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Glucose Transport by Mixed Ruminal Bacteria from a Cow

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The glucose transport of mixed ruminal bacteria harvested from a holstein cow fed 5.0 kg of Italian ryegrass and 1.5 kg of flaked corn a day was investigated. The Eadie-Hofstee plot characterized two transport systems: a high-affinity, low-velocity system and a low-affinity, high-velocity system. The former system (K_m = 16 μM; V_{max} = 2.2 nmol/min/mg of protein) is considered dominant under this feeding condition based on the glucose concentration in the rumen (<1 mM). In light of the facts that the protonophore SF6847 and the lipophilic triphenylmethyl phosphonium ion had no effect on the high-affinity system and an artificially generated proton gradient and electrical potential across the cell membrane did not increase glucose transport, a proton motive force is not be involved in the system. On the other hand, from the facts that chlorhexidine inhibited about 90% of the high-affinity system while iodoacetate showed no significant effect, and a high phosphoenolpyruvate-dependent phosphorylation of glucose was actually shown, the phosphoenolpyruvate-dependent phosphotransferase system is considered the main system in the high-affinity system. Moreover, as shown by the facts that harmaline inhibited about 30% of the high-affinity system and the artificially generated sodium gradient across the cell membrane significantly stimulated glucose transport, this system also includes sodium symport to some degree. The high-affinity system was sensitive to a decrease in pH (<6.5) and was inhibited by the presence of sucrose, mannose, and fructose.

The concentration of soluble nutrients in the rumen is usually very low, and the ruminal bacteria, in many cases, should take up the nutrients by using active transport systems which require some kind of energy (36). Transport, the first energy-requiring process, could often be rate limiting in the nutrient metabolism of bacteria (5, 25).

In the study of nutrient transport in the microbial ecosystem, two processes should be considered. The first is to investigate the mechanism and characteristics of transport for individual dominant microbial species; the second is to specify the major transport system in the ecosystem as a whole. A combination of the two processes would enable one to manipulate the ruminal ecosystem through the control of nutrient transport in the ruminal bacteria. Of the carbohydrate transport systems in bacteria, proton symport, sodium symport, primary active transport, group translocation, and facilitated diffusion have been identified (7, 35). There have been many reports about carbohydrate transport systems in some individual species of ruminal bacteria (20), but it is difficult to infer what type of system would actually work and what factors would affect these transport systems in the whole rumen ecosystem under certain specific environmental conditions, since the contribution of each species to the system as a whole is not clear.

Among the carbohydrates, glucose is considered one of the most important energy sources for microorganisms in the rumen because it is a major constituent in plant tissues (40). This importance of glucose is enhanced when cereal grains are added to the diets because starch, which constitutes 50 to 70% of grains (1), is rapidly converted to glucose (14).

This study was conducted in order to investigate the mode and properties of the major transport system of glucose which would be active in mixed bacteria in the rumen of a cow fed a grain-containing forage-based diet.

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MATERIALS AND METHODS

Animal and diet. A ruminally fistulated, nonlactating holstein cow (body weight, 591 kg) was given 5.0 kg of first-cut, early-vegetative Italian ryegrass hay and 1.5 kg of flaked corn per day at 0900. The amount of the diet was equivalent to the calculated maintenance energy requirement (2). The chemical composition of the diet was as follows (in percentages on a dry matter basis): neutral detergent fiber, 49.9; acid detergent fiber, 27.3; starch, 14.1; crude protein, 12.1; ether extract, 2.7; and crude ash, 9.1. Water was freely given.

Preparation of mixed ruminal bacteria. Ruminal contents were taken 2 h after feeding through the fistula by a suction pump for a liquid portion and by hand grasp for a solid portion and were brought back to the laboratory in filled packed vessels. Equal amounts (wt/wt) of these portions were mixed, ground by a homogenizer (NM-2; Nihon Seiki Co., Tokyo, Japan) at 250 W for 1 min, and squeezed through four layers of gauze. The squeezed sample was left undisturbed for 30 min at 38°C, during which the feed particles were floated due to gas production. After the fluid obtained from the middle section of the bottle (about 40% of the total volume) was slowly centrifuged (at 750 × *g* for 10 min at 10°C) in order to remove the protozoa, mixed ruminal bacteria were harvested by another centrifugation (at 10,000 × *g* for 15 min at 10°C) (30, 47). Every procedure was anaerobically performed under an N_2 stream. No protozoa and feed particles were detected in the bacterial fraction by microscopic inspection. A previous study indicated that more than half of the viable bacteria would be derived from the solid portion when these procedures were used (24).

