Ruminants can utilize structural carbohydrates in plant tissues, including cellulose and hemicellulose, through microbial activities in the rumen, although these components cannot be degraded by mammalian digestive enzymes. Cellobiose, one of the major products of cellulose degradation (38), can be metabolized by many cellulolytic and noncellulolytic species of ruminal bacteria (14, 32, 44). Transport of a nutritional substrate, the first energy-requiring process in a substrate-limiting environment, can often be rate limiting in the nutrient metabolisms of bacteria (7, 26). In order to understand the final steps in fiber utilization, it is necessary to investigate the dominant cellobiose transport system in the rumen as a whole. There are many reports on the carbohydrate transport systems of each species of ruminal bacterium (24), but only a few systems (i.e., Fibrobacter succinogenes [22], Ruminococcus flavefaciens [12], and Streptococcus bovis [25]) have been studied with regard to cellobiose transport. These studies have shown that the phosphoenolpyruvate-dependent phosphotransferase system (PEP-PTS) and active transport systems can be used for the cellular uptake of cellobiose. In addition to the problem of the scarcity of studies, there is the difficulty of accurately estimating the dominant transport systems in the rumen from the results of transport studies of each species, because it has been a long time since the first isolation of many of the ruminal type strains (4, 14) and also because it is difficult to determine the actual population size of a specific strain in the rumen. It is not clear that cellobiose indeed can be transported into the ruminal microbial cells in its intact form, since β-glucosidase activities have been found in some ruminal bacteria (5, 35, 52) as well as in the fluids of actual and artificial rumens (8, 27, 51). Our study determined the relative contribution of cellobiose transport in the apparent utilization of cellobiose and characterized the major cellobiose transport systems in the rumen as a whole by using mixed ruminal bacteria taken from a cow fed a forage diet.

**Materials and Methods**

**Animal and Diet.** A ruminally fistulated, nonlactating holstein cow (body weight, 470 kg) was fed 6.0 kg of first-cut, prebloom Italian ryegrass hay every day at 9:00 a.m., which was equivalent to the energy requirement for maintenance (28). The chemical composition of the hay (on a dry-matter basis), analyzed by the proximate method (10) and detergent analysis (48, 49), was as follows: neutral detergent fiber, 56.2%; acid detergent fiber, 31.1%; crude protein, 15.4%; ether extract, 3.3%; and crude ash, 10.5%. Water was freely given.

**Preparation of Mixed Ruminal Bacteria.** Liquid and solid portions of the ruminal content, taken by suction pump and by hand grasp, respectively, were taken 2 h after feeding the cow through a fistula. Equal amounts (wt/wt) of these portions were mixed, ground with a homogenizer (model MN-2; Nihon Seiki Co., Tokyo, Japan) at 250 W for 1 min, and squeezed through four-layered gauze. The squeezed fluid was left undisturbed for 30 min at 38°C to separate the feed particles (53). The fluid obtained from a middle portion of the undisturbed sample was slowly centrifuged (at 750 × g for 10 min at 10°C) to remove protozoa (32) and then centrifuged again (at 10,000 × g for 15 min at 10°C) to harvest the mixed ruminal bacteria, in which no protozoa were microscopically detected. Anaerobic conditions were maintained through the whole procedure by using an N₂ gas stream.

**Measurements of Cellobiose and Glucose Transports.** The mixed ruminal bacteria were washed twice and resuspended in NKMP buffer (15). The transport was initiated by the addition of cellobiose or glucose containing [3H]cellobiose (329 MBq/μmol) or [β-3H]glucose (11.2 MBq/μmol), respectively, to the cell suspension. The reaction was terminated by dispersing the suspension (100 μl) into ice-cold NKMP buffer (2.0 ml). After the cell suspension was sent through a membrane filter (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 166TR; Packard Instrument Co., Meriden, Conn.). Transport rates were calculated from the difference between the levels of uptake at 38 and 0°C at 60 s, which nearly matched the result obtained with a regression coefficient from the values at 10, 30, and 60 s at 38°C. In the inhibitor experiments, the cells were incubated with 3.5-di-tert-butyl-4-hydroxybenzilidene-malononitrile (SF6847), triphenylmethyl phosphonium (TPMP) bromide, iodacetate, chlorohexidine, and/or harmaline for 10 min prior to the addition of cellobiose (0.01 mM). Some inhibitors were dissolved in ethanol (up to 4% in final concentration), which exhibited no significant effect on cellobiose uptake.

