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# Modulation of urea transport across sheep rumen epithelium in vitro by SCFA and $CO_2$

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**Abdoun K, Stumpff F, Rabbani I, Martens H.** Modulation of urea transport across sheep rumen epithelium in vitro by SCFA and $CO_2$. *Am J Physiol Gastrointest Liver Physiol* 298: G190–G202, 2010. First published November 19, 2009; doi:10.1152/ajpgi.00216.2009.—Urea transport across the gastrointestinal tract involves transporters of the urea transporter-B group, the regulation of which is poorly understood. The classical stimulatory effect of $CO_2$ and the effect of short-chain fatty acids (SCFA) on the ruminal recycling of urea were investigated by using Ussing chamber and microelectrode techniques with isolated ruminal epithelium of sheep. The flux of urea was found to be phloretin sensitive and passive. At a luminal pH of 6.4, but not at 7.4, the addition of SCFA (40 mmol/l) or $CO_2/HCO_3^-$ (10% and 25 mmol/l) led to a fourfold increase in urea flux. The stepwise reduction of luminal pH in the presence of SCFA from 7.4 to 5.4 led to a bell-shaped modification of urea transport, with a maximum at pH 6.2. Lowering the pH in the absence of SCFA or $CO_2$ had no effect. Inhibition of $Na^+/H^+$ exchange increased urea flux at pH 7.4, with a decrease being seen at pH 6.4. In experiments with double-barreled, pH-sensitive microelectrodes, we confirmed the presence of an apical pH microclimate and demonstrated the acidifying effects of SCFA on the underlying epithelium. We confirm that the permeability of the ruminal epithelium to urea involves a phloretin-sensitive pathway. We present clear evidence for the regulation of urea transport by strategies that alter intracellular pH, with permeability being highest after a moderate decrease. The well-known postprandial stimulation of urea transport to the rumen in vivo may involve acute pH-dependent effects of intraruminal SCFA and $CO_2$ on the function of existing urea transporters.

$pH_i$; urea transporter-B; short-chain fatty acids; microclimate; volatile fatty acid

UREA, POSSIBLY BECAUSE OF its small size, was long thought to move passively across epithelia, depending only on the rate of delivery via blood. The urea permeability of cellular membranes has now been established to be several orders of magnitude above that of lipid membranes (11, 92) and is coupled to the expression of specific urea-transporting proteins with channel-like kinetics (7, 46, 71, 81, 82, 95). Whereas the role that these proteins play in the elegant renal concentrating mechanism has received much attention, their function and regulation in other parts of the body, such as the gut (39), continues to be poorly understood.

In contrast to the paucity of our knowledge concerning extrarenal urea transport in humans, we have long known of the ability of camels, cows, or sheep to shift the excretion of urea from the kidney (62, 72) to the gastrointestinal tract (79). The transport of urea through the rumen epithelium was first demonstrated many years ago in vivo and in vitro (16, 29, 79, 89) and the physiological significance is clear: in the rumen, dietary cellulose is broken down by bacteria that utilize urea-nitrogen for the synthesis of microbial proteins. After passage into the duodenum, the amino acids of these proteins are absorbed and reach the liver, where new urea for secretion into the rumen can be formed. Recycling of nitrogen via urea secretion into the rumen thus allows these animals to survive on low-protein diets while producing milk and meat for human consumption (1, 41, 67). The quantities of nitrogen recycled vary widely and might account for up to 25% of the nitrogen ingested (60) or up to 90% of urea turnover (for reviews, see Refs. 35, 44, 50, 67).

Our understanding of the mechanisms by which urea is transported across the rumen epithelium has evolved gradually. Gärtner and coworkers (16, 28, 29) originally postulated a saturable carrier for urea on the basis of observations of an inverse correlation between renal and ruminal urea excretion rates with no clear correlation to plasma urea levels. These studies were later challenged (38, 89), and, for a number of years, an uneasy consensus supporting ruminal urea transport via lipid diffusion reemerged. However, this hypothesis could not explain the outcome of feeding studies (13, 34, 36, 41, 59, 60), leading to the suggestion that the recycling of urea into the rumen is not determined by plasma urea levels but involves changes in the permeability of the rumen epithelium in response to dietary stimuli (35).

