_{app}) was approximately 2 (Fig. 4b).

Sodium-loaded cells. Since valinomycin has the ability to translocate sodium as well as potassium, it was possible to preload the cells with potassium and/or sodium (data not shown). When deenergized cells were loaded with 100 mM potassium phosphate plus 100 mM sodium phosphate and

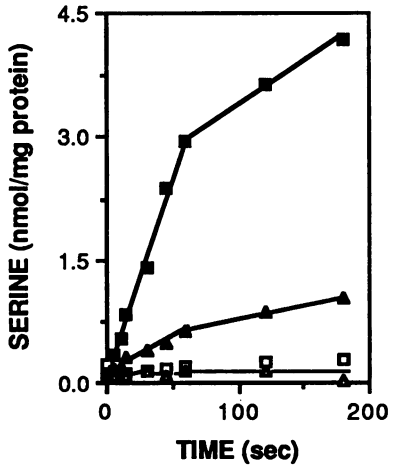
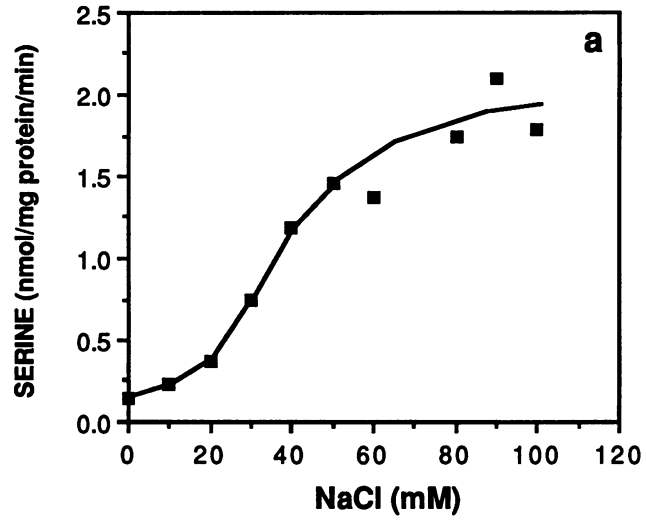


FIG. 3. Transport of serine by deenergized cells which were either treated with valinomycin and loaded with potassium (■ and □) or not treated (▲ and △). The cells were diluted into 100 mM sodium phosphate (pH 6.5) (closed symbols) or 100 mM potassium phosphate (pH 6.5) (open symbols).



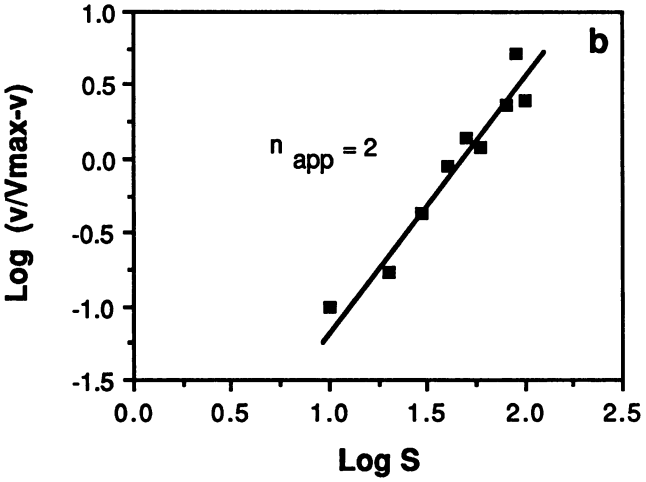


FIG. 4. (a) Effect of added sodium on the rate of serine transport by deenergized cells of *S. bovis*. Cells were washed in 100 mM potassium phosphate (pH 6.5), treated with valinomycin, and diluted into 100 mM choline chloride supplemented with 0 to 100 mM sodium chloride. A Hill plot of the data are shown in panel b, where S is the concentration of NaCl (millimolar) and v is the rate of serine transport (nanomoles per milligram of protein per minute).

diluted into 100 mM sodium phosphate, there was rapid uptake of serine. Under these conditions, the $\Delta\psi$, and not the $\Delta\tilde{\mu}_{Na}$, was driving transport. When the cells were loaded only with potassium and diluted into potassium plus sodium (only $\Delta\tilde{\mu}_{Na}$), serine was transported, but the rate was half as fast as that observed with a $\Delta\psi$. When the cells were loaded with both sodium phosphate and potassium phosphate and diluted into buffer containing sodium phosphate as well as potassium phosphate, there was no transport of serine.