**Determinations of PEP-PTS and Cellobiase Activities.** The activity of the PEP-PTS in the presence of 0.01 mM cellobiose was assayed spectrophotometrically (15) and contrasted with the activity in the reaction mixture without cellobiose. The cell-associated cellobiase activity was measured by examining the generation of glucose from cellobiose in NKMP buffer at 38°C by the washed cells for 1 min. Both chlorohexidine (200 μM) and iodacetate (2 mM) were added to the buffer 10 min prior to the addition of cellobiose in order to inhibit the uptake of cellobiose (as shown in Results) and glucose (15) into the cells. The extracellular cellobiose in the rumen was estimated by comparing the activities of a centrifuged ruminal fluid (at 10,000 × g for 15 min) to those of the cell fraction. Glucose in NKMP buffer with the washed cells was measured spectrophotometrically by an enzymatic method using hexokinase and glucose-6-phosphate dehydrogenase (18), but glucose and other sugars in the ruminal fluid were analyzed...
with a high-performance liquid chromatograph (HPLC) equipped with a pulsed electrochemical detector and a pellicular anion-exchange column as described previously (15), because some colored elements in the ruminal fluid could have disturbed the spectrophotometric assays.

Formation of artificial membrane potentials and determination of PMF. The artificial potentials were generated as described previously (15). An artificial proton gradient (change in pH [ΔpH]) was created by diluting the acetate-loaded cells in a potassium buffer. An artificial electrical potential (Δφ) was generated by loading the valinomycin-treated cells with potassium and diluting them 50-fold in a bis-Tris buffer. A chemical sodium gradient (ΔNa) was created by diluting the potassium-loaded cells 50-fold in a sodium-potassium buffer. The proton motive force (PMF) was also determined as described previously (15). After the cells were incubated with H_3O^+, [carboxyl-14C]inulin, [7,14C]benzoic acid, or [phenyl-3H]tetraphenyl phosphonium ([3H]TPP) bromide, the radioactivities of the cells and extracellular water, being separated from each other by centrifugation through a silicon oil (a mixture of KF-961 and CH510; Shin-Etsu Chemical Co. and Toray Dow Corning, respectively, both in Tokyo, Japan), were counted by liquid scintillation. The intracellular volume (2.7 μl/mg of protein) was estimated by measuring the difference between levels of 3H_2O and [carboxyl-14C]inulin. The Δφ and ΔpH were calculated by determining the levels of uptake of [7,14C]benzoic acid and [3H]TPP by using the Henderson-Hasselbalch and the Nernst equations, respectively. Nonspecific binding of [3H]TPP was calculated from examining the cells treated with 0.1% toluene.

Other analyses. The protein content in the mixed ruminal bacteria was measured by the method of Lowry et al. (21); it was 166 μg/ml of the suspension when the solution's optical density at 600 nm was 1.0. Intracellular sodium was measured as described previously (15) with an atomic absorption spectrophotometer (model Z-8000; Hitachi Ltd., Tokyo, Japan) after a correction for extracellular contamination. Intracellular ATP was measured with a luminometer (model Z-8000; Hitachi Ltd., Tokyo, Japan) with a correction for extracellular contamination. Artificial electrical potentials were generated as described previously (15). After the cells were incubated with 3H_2O, [carboxyl-14C]inulin, [7,14C]benzoic acid, or [phenyl-3H]tetraphenyl phosphonium ([3H]TPP) bromide, the radioactivities of the cells and extracellular water, being separated from each other by centrifugation through a silicon oil (a mixture of KF-961 and CH510; Shin-Etsu Chemical Co. and Toray Dow Corning, respectively, both in Tokyo, Japan), were counted by liquid scintillation. The intracellular volume (2.7 μl/mg of protein) was estimated by measuring the difference between levels of 3H_2O and [carboxyl-14C]inulin. The Δφ and ΔpH were calculated by determining the levels of uptake of [7,14C]benzoic acid and [3H]TPP by using the Henderson-Hasselbalch and the Nernst equations, respectively. Nonspecific binding of [3H]TPP was calculated from examining the cells treated with 0.1% toluene.