Current models of ruminal urea transport suggest that, whereas the concentration gradient serves as a driving force for the entry of urea from the blood into the rumen, the rate of influx is regulated in a far more complex manner (1, 35, 41, 44, 67, 90), involving both long-term adaptation and a system of short-term regulation corresponding to a postprandial increase in the extraction rate of arterial urea by the rumen (44, 50, 64, 65). Thus, when feed is supplemented with a rapidly fermentable energy source, the daily flux of urea across the rumen wall increases up to twofold (41, 58–60, 86) and, in the 3-h interval after a meal, urea flux into the rumen can rise even further (64). This mechanism allows the animal to capitalize on available carbohydrates by an endogenous supply of nitrogen (33). As the blood urea level decreases when these energy supplements are added, the increase in urea flux must be mainly attributable to modified rumen epithelial permeability via changes in the expression level or permeability of urea-transporting membrane proteins.

Cytohistochemical studies have led to the model of a functional syncytium of the multilayered rumen epithelium with an epithelial permeability barrier (with zonae occludentes) at the level of the stratum granulosum. The cell layers are interconnected by gap junctions from the stratum granulosum via the

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stratum spinosum to the stratum basale, where expression of $Na^+$-$K^+$-ATPase is concentrated (32). Recent studies have shown that urea transporter mRNA and protein corresponding to urea transporter-B (UT-B; splice variants 1 and 2) (51, 52) are present in the epithelium of the rumen and colon of sheep (68, 69). In addition, staining for UT-B has been demonstrated in the membranes of all epithelial layers of the bovine rumen with the exception of the stratum corneum (80, 83).

Intriguingly, a recent study has shown the stimulatory effects of a 2-wk diet with induction of low ruminal pH and an elevation of intraruminal butyrate on the localization and expression of UT-B in the rumen (80). The future will show whether long-term effects of butyrate on urea transport are indeed related to a higher density of urea transporters in the ruminal tissue or whether concomitant increases in ruminal surface area are sufficient to explain these effects (24, 90). In general, however, the demonstration of an effect of dietary changes on the expression of gastrointestinal urea transporters in ruminants has been challenging (18, 48, 51, 61, 80).

Whatever the ultimate reasons for long-term changes in urea transport, changes in surface area cannot explain the rapid short-term diurnal variations in urea transport in response to fermentational processes with the release of $CO_2$ and SCFA. Thus the bubbling of $CO_2$ into the rumen (65, 87, 90) leads to a rapid rise in urea transfer to the rumen, with effects seen after as little as 15 min (65). Increases in blood flow have been discussed but cannot explain the magnitude of the effect (19, 66). Conversely, the effects of SCFA on urea transport are less clear. Whereas acute stimulatory effects of butyrate were observed in two studies of urea transport into the rumen in toto (87) and into an isolated ruminal pouch (90), an inverse effect was observed in a later study (65). Difficulties in controlling various parameters in vivo with an impact on urea transport (such as ammonia) may explain these discrepancies (65, 87, 90).

The aim of the present study has therefore been directly to examine the effect of SCFA and $CO_2$ on urea fluxes across the freshly isolated rumen epithelium of sheep in the controlled environment of the Ussing chamber. Depending on mucosal pH, we were able to observe a fourfold stimulation of ruminal urea transport by SCFA and $CO_2$ under in vitro conditions. The effects of SCFA are maximal in the pH range from ~6.2 to 6.4 (found physiologically in the rumen) and decrease with alkaline or acidic deviation from this value. In experiments with pH-sensitive microelectrodes, we were able to demonstrate that, at a pH of 6.4, the addition of SCFA has a significant acidifying effect on the cytosolic space of the transporting layer of cells. We suggest that the rapid increase in urea transport that is observed postprandially in vivo occurs in response to SCFA and $CO_2$ and is functional rather than transcriptional, involving acute effects of pH on the activity of a urea-transporting protein that is likely to be UT-B (51, 52, 68, 69, 80, 83).

## MATERIALS AND METHODS

### *Flux Measurements in the Ussing Chamber*

*Isolation and incubation of rumen epithelium.* The incubation of rumen epithelium has been described in detail by Abdoun et al. (2). Briefly, sheep were killed in a local slaughterhouse, and the reticulorumen was removed from the abdominal cavity within 2–3 min. A 250-$cm^2$ piece of rumen wall was taken from the ventral sac, cleaned in a buffer solution, stripped from the muscle layer, taken (duration: ~20 min) to the laboratory in a physiological buffer solution, and maintained at 38°C. The buffer was gassed with 95% $O_2$-5% $CO_2$ during transportation. Pieces of the epithelium (3 × 3 cm) were mounted between the two halves of an Ussing chamber to give an exposed area of 3.14 $cm^2$. The mounted tissues were bathed on each side with 16 ml of buffer solution by using a gas-lift system and were gassed with 95% $O_2$-5% $CO_2$, 90% $O_2$-10% $CO_2$, or 100% $O_2$ ($HCO_3^-$-free buffer solutions) at 38°C.