Measurement of glucose transport. The mixed ruminal bacteria were washed twice and resuspended (2 ml) in NKMP buffer (50 mM Na_2HPO_4, 10 mM KH_2PO_4, 5 mM $MgCl_2$, and 3.4 mM cysteine-HCl [pH 6.8]). The cells (0.82 ± 0.05 [standard error {SE}] mg of protein/ml) were anaerobically incubated in 500 μl of NKMP buffer at 38°C under an N_2 stream, and transport was started by the addition of glucose containing D-[U-^{14}C]glucose (0.1 to 0.4 μCi, and 302 μCi/μmol). Samples (100 μl each), which were taken at various time points, were immediately dispersed into 2.0 ml of ice-cold NKMP buffer, filtered through membrane filters (0.45-μm pore size), and washed with 2.0 ml of ice-cold NKMP buffer. The radioactivity of the cells was measured by using a liquid scintillation counter (Tri-Carb 1600TR; Packard Instrument Co., Meriden, Conn.). Because the glucose uptake continued linearly for 1 min, the velocity of the transport was determined from the regression coefficient obtained from the values at 10, 30, and 60 s. In the inhibitor experiments, the cells were incubated with 3,5-di-*tert*-butyl-4-hydroxybenzilidene malononitrile (SF6847), triphenylmethyl phosphonium (TPMP) bromide, iodoacetate, chlorhexidine, or harmaline for 10 min before the initiation of transport.

Artificial membrane potentials. The cells were incubated with 12 mM 2-deoxy-D-glucose (2-DG) for 1 h at 38°C in advance of the creation of the artificial membrane potentials. Acetate-loaded cells (80 mM potassium acetate–10 mM

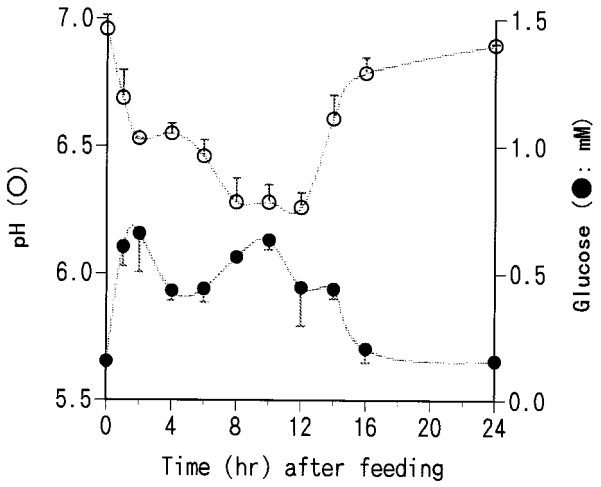

FIG. 1. Diurnal changes in pH and glucose concentration in the rumen of a holstein cow fed 5.0 kg of Italian ryegrass and 1.5 kg of flaked corn a day. The lowest pH value was 6.26, and the highest glucose concentration was 0.66 mM. Vertical bars, SEs.

K_2HPO_4) were diluted 50-fold into 50 mM K_2HPO_4 buffer to generate an artificial proton gradient (ΔpH). An artificial electrical potential ($\Delta\psi$) was created by loading valinomycin (10 μM at 0°C for 30 min)-treated cells with potassium (50 mM K_2HPO_4 buffer) and diluting them 50-fold into 100 mM Bis-Tris buffer (Sigma Chemical Co., St. Louis, Mo.). A chemical sodium gradient (ΔpNa) was generated by a 50-fold dilution of potassium-loading cells (50 mM K_2HPO_4 buffer) into a 50 mM Na_2HPO_4–50 mM K_2HPO_4 buffer. All the above-mentioned buffers were anaerobic buffers including 3.4 mM cysteine-HCl and were adjusted to pH 6.8 with HCl.