RESULTS

Monosaccharides were detected in the rumen in concentrations up to 0.49, 0.02, 0.02, and 0.12 mM for glucose, galactose, arabinose, and xylose, respectively, but cellobiose could not be detected at any time under these feeding conditions. The lower limits obtained by HPLC analysis of sugars in the ruminal fluid were 0.01 mM for the monosaccharides and 0.02 mM for cellobiose because of a high background noise. The diurnal fluctuation of the ruminal pH ranged between 6.3 and 7.2 (data not shown).

Values for kinetic parameters of the cell-associated cellobiase of the ruminal bacteria were 0.25 mM for the K_m and 26 nmol/min/mg of protein for the V_max. The extracellular cellobiase activity in the ruminal fluid was estimated to have a K_m of 1.0 mM and a V_max of 23 nmol/min/mg of protein in the corresponding bacterial cells. Several chemicals known to be β-glucosidase inhibitors (9, 30, 33) were examined for their effects on cell-associated cellobiase activity in the presence of 0.1 mM cellobiose. The cellobiase activity was almost completely inhibited (>95%) by 10 and 100 μM nojirimycin, and a similar degree of inhibition was also shown at a lower concentration of cellobiose (0.02 mM) with 10 μM nojirimycin. Glucocone-δ-lactone (10 and 100 μM), on the other hand, showed only a partial inhibition (40 to 60%), and iodoacetamide (10 and 100 μM) did not significantly suppress (<10%) the cellobiase activities of the ruminal bacteria (data not shown).

Figure 1 shows the Eadie-Hofstee plots of levels of uptake of cellobiose with and without 20 μM nojirimycin and of glucose into the cells at various substrate concentrations. The levels of uptake of cellobiose without nojirimycin and of glucose showed biphasic kinetics indicative of high-affinity–low-velocity and low-affinity–high-velocity systems. The high-affinity system for cellobiose and glucose uptake showed 7.4 and 20 μM for the K_m and 1.2 and 2.1 nmol/min/mg of protein for the V_max, respectively. The kinetic constants of the low-affinity system were difficult to correctly determine because of isotope dilution, but they likely were 1 to 3 mM (for cellobiose) or 3 to 5 mM (for glucose) for the K_m and more than 10 nmol/min/mg of protein for the V_max for both sugars. The uptake of cellobiose in the presence of nojirimycin, on the other hand, showed only one system, having a K_m of 14 μM and a V_max of 1.0 nmol/min/mg of protein.

The effects of inhibitors on cellobiose uptake with 20 μM nojirimycin are shown in Table 1. The table also shows the bioenergetic properties of the cells in the presence of the inhibitors. A proton-conducting uncoupler, SF6847 (13), and harmaline, which is known to be an inhibitor of sodium-dependent transport systems (6, 42), did not affect cellobiose uptake. Harmaline did not change any of the bioenergetic properties, while SF6847 decreased the ΔpH by about 80% compared with that of the control. A lipophilic ion, TPMP³⁻, which is known to dissipate the membrane potential (37),

TABLE 1. Effects of various inhibitors on cellobiose transport and bioenergetic properties of the cells