The standard electrolyte solution contained (in mmol/l) 140 $Na^+$, 5 $K^+$, 1 $Ca^{2+}$, 1 $Mg^{2+}$, 104 $Cl^-$, 1 $H_2PO_4^-$, 2 $HPO_4^{2-}$, 10 glucose, and 40 gluconate, with 1 phenyl-phosphorodiamidate (urease inhibitor) and urea added at 1 mmol/l or as indicated. In $HCO_3^-$-containing solutions, 25 mmol/l gluconate was replaced by 25 mmol/l $HCO_3^-$. In SCFA-containing solutions, 40 mmol/l gluconate was replaced by 25 mmol/l acetate, 10 mmol/l propionate, and 5 mmol/l butyrate. These concentrations are well tolerated by the tissue in vitro and reflect the relative proportions found in the rumen physiologically.

In solutions containing both $HCO_3^-$ and SCFA, the concentration of chloride was lowered by 25 mmol/l. Mannitol was used to adjust the osmolarity of all solutions to 305 mosmol/l (Osmomat 030-D, GONOTEC, Berlin). In experiments in which mannitol fluxes were measured, 1, 3, 5, and 7 mmol/l mannitol were added to the corresponding buffers without renewed adjustment for osmolarity, which thus differed by small amounts (<7 mmol/l).

Bicarbonate-free solutions were buffered with 8 mmol/l MOPS ($C_7H_{15}NO_4S$). All solutions were adjusted to a standard pH of 7.4 or as indicated by using Tris [$(HOCH_2)_3CNH_2$] or MES ($C_6H_{13}NO_4S$) buffers, and antibiotics were added [penicillin G, kanamycin, and 5-fluorocytosine (55)]. Antibiotics, amiloride, and phloretin were obtained from Sigma (Munich, Germany). Phenyl phosphorodiamidate was obtained from ABCR (Karlsruhe, Germany). Radioisotope $^{14}C$-urea was purchased from Hartmann Analytik (Braunschweig, Germany), $^{36}Cl$ was from Amersham (Braunschweig, Germany), and $^3H$-labeled mannitol was purchased from Perkin Elmer, Germany (Rodgau-Jügesheim). All reagents were of analytical grade.

*Electrical measurements and measurement of flux rates.* The transepithelial conductance ($G_t$) was determined by applying a 100-μA current for 1 s across the tissue in both directions and measuring the resulting change in transmural potential difference ($PD_t$), from which $G_t$ can be calculated by using Ohm's law (Mussler Scientific Instruments, Aachen/Germany). The sum of all electrogenic ions moving across the tissue was determined by measuring the external current

Table 1. *Effect of SCFA on unidirectional urea flux rates across the rumen epithelium under control conditions (pH 7.4) in absence of $CO_2$/$HCO_3^-$*

| Group | $J_{ms}^{urea}$, nmol·cm$^{-2}$·h$^{-1}$ | $J_{sm}^{urea}$, nmol·cm$^{-2}$·h$^{-1}$ | $J_{net}^{urea}$, nmol·cm$^{-2}$·h$^{-1}$ | $I_{sc}$, μeq·cm$^{-2}$·h$^{-1}$ | $G_t$, mS·cm$^{-2}$ | *N*/*n* |
|---|---|---|---|---|---|---|
| pH 7.4−SCFA | 20.8 ± 1.8 | 21.8 ± 2.1 | +1.0 ± 1.4 | 0.7 ± 0.1* | 2.7 ± 0.1 | 3/9 |
| pH 7.4+SCFA | 22.8 ± 2.4 | 22.6 ± 2.4 | +0.1 ± 2.5 | 1.0 ± 0.1† | 2.8 ± 0.1 | 3/9 |

Values are means ± SE. SCFA, short-chain fatty acids; $J_{ms}^{urea}$, mucosal-to-serosal urea flux rate; $J_{sm}^{urea}$, serosal-to-mucosal urea flux rate; $J_{net}^{urea}$, net urea transport; $I_{sc}$, short-circuit current; $G_t$, transepithelial conductance. *N* = number of animals; *n* = number of tissues for each treatment. Values in the same column bearing different symbols are significantly different at $P < 0.05$ (paired *t*-test).