Determination of PEP-PTS activity. The phosphoenolpyruvate-dependent phosphotransferase system (PEP-PTS) activity was spectrophotometrically assayed by basically using the method of Kornberg and Reeves (15). Toluenated cells (0.7 mg of protein) treated with 0.1% toluene (i.e., 1% of a toluene-ethanol [1:9, vol/vol] solution) were incubated in 2.0 ml of NKMP buffer with 2 mM PEP, 0.1 mM NADH, and lactate dehydrogenase (activity, 5 μmol/min/ml) (Oriental Yeast, Osaka, Japan) at 38°C. The oxidation of NADH was measured by the decrease in A_{340} after the addition of 0.05 mM glucose in contrast to the control without glucose. The value of $6.22 \cdot mM^{-1} \cdot cm^{-1}$ was used as the NADH extinction coefficient.

Proton motive force determination. Cells were incubated in a buffer (1.0 ml) containing either ^3H_2O (4 μCi), [carboxyl-^{14}C]inulin (0.5 μCi; molecular weight, 5,000 to 5,500; 2.58 μCi/mg), [7-^{14}C]benzoic acid (0.5 μCi; 22 μCi/μmol), or [phenyl-^{3}H]tetraphenyl phosphonium bromide ([^{3}H]TPP$^+$) (0.5 μCi; 37.4 μCi/nmol) for 5 min at 38°C. The cell suspensions were centrifuged (at 10,000 $\times$ *g* for 5 min) through silicon oil (KF-961; Shin-Etsu Chemical Co., Tokyo, Japan). After the supernatant (20 μl) was removed, the remaining sample was frozen and the pellet was removed from the bottom of the tube with dog nail clippers. Supernatant and pellet samples were counted by liquid scintillation. The ΔpH was determined from the uptake of [7-^{14}C]benzoic acid by using the Henderson-Hasselbalch equation. Intracellular volume (2.8 μl/mg of protein) was estimated from the difference between ^3H_2O and [carboxyl-^{14}C]inulin. The $\Delta\psi$ was calculated from the distribution of [^{3}H]TPP$^+$ by using Nernst's equation. Nonspecific binding of [^{3}H]TPP$^+$ was estimated from the cells which were treated with 0.1% toluene (1% of a toluene-ethanol [1:9, vol/vol] solution).

Other analyses. The chemical composition of the feeds was analyzed by the proximate method (3) and detergent analysis (45, 46). Starch in the feeds was determined as the glucose content after the extraction and hydrolysis of starch with 4.6 M perchloric acid following the removal of soluble sugars from the feeds by boiling with 80% ethanol (1). Glucose in the starch hydrolysate was measured by the colorimetric method with glucose oxidase, peroxidase, and 2,2′-azino-di-(3-ethylbenzthiazoline)-6-sulfonate (Boehringer Mannheim) (16). The glucose concentration in the rumen was determined as follows. After centrifugation (10,000 $\times$ *g* for 10 min at 4°C) the supernatant of the ruminal fluid was deproteinized with 80% ethanol, dried by a centrifugal concentrator (VC-960; TAITEC Corp., Saitama, Japan), and diluted in distilled water. Glucose in the solution was measured by a high-pressure liquid chromatograph equipped with a pulsed electrochemical detector (DX-300 system; Dionex, Sunnyvale, Calif.) and a pellicular anion exchange column (CarboPac PA1; Dionex) (11). Sodium hydroxide solution (16 mM) was used as the eluent, and 2-DG was used as the internal standard. Protein content in the mixed ruminal bacteria was measured by the method of Lowry et al. (17) and was 192 μg/ml at an optical density (at 600 nm) of 1.0. Intracellular sodium was measured with an atomic absorption spectrophotometer (Z-8000; Hitachi Ltd., Tokyo, Japan) after the cells were centrifuged (10,000 $\times$ *g* for 5 min) through silicon oil (KF-961; Shin-Etsu Chemical Co.) and dissolved in 3 N HNO_3 for 24 h. Values were corrected for extracellular contamination. Intracellular ATP was measured with a luminometer (Model 1250; LKB-Pharmacia, Turku, Finland) by using a luciferin-luciferase mix (Sigma Chemical Co.) after extraction with 14% ice-cold perchloric acid (42).