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Inhibition of cellobiose transport (%a)</th>
<th>ΔpH (mV)b</th>
<th>Δφ (mV)b</th>
<th>Intracellular ATP (mM)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>No inhibitor</td>
<td>0</td>
<td>0</td>
<td>17</td>
<td>102</td>
<td>0.78</td>
</tr>
<tr>
<td>SF6847</td>
<td>10</td>
<td>0</td>
<td>3</td>
<td>105</td>
<td>0.75</td>
</tr>
<tr>
<td>TPMP bromide</td>
<td>1</td>
<td>8</td>
<td>14</td>
<td>78**</td>
<td>0.78</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>10</td>
<td>25**</td>
<td>17</td>
<td>45**</td>
<td>0.45**</td>
</tr>
<tr>
<td>Chlorohexidine</td>
<td>1</td>
<td>43**</td>
<td>5**</td>
<td>110</td>
<td>0.25**</td>
</tr>
<tr>
<td>Harmaline</td>
<td>2</td>
<td>50**</td>
<td>98</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>Chlorohexidine + Iodoacetate</td>
<td>2</td>
<td>97**</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* In the presence of 0.01 mM cellobiose and 20 μM nojirimycin-1-sulfonate. ** and *** indicate significant inhibition (P < 0.05 and 0.01, respectively). ND, not determined.
showed no effect on cellobiose uptake at 1 mM, but it significantly decreased the uptake at 10 mM. The $\Delta \psi$ decreased significantly with both 1 and 10 mM TPMP bromide, by 23 and 55%, respectively, but only with 10 mM TPMP bromide was there a significant decrease in the intracellular ATP (by 42%) compared with the level in the control. Iodoacetate, an inhibitor of glyceraldehyde-3-phosphate dehydrogenase (17), showed a significant inhibition of cellobiose uptake when 2 mM was added, and it also showed a slight but significant inhibition when 500 $\mu$M was added. Generation of intracellular ATP was inhibited by 68 and 28% when 2 mM and 500 $\mu$M iodoacetate were added, respectively. Iodoacetate also significantly decreased the $\Delta$H at both concentrations. Chlorhexidine, an inhibitor of the PEP-PTS (23), showed a more than 80% inhibitory effect on cellobiose uptake, and it almost completely inhibited uptake when it was added with 2 mM iodoacetate.

The effects of various artificial potentials on cellobiose uptake in the presence of 20 $\mu$M nojirimycin were investigated. However, none of the artificially generated $Z\Delta$H (45 mV), $\Delta \psi$ (93 mV), or $Z\Delta$pNa (61 mV) showed significant promotion of cellobiose uptake compared with that of the control, which had no artificial potentials (data not shown).

The PEP-dependent oxidation of NADH, an index of the PEP-PTS, with 20 $\mu$M nojirimycin occurred at the rate of 0.35 nmol/min/mg of protein, while no oxidation was shown in the presence of 200 $\mu$M nojirimycin and those of the cellobiases. When the cellobiose concentration was below 0.02 mM, more than 20% of the cellobiose could be incorporated into the cells in its intact form by the real cellobiose transporter.

In the inhibitor experiment, chlorhexidine showed the strongest inhibitory effect on cellobiose uptake, which suggests that the PEP-PTS is the most direct source of ATP generation in the rumen. Although chlorhexidine possibly affects some properties other than the PEP-PTS (1, 25), no effect on the bioenergetic properties (i.e., intracellular ATP and PMF) was detected for the mixed ruminal bacteria in this study. Moreover, the PEP-dependent oxidation of NADH, which showed a rate similar to that of cellobiose transport (0.35 versus 0.41 nmol/min/mg of protein) and was mostly inhibited by chlorhexidine, also supports the inclusion of the PEP-PTS in cellobiose transport. Other than chlorhexidine, iodoacetate and a higher concentration of TPMP bromide (10 mM) significantly inhibited cellobiose transport. Since intracellular ATP generation was the common property inhibited by these inhibi-

**FIG. 2.** Effect of pH on cellobiose transport by mixed ruminal bacteria. Cells were incubated in the presence of cellobiose (0.01 mM) and nojirimycin-1-sulfate (20 $\mu$M) in buffers containing 25 mM Na$_2$HPO$_4$ and 50 mM MES (2-[N-morpholino]ethanesulfonic acid), which were adjusted at various pH levels. Circles with different letters indicate significant differences ($P < 0.05$).
An ATP-dependent active transport system for uptake of cellobiose has been reported in several genetic studies of nonruminant species such as Escherichia coli (34), Bacillus stearothermophilus (19), and Erwinia spp. (3, 11). The cellobiose PEP-PTS has been reported only in S. bovis (25), and no ATP-dependent transport system which was also independent of PMF has been found so far. On the other hand, the PMF seems to be concerned little with cellobiose transport, because transport was not affected by the addition of SF6847 and TPMP bromide (1 mM), in the presence of which significant reductions in the ΔpH and Δφ, respectively, occurred without there being a decline in ATP production. Furthermore, the absence of PMF involvement in cellobiose transport by the mixed ruminal bacteria is supported by the fact that neither artificially generated ΔpH nor Δφ promoted cellobiose transport. The sodium symport system is not thought to be concerned with cellobiose transport either, because cellobiose uptake was not affected by harmaline and also not promoted by an artificial ΔNa at all.