Table 2. *Effect of $CO_2/HCO_3^-$ on unidirectional urea flux rates across the rumen epithelium under control conditions (pH 7.4)*

| Group | $J_{ms}^{urea}$, nmol·cm$^{-2}$·h$^{-1}$ | $J_{sm}^{urea}$, nmol·cm$^{-2}$·h$^{-1}$ | $J_{net}^{urea}$, nmol·cm$^{-2}$·h$^{-1}$ | $I_{sc}$, μeq·cm$^{-2}$·h$^{-1}$ | $G_t$, mS·cm$^{-2}$ | |
|---|---|---|---|---|---|---|
| Control | 20.7 ± 4.9* | 23.1 ± 6.9* | −3.1 ± 9.6 | 0.6 ± 0.1* | 2.5 ± 0.1 | 3/9 |
| $CO_2$ | 31.5 ± 6.5† | 31.1 ± 9.3† | 0.4 ± 15.1 | 1.2 ± 0.1† | 3.5 ± 0.2 | 3/12 |

Values are means ± SE. Absence of SCFA; $N$ = number of animals; $n$ = number of tissues for each treatment. Values in the same column bearing different symbols are significantly different at $P < 0.05$ (unpaired $t$-test).

[equivalent to short-circuit current ($I_{sc}$)] necessary for clamping $PD_t$ to zero.

$^{14}$C-labeled urea (46.25 kBq) was added to the "hot" side of the epithelium, and three flux periods of 20 min were performed after an equilibration time of 40 min. Samples from the hot side were taken before the first and after the last flux period for the calculation of the specific radioactivity and assayed by using a β-counter (LKB Wallace-Perkin-Elmer; überlingen, Germany). The sample volume was replaced by the corresponding buffer.

Chloride and mannitol fluxes were measured essentially as described previously (2, 73, 74). All measurements were performed under classical Ussing chamber conditions without chemical gradients and with transepithelial potential clamped to 0 mV in the short-circuit mode. In accordance with these requirements and in correspondence to previous studies with SCFA (26, 77), SCFA were added to both sides of the tissue.

*Patch-Clamp Measurements*

Patch-clamp measurements were performed as reported previously (84, 85). In brief, ruminal epithelial cells were isolated by fractional trypsinization and cultured on coverslips. Extracellular NaCl solution contained (in mmol/l) 115 NaCl, 1 $NaH_2PO_4$, 5 KCl, 10 HEPES, 1.7 $CaCl_2$, and 0.9 $MgCl_2$, to which 30 mmol/l urea or mannitol was added as indicated. Cells were filled with a solution that contained (in mmol/l) 123 K-gluconate, 10 NaCl, 1 $KH_2PO_4$, 10 HEPES, 0.8 $CaCl_2$, 0.9 $MgCl_2$, and 5 EGTA. From a holding potential of −40 mV, the voltage was stepped from −120 to 100 mV, with a step size of 10 mV, and currents were measured with an EPC 9 patch-clamp amplifier (HEKA Elektronic, Lambrecht, Germany).

*Microelectrode Experiments*

*Electrical measurements.* Fresh ruminal epithelium was introduced into a small microelectrode chamber, apical side up. The apical and basolateral sides of the chamber were continuously perfused (MS/CA4/840, Ismatec, Glattbrugg-Zürich, Switzerland) with solution warmed to 37°C essentially as previously described (84, 85). The composition of the solutions was the same as in the bicarbonate-free solutions used in the Ussing chamber experiments, with MOPS being used to buffer pH and gluconate being replaced by SCFA as indicated. Solutions were bubbled with $O_2$ throughout. The pH of the experimental solutions was monitored at hourly intervals by using a conventional pH meter (Inolab pH 720, Weilheim, Germany) and adjusted if necessary.

The transepithelial voltage, resistance, and $I_{sc}$ of the tissues were controlled via voltage clamp (Biomedical Instruments, Munich, Germany) (43). The potentials of the two barrels of the microelectrode were measured with an FDA223 Dual Electrometer (World Precision Instruments, Sarasota, FL) and referenced to the mucosal side of the bath via a KCl bridge. All parameters were recorded by using Chart 5 for Windows (ADInstruments, Bella Vista, NSW, Australia) and filtered with digital median filters. The fractional apical resistance $f(R_a)$ was calculated from the potential response ($\Delta V_a$) of the microelectrode to 10 mV transepithelial voltage pulses of 0.3 s duration ($\Delta V_t$) (23), i.e., [$f(R_a)$ (%) = $\Delta V_a/\Delta V_t \times 100$] (Fig. 7).