Membrane vesicles. Membrane vesicles which were treated with valinomycin, loaded with potassium phosphate (pH 6.5), and diluted into 100 mM choline or lithium chloride were unable to take up serine (Fig. 5). However, rapid uptake was observed when the same vesicles were diluted into 100 mM sodium phosphate ($\Delta\psi$ and $\Delta\tilde{\mu}_{Na}$). When the vesicles were loaded with potassium phosphate plus sodium chloride (each 100 mM) and diluted into 100 mM sodium

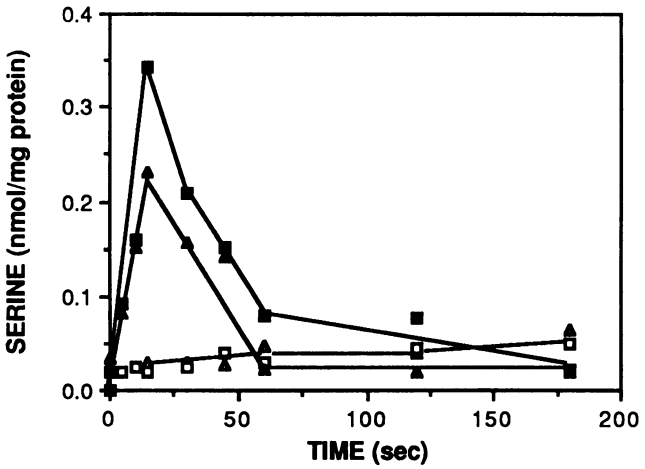


FIG. 5. Transport of serine by membrane vesicles of *S. bovis*. Vesicles were loaded with either 100 mM potassium phosphate (■, □, and △) or 100 mM potassium phosphate plus 100 mM sodium chloride (▲) and diluted (50-fold) into 100 mM choline chloride (□), 100 mM lithium chloride (△), or 100 mM sodium phosphate (■ and ▲). The pH was always 6.5.

phosphate, the accumulation of serine decreased by 33% (no $\Delta\tilde{\mu}_{Na}$, only $\Delta\psi$). Sodium-dependent transport occurred rapidly over the first 15 s of incubation and was followed by the efflux of serine.

Competition. When either glucose-energized cells or frozen membrane vesicles were incubated with a 70-fold excess of unlabeled threonine, the uptake of labeled serine was completely inhibited. In contrast, unlabeled alanine or leucine did not affect serine uptake (data not shown).

DISCUSSION

No special precautions were taken to eliminate the sodium contamination that is always associated with laboratory glassware, but a simple potassium wash of *S. bovis* cells virtually eliminated the uptake of serine, threonine, and alanine (Fig. 1). Since cells which were incubated in sodium buffers showed rapid rates of amino acid transport, it appeared that sodium was necessary for transport. The sodium dependency was further corroborated by the following observations: (i) glucose-energized and deenergized cells took up serine in the presence of sodium but not potassium (Fig. 3), (ii) nigericin and monensin treatments inhibited sodium-dependent transport (Fig. 2), (iii) sodium-loaded and deenergized cells were unable to transport serine, and (iv) membrane vesicles showed the same dependency for sodium as whole cells did (Fig. 5).

Because deenergized cells which were diluted into sodium transported serine, it appeared that the $\Delta\tilde{\mu}_{Na}$ was a driving force for uptake (Fig. 3). However, the $\Delta\tilde{\mu}_{Na}$ drove serine transport half as fast as did an artificial $\Delta\psi$, and the uptake rate with a $\Delta\psi$ plus a $\Delta\tilde{\mu}_{Na}$ was more than three times that observed with only a $\Delta\tilde{\mu}_{Na}$ (Fig. 3). These observations with whole cells indicated that the $\Delta\psi$ (initially 100 mV) was greater than the $\Delta\tilde{\mu}_{Na}$. Results with membrane vesicles also showed that the $\Delta\psi$ allowed 66% of the accumulation seen with both the $\Delta\psi$ and the $\Delta\tilde{\mu}_{Na}$ (Fig. 5). Direct and accurate calculations of $\Delta\tilde{\mu}_{Na}$ were prevented by the interference of extracellular sodium with intracellular sodium determinations (see below).

S. bovis cells required more than 30 mM sodium for half-maximal rates of serine transport (Fig. 4). This extremely poor affinity for sodium prevented a direct demonstration of simultaneous sodium and serine uptake and a determination of the sodium and serine stoichiometry. The specific activities (approximately 200 μCi/ml, 0.6 μg/ml) of commercial sources of ^{22}Na were not great enough for realistic estimates of transport at low sodium concentrations. Efflux and counterflow experiments were confounded by extracellular or extravesicular contaminations of sodium and the inability of the cells or membrane vesicles to take up labeled amino acids at low concentrations of sodium.

While it was difficult, if not impossible, for us to determine the stoichiometry of sodium and serine transport, a Hill plot indicated that the serine transport system had more than one binding site for sodium. The n_{app} was actually 2.0, but Hill plots often have slopes which are not whole numbers. In these whole-cell experiments, the extracellular concentration of sodium varied from 0 to 100 mM, but this change should not have drastically affected the total driving force across the cell membrane (see above). Based on the Hill derivation, even a 25% increase in the transport rate at high sodium concentrations would have affected the n_{app} less than 6%. Similar experiments were performed with membrane vesicles which had a constant $\Delta\tilde{\mu}_{Na}$ (approximately 100 mV), but the rates of serine uptake were very low at sodium concentrations lower than 40 mM. Low counts prevented a precise estimate of n_{app}, but the calculated value was once again greater than 1 (data not shown).