The difference between two averages was tested using the Student *t* test (41). Every measurement was repeated at least three times.

Materials. Radiolabelled glucose was supplied by Amersham, Buckinghamshire, England. All other radiolabelled chemicals were supplied by NEN, Wilmington, Del. SF6847 and TPMP Br were obtained from Wako Pure Chemicals, Osaka, Japan, and Nakalai Tesque, Kyoto, Japan, respectively. Chlorhexidine and harmaline were obtained from Sigma Chemical Co.

RESULTS

The diurnal changes in pH and glucose concentration in the rumen are shown in Fig. 1. The ruminal glucose concentration was always below 0.7 mM under this feeding condition. The Eadie-Hofstee plot of glucose uptake by the ruminal bacteria is shown in Fig. 2, and this plot characterized two obviously different transport systems: a high-affinity, low-velocity system and a low-affinity, high-velocity system, which bordered at about 1 mM glucose. The high-affinity system showed a K_m value of 16 $\pm$ 4.2 (SE) μM and a V_{max} value of 2.2 $\pm$ 0.5 (SE) nmol/min/mg of protein. The low-affinity system likely had values higher than 5 mM and 10 nmol/min/mg of protein as the K_m and V_{max}, respectively, estimated from the Eadie-Hofstee plot at glucose concentrations below 5 mM. These kinetic constants would be difficult to determine correctly at higher concentrations because of isotope dilution.

The effects of inhibitors on glucose uptake in the presence of 0.05 mM glucose are shown in Table 1. The rate of glucose uptake by the ruminal bacteria was 1.8 $\pm$ 0.4 (SE) nmol/min/mg of protein when 0.05 mM glucose was added. Both SF6847, the proton-conducting uncoupler (13), and TPMP$^+$, the lipophilic ion which dissipates the membrane potential (33), showed no effect on glucose transport in spite of their actual inhibitory effects on ZΔpH and $\Delta\psi$. Higher and lower concentrations of SF6847 (100 μM and 1 μM) and TPMP$^+$ (10 mM and 0.1 mM) had no significant effect on glucose transport (data not shown). Iodoacetate, which inhibited about 80% of ATP generation in the cells, also showed no effect on glucose transport. On the other hand, chlorhexidine, an inhibitor of the PEP-PTS (19), showed a 90% inhibitory effect on glucose uptake. Harmaline, which is known as an inhibitor of the sodium-dependent transport system (4, 39), also showed an inhibitory

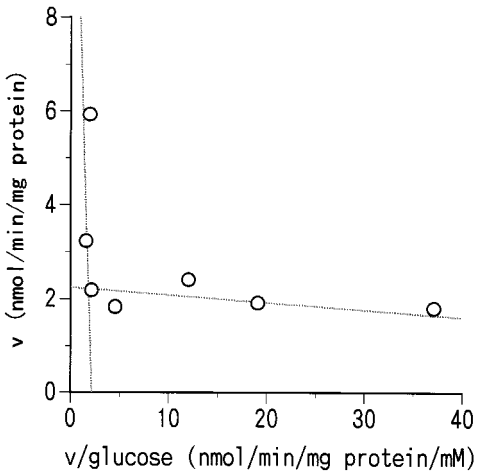

FIG. 2. Eadie-Hofstee plot of glucose transport by mixed ruminal bacteria.