*Manufacture of double-barrelled pH-sensitive microelectrodes.* Piggy-back double-barreled microelectrodes were prepared from filamented GC120F 10 and GC150F 15 borosilicate glass tubing (Harvard Apparatus, Kent, UK). One end of the smaller diameter tubing (GC120F 10) was heated and gently bent at a small angle from the main shaft (17). Two core cable ends (4 × 10, 611889, Conrad Bauelemente, Conrad Elektronik, Hirschau, Germany) were pushed into tightly fitting shrink tubing (Deray-H-set 1/8″, DSG-Canusa, Meckenheim, Germany) and used to join this bent glass tube with the larger diameter tube, leaving the middle section free of tubing. After being baked (190°C) until firmly adjoined, the piggyback electrodes were pulled by using a programmable multipipette puller (PMP-107, Microdata Instrument, South Plainfield, NJ) to give a resistance of

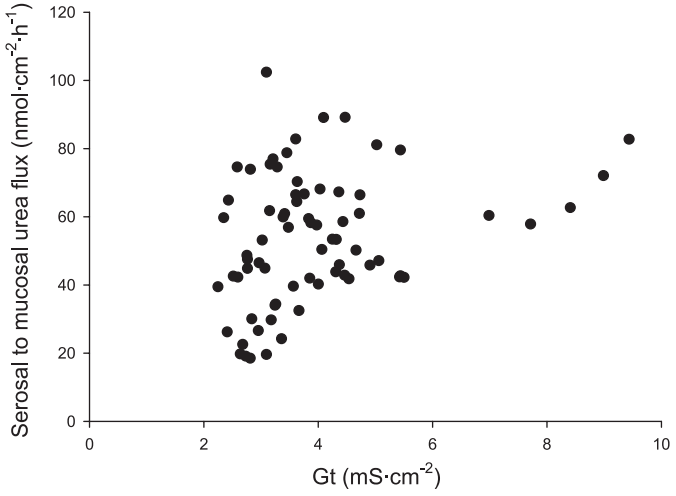


Fig. 1. Plot of the serosal-to-mucosal urea flux rates across the rumen epithelium against transepithelial conductance ($G_t$) in the presence of short-chain fatty acid (SCFA; $n$ = 73). There is no significant correlation.

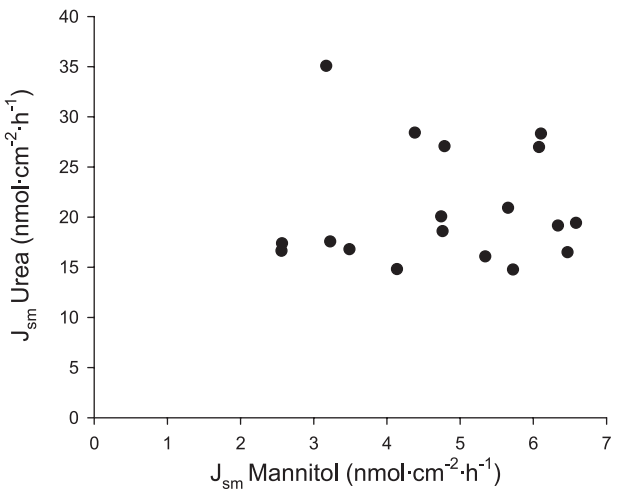


Fig. 2. Serosal-to-mucosal ($J_{sm}$) transport rates of mannitol and urea across the isolated rumen epithelium of sheep at pH 7.4 in the presence of SCFA ($N$ = 3, $n$ = 18), showing no correlation.