The cellobiose PEP-PTS has been reported in several genetic studies of nonruminant species such as Escherichia coli (34), Bacillus stearothermophilus (19), and Erwinia spp. (3, 11). An ATP-dependent active transport system of cellobiose has also been observed in some clostridia (29, 31, 45). For ruminal species, however, the PEP-PTS has been shown only in S. bovis (25), and no ATP-dependent transport system which was also independent of PMF has been found so far. On the other hand, inhibitor experiments with R. flavefaciens and F. succinogenes showed that these species had some active transport in which both PMF and ATP were involved (12, 22). The presence of a sodium symport system with cellobiose was also suggested for F. succinogenes (22). Because more than 25 species can ferment cellobiose in the rumen (14, 32, 44), other ruminal bacteria not yet investigated may use the PEP-PTS and ATP-dependent active transport system for uptake of cellobiose. Similarly, PMF-dependent active transport and sodium symport systems would not have much significance in cellobiose utilization among many cellobiose-utilizing species in the whole rumen.

Cellobiose transport is regarded as energetically more efficient than glucose transport. When we consider the PEP-PTS, 1 mol of PEP and 1 mol of ATP are needed to produce 2 mol of glucose phosphate from 1 mol of cellobiose when cellobiose is transported by the cellobiose PEP-PTS, while 2 mol of PEP is required when cellobiose is extracellularly hydrolyzed and transported by the glucose PEP-PTS. When PEP and ATP are compared, the change in Gibbs free energy of PEP to pyruvate is higher than that of ATP to ADP (−62 versus −35 kJ/mol at pH 7.0), and only 2 mol of PEP is generated from 1 mol of hexose via the Embden-Meyerhof-Parnas pathway. On the other hand, if we assume that cellobiose and glucose are transported by active transport systems, intracellular phosphorylation by cellobiose phosphorylase, which has been observed in several ruminal bacteria (2, 47, 50), may conserve ATP compared with the ATP conserved after phosphorylation of two glucose molecules by glucokinase (41). A higher growth efficiency of cellobiose-grown cells than that of glucose-grown cells was actually observed for Ruminococcus albus (47), which might be attributed to a lower energy requirement of the substrate transporter. Such an efficient transport of cellobiose would be more beneficial when the cellobiose concentration is low, when the competition for this substrate might be intense. This conclusion is consistent with there being a higher proportion of true cellobiose transport in the apparent utilization of cellobiose when there is a low level of cellobiose, as shown in this study. When a substrate is abundant, however, the decisive quality of its transport system may shift from efficiency to velocity. Since glucose transport has a high-velocity system in the rumen, presumably a facilitated diffusion (15), transport of glucose after the extracellular degradation of cellobiose may be advantageous when the cellobiose concentration is high.

In our study, cellobiose transport activity decreased as the medium pH declined. A decline in ruminal pH usually occurs when an animal is fed a diet rich in soluble carbohydrates and starch (14), when the ruminal microbes possibly access a sufficient amount of soluble sugars other than cellobiose. Although some celluloslytic species show a preference for cellobiose over other sugars (12, 47), these bacteria could hardly survive a low ruminal pH (40). Cellobiose transport in the rumen, therefore, seems to have relatively little significance when the pH is low, although it is not certain that the proportion of true cellobiose transport indeed decreases at a low ruminal pH, because β-glucosidase activity in the rumen also decreases at a pH below 5.5 (8).

Cellobiose uptake was inhibited by glucose in this study. The inhibitory effect of glucose on cellobiose transport was also shown for R. flavefaciens (12). Since the dominant transport system for both glucose and cellobiose in the mixed ruminal bacteria was the PEP-PTS, the inhibition might be due to competition for the same component of the system (presumably enzyme II) (36). No sugar other than glucose, however, inhibited cellobiose uptake in this study, although sucrose and maltose inhibit cellobiose utilization by catabolite regulatory mechanisms in several noncellulosytic ruminal bacteria (39). To understand the reason for this discrepancy, further studies of the other species capable of fermenting cellobiose are required. In addition, since transport and utilization of cellobextrins (i.e., cellotriose, cellotetraose, and cellopentaose) were observed in R. albus (20) and Clostridium thermocellum (45), further investigation is also needed to clarify the presence and extent of cellobextrin transport in the rumen.

REFERENCES