TABLE 1. Effect of inhibitors on glucose transport

Inhibitor[b]	Inhibition[a] (%)
SF6847[c] (10 μM)	0
TPMP Br[d] (1 mM)	0
Iodoacetate[e] (500 μM)	6
Chlorhexidine (200 μM)	88**
Harmaline (4 mM)	31*

[a] * and **, significant inhibitions ($P < 0.05$ and $P < 0.01$, respectively).
[b] Inhibitors were added 10 min prior to glucose (0.05 mM) addition. All inhibitors except iodoacetate were dissolved in ethanol, and the final concentration of ethanol was 1 to 4% (vol/vol). Ethanol (4%) showed no significant effect (<3%) on glucose transport.
[c] SF6847 inhibited ZΔpH generation by 69% (control = 15 mV).
[d] TPMP Br inhibited Δψ generation by 60% (control = 144 mV).
[e] Iodoacetate inhibited intracellular ATP generation by 77% (control = 2.2 mM).

effect, which was weak but significant. When 5 mM glucose was added, none of the inhibitors noted above showed a significant inhibitory effect on glucose uptake (data not shown).

The effects of various artificial potentials on glucose transport are shown in Table 2. Even when 50 mV more ZΔpH or 100 mV more Δψ was created, glucose uptakes were almost the same as with the control treatment. With the artificial sodium gradient, on the other hand, a significantly higher uptake of glucose occurred compared with the control treatment.

The PEP-dependent oxidation of NADH with lactate dehydrogenase as an index of PEP-PTS activity is shown in Fig. 3. The oxidation of NADH occurred at the rate of 2.0 mol/min/mg of protein, and this oxidation was largely inhibited (>90%) by 200 μM chlorhexidine. Chlorhexidine, on the other hand, did not show any inhibitory effect on lactate dehydrogenase activity, which was measured by the oxidation of NADH when pyruvate was used as the substrate (data not shown).

The effect of pH of the buffer on glucose transport in the presence of 0.05 mM glucose is shown in Fig. 4. Glucose uptake decreased significantly under pH 6.5, and the uptake at pH 5.5 was lower than half the value at pH 6.5.

The effects of the presence of various sugars (0.10 mM) on the uptake of glucose (0.05 mM) are shown in Table 3. Glucose itself showed about a 60% inhibitory effect, which is almost the same as the theoretical value. The effect of sucrose was similar to that of glucose. Mannose and fructose also showed significant inhibitory effects, although the effects were about half that of glucose.

DISCUSSION

The Eadie-Hofstee plot of glucose uptake by the mixed ruminal bacteria apparently characterized two transport systems. Under the feeding conditions of this study, in which the ruminal glucose concentration was always low, below 1 mM, the high-affinity system is considered dominant for glucose transport by ruminal bacteria because the low-affinity system would rapidly decrease as glucose concentration decreased while the capacity of the high-affinity system is relatively constant.

TABLE 2. Effect of artificial potentials on glucose transport

Potential	Glucose transport[a] (nmol/min/mg of protein)
Control[b]	0.35
ZΔpH (39 mV)	0.34
Δψ (118 mV)	0.34
ZΔpNa (63 mV)	0.75**

[a] The glucose concentration in the buffer was 0.05 mM. **, significant promotion of glucose transport ($P < 0.01$).
[b] ZΔpH = −15 mV; Δψ = 19 mV.

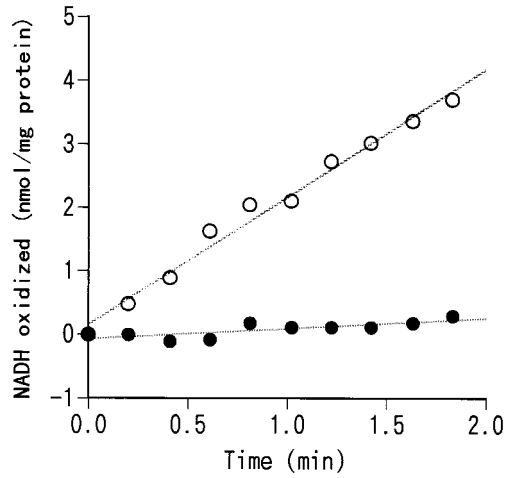

FIG. 3. PTS-dependent NADH oxidation catalyzed by lactate dehydrogenase with (●) or without (○) chlorhexidine (200 μM) in the presence of 0.05 mM glucose.