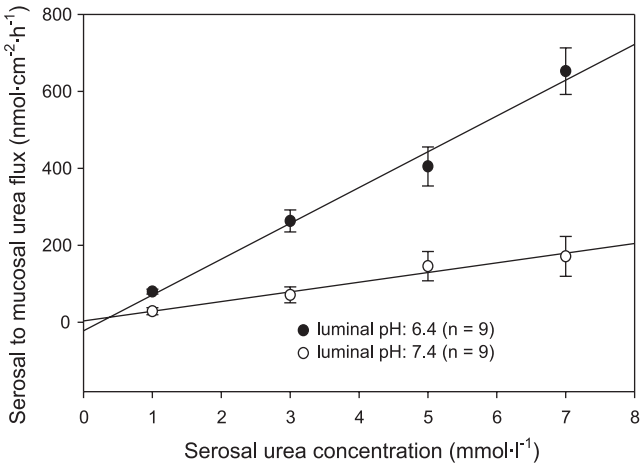


Fig. 3. Serosal concentrations of urea and $J_{sm}$ urea across the rumen epithelium at a luminal pH of 7.4 and 6.4 in the presence of SCFA. Each point corresponds to the mean value of flux data from 9 epithelia from 3 different animals ($N = 3$; $n = 9$). The data in the 2 pH groups were significantly different ($P < 0.05$, paired $t$-test) and correlated linearly with serosal urea concentration according to

$$J_{sm}(7.4) = 25.1 \cdot 10^{-6} \cdot [\text{urea}] \cdot 1 \cdot \text{cm}^{-2} \cdot \text{h}^{-1} + 3.7 \text{ nmol} \cdot \text{cm}^{-2} \cdot \text{h}^{-1} (r^2 = 0.98)$$

and

$$J_{sm}(6.4) = 93 \cdot 10^{-6} \cdot [\text{urea}] \cdot 1 \cdot \text{cm}^{-2} \cdot \text{h}^{-1} - 22 \text{ nmol} \cdot \text{cm}^{-2} \cdot \text{h}^{-1} (r^2 = 0.99).$$

[urea], Urea concentration.

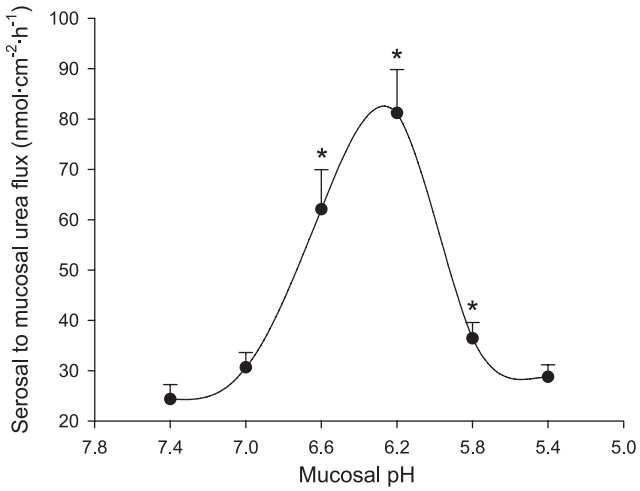


Fig. 4. Effect of decreasing mucosal pH on $J_{sm}$ urea flux across the rumen epithelium in the presence of SCFA [absence of $CO_2/HCO_3^-$, $N = 2$; $n = 8$, $^*P < 0.5$ vs. pH 7.4 (paired $t$-test)].

~50 MΩ (measured with EPC 9, Heka Elektronik, Lambrecht, Germany).

For silanization, the bent ends were connected to a pressure source and perfused with dried air, while the straight ends were pushed into the rubber lid of a heated glass jar and exposed to the vapor formed by a drop (200 μl) of dichlorodimethylsilane (Sigma-Aldrich) for 30 min. Pipettes were then baked at 190°C for 2 h, after which time the silanized channel was immediately backfilled with 3 μl Hydrogen Ionophore I-Cocktail A (Sigma Aldrich) via a modified Hamilton syringe (type 7000.50C, 0.5 ml, MedChrom, Germany). In some experiments, Theta glass tubing (TGC150–7.5, Harvard Apparatus) was used but, in this case, it proved more difficult to silanize one channel selectively.

The filled pipettes were placed tip downward in a jar containing a dehydrating agent at least overnight to allow the hydrogen ionophore to diffuse into the tip of the electrode. On the day before use, the hydrogen-sensitive barrel was backfilled with a solution containing 100 mmol/l KCl, 20 mmol/l HEPES, pH 7.4. The reference channel was filled with 0.5 mmol/l KCl. Both solutions were degassed by heating at 40°C before use.

To achieve functional microelectrodes, residual clogging silane had to be removed. Controlled breakage (17, 88) yielded unsatisfactory results. Use of a micromanipulator to push the electrode through a piece of tissue paper (Kimwipe Lite 