During the course of our experiments, Hama et al. (12) reported that serine and threonine transport in *E. coli* was coupled to sodium. A striking difference between the *E. coli* system and the one described here is the affinity for sodium. The sodium-dependent transport system of *E. coli* had an affinity constant for sodium of 21 μM. Given the observation that the $\Delta\psi$ was the largest driving force for serine uptake in *S. bovis*, it is difficult to explain the extremely large requirement for sodium. Clearly, only micromolar quantities of sodium should have been required if sodium were only serving as a cosubstrate for transport. Since the transport system exhibited positive cooperativity and appeared to have more than one site for sodium (n_{app}, 2), one could speculate that there is a low-affinity allosteric site for sodium as well as a catalytic site.

The affinity for sodium was extremely poor, but *S. bovis* has evolved in an environment in which sodium concentrations are rarely, if ever, lower than 90 mM. Furthermore, not all amino acid transport systems in *S. bovis* showed a similar sodium dependency. Cells incubated in potassium buffer were able to take up glutamine and leucine at rapid rates (data not shown). The proline and sodium transport system of *E. coli* can be coupled to lithium (4), but in *S. bovis* no uptake was observed when sodium was replaced by lithium. The serine and threonine transport system of *E. coli* showed a similar specificity for sodium (12).

Sodium-dependent amino acid transport has not been previously described for streptococci. In *Streptococcus cremoris*, serine-threonine and glycine-alanine were cotransported with protons (9). A single H^+-amino acid transport system for glycine, alanine, serine, and threonine was observed in *Streptococcus faecalis*, and there was "no evidence for an obligatory involvement" of sodium (2). Reizer and Panos also noted that sodium had no effect on α-aminoisobutyric acid (an alanine analog) in *Streptococcus pyogenes* (23). Sodium even inhibited alanine transport by *S. faecalis* protoplasts (18), and a similar inhibition of α-aminoisobutyric acid transport was observed in *Streptococcus lactis* (30).

Neutral amino acid transport in *S. bovis* was strongly sodium dependent, but this bacterium was able to grow in sodium-deficient medium containing purified amino acids (data not shown). Because *S. bovis* is able to grow with ammonia as its sole nitrogen source (33), neutral amino acid transport is probably not essential for growth. Recently, the importance of peptide transport systems has also been recognized (13, 20, 32), and *S. bovis* grows faster with peptides than with amino acids (5). However, amino acid systems may provide a means of scavenging residual amino nitrogen. Within the rumen, amino acid concentrations are generally low (34), and the affinity constants of *S. bovis* for threonine, serine, and alanine were also low, 8.8 to 6.3 μM. These affinity constants were lower than those reported for *S. cremoris* (9).

The ionophores monensin and nigericin at concentrations greater than 5 μM virtually eliminated serine transport, but nigericin was somewhat more effective at low concentrations (Fig. 2). Nigericin is usually described as a potassium-proton antiporter, but either ionophore can also exchange sodium for protons (21). Monensin caused a decrease in intracellular potassium and pH in *S. bovis*, but, as expected, it had no effect on the $\Delta\psi$ (24). Once the intracellular pH was reversed, there was an increase in sodium. Since there was no external potassium in these ionophore experiments (Fig. 2) and since the downward gradient of potassium was very large, it is likely that the $\Delta\tilde{\mu}_{Na}$ was greatly reversed (higher Na inside). Based on these assumptions, serine and sodium transport had to proceed against an upward sodium gradient after monensin or nigericin treatment. If the reversed $\Delta\tilde{\mu}_{Na}$ were greater than the $\Delta\psi$, transport would cease. A decrease in intracellular pH could also have decreased serine transport, but sodium-loading experiments with valinomycin, an ionophore not capable of translocating protons, indicated that sodium influx was affecting uptake more than proton influx was.

Monensin is commonly used as a feed additive in the beef cattle industry to inhibit the growth of gram-positive bacteria (6, 7), but little is known about its mechanism of action in ruminal bacteria. The expenditure of additional ATP to maintain favorable ion gradients is obviously an important factor limiting the growth of monensin-sensitive species (24), but the effects of this sodium ionophore on sodium-dependent transport systems have not previously been demonstrated. Franklund and Glass (11) noted that sodium stimulated glucose uptake in the ruminal bacterium *Bacteroides succinogenes*; however, they could not demonstrate an absolute dependence of transport on sodium. To our knowledge, the work presented here is the first demonstration of sodium-dependent, monensin-sensitive transport in ruminal bacteria.

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