The absence of any effects due to the protonophore and the lipophilic ion on glucose transport in the presence of 0.05 mM glucose, which represents the high-affinity system, suggests that a proton motive force is not involved in the system. This suggestion was also supported by the fact that an artificial proton gradient and electrical membrane potential did not promote glucose uptake.

The strong inhibitory effect of chlorhexidine suggests that the PEP-PTS appears to be involved in the glucose transport system, although it was doubted that chlorhexidine was a "specific" inhibitor of the PEP-PTS (22, 23). The fact, however, that the PEP-dependent oxidation of NADH by lactate dehydrogenase, which was mostly inhibited by chlorhexidine, was

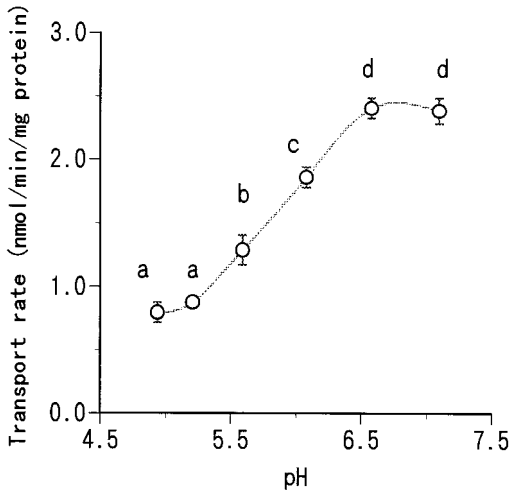

FIG. 4. Effect of pH on glucose transport by mixed ruminal bacteria. Cells were incubated in a buffer containing 25 mM Na_2HPO_4, 5 mM KH_2PO_4, and 50 mM MES (morpholineethanesulfonic acid) with 0.05 mM glucose. Circles with different letters differ significantly. Vertical bars, SEs.

TABLE 3. Effects of various sugars on glucose transport

Sugar[b]	Inhibition of glucose transport (%)[a]
Glucose	64**
Galactose	13
Mannose	31**
Fructose	28*
Xylose	0
Arabinose	0
Maltose	13
Cellobiose	9
Lactose	7
Sucrose	57**

[a] * and **, significant inhibitions ($P < 0.05$ and $P < 0.01$, respectively).
[b] Each sugar (0.10 mM) was added to a buffer containing 0.05 mM glucose.

almost equivalent to glucose transport by the intact cells (2.0 versus 1.8 nmol/min/mg of protein at 0.05 mM glucose) suggests that the PEP-PTS is indeed the major transport system for glucose.

On the other hand, harmaline also inhibited glucose transport, less effectively but significantly, and the artificial sodium gradient also promoted glucose uptake. These findings suggest that the high-affinity system may include a sodium symport.

Iodoacetate, an inhibitor of glyceraldehyde-3-phosphate dehydrogenase, did not affect the high-affinity transport system in spite of its highly inhibitory effect on ATP production. This suggests that the ATP-dependent transport system does not have much importance in glucose transport in the rumen. The de-energizing of the cells with 2-DG decreased glucose transport activity by 80%, as estimated from the difference between the nonenergized cells and the control treatment in Table 2 (1.8 versus 0.35 nmol/min/mg of protein). This decrease could be attributed to the inhibitory effect of 2-DG on PEP-PTS activities (6). Preliminary experiments indicated that cells of mixed ruminal bacteria treated with 2-DG (12 mM) for 1 h could create only 15% of the amount of intracellular ATP created by untreated cells in the presence of 0.05 mM glucose. The degree of de-energization (85%) was kept unchanged even when the cells were incubated for 8 h with 2-DG. The remaining activity of glucose transport (about 20%) after 2-DG treatment suggests that other systems may work by using the retained energy which could not be inhibited by 2-DG.

Some grain was added to a forage-based diet in this study because grains are now generally used as feeds for beef and dairy cattle in Japan and the United States (28, 44). The glucose concentration in the rumen, however, is still kept at a low level under this condition, which would make for a highly competitive environment for the acquisition of this substrate. The PEP-PTS is known to be energetically efficient because of the simultaneous uptake and phosphorylation of 1 mol of sugars during the consumption of 1 mol of PEP (12, 35). Among the ruminal bacteria, some of the predominant sugar- and starch-utilizing bacteria, i.e., *Streptococcus bovis*, *Selenomonas ruminantium*, and *Megasphaera elsdenii*, have the PEP-PTS for glucose transport (21–23). The highly competitive environment for glucose may have facilitated the evolution of the PEP-PTS in ruminal bacteria.

A fibrolytic ruminal bacterium, *Fibrobacter succinogenes*, was reported to have a sodium symport system for glucose (9). In such species capable of using various sugars, glucose utilization may occasionally occur during the process of fiber degradation. It does not seem necessary for these bacteria to possess the PEP-PTS, an efficient but very large system comprising three to four components with molecular sizes of more than 100 kDa (7, 32). On the other hand, the sodium symport system, which has a smaller molecular size of about 50 kDa (18), seems to be a convenient system for the occasional use of a substrate. The sodium gradient across the cell membrane is a readily available force because cells usually maintain a low intracellular concentration of sodium, in contrast to potassium, which has a high internal concentration (12).

As regards the production of the sodium gradient, a sodium/proton antiport has been reported in many bacteria (31). The activity of this antiport, however, may not be indispensable for keeping a sodium gradient in ruminal bacteria, because proton-conducting uncouplers did not affect glucose transport. *S. bovis* was reported to have a sodium pump (42), and some primary system may also be involved in sodium extrusion from the cells of mixed ruminal bacteria.

It has been reported that the activity of the PEP-PTS decreased as glucose concentration increased and as the medium pH decreased for *Streptococcus mutans* (8, 10) and *S. bovis* (29). In the present study, the high-affinity system also decreased as the pH in the buffer decreased. Feeding cattle high-grain diets would decrease the activity of this system because such diets usually make the ruminal pH lower. The glucose concentration in the rumen seems to be kept at a low level (<1 mM) even when some grains are used as feeds (38, 43), but are expected to increase sometimes when high-grain diets are given (37, 38). At that time, the major transport system for glucose in the rumen would shift from the high-affinity, low-velocity system to the low-affinity, high-velocity system. This shift means a change in the strategy of glucose utilization from high efficiency to high velocity on those diets. The low-affinity, high-velocity transport system would probably include facilitated diffusion, which was reported in *S. bovis* (34), because the glucose transport system of mixed ruminal bacteria was not inhibited by any of the inhibitors used in this experiment when 5 mM glucose was added. Bacteria having such a high-velocity system would be advantageous when high-grain diets are given. Even when a low-grain diet is given, the glucose concentration in a microenvironment may be high enough for the low-affinity system because amylolytic bacteria tend to attach to starch granules (26, 27). Moreover, monosaccharides, like glucose, may not be the only form of carbohydrate transported by ruminal bacteria. The transport of oligomeric forms of carbohydrates requires further investigation.

In this study, sucrose, fructose, and mannose inhibited glucose transport by the high-affinity, low-velocity system. Glucose, fructose, and sucrose are the major components of the soluble carbohydrates in plant tissues (40). It seems reasonable that bacteria capable of utilizing these sugars could use common components of the PEP-PTS for the transport of these different sugars, considering the large size of this system. Mannose also competitively inhibited glucokinase activity in *S. ruminantium* (21). This sugar, however, is a minor component in the soluble fraction of plants, although it is definitely included in the cell wall fraction as mannan. The reason mannose affects glucose transport, other than an analogous chemical structure, is not clear. Among the three species of ruminal bacteria having the PEP-PTS as noted above, *S. bovis* and *S. ruminantium* can utilize all these sugars and *M. elsdenii* can utilize sucrose and fructose (14), which suggests that these bacteria play important roles in glucose transport in the whole rumen. Other bacteria, however, might be involved, in light of the fact that sucrose did not inhibit the glucose PEP-PTS for *S. ruminantium* HD4 (23) and *S. bovis* JB1 (22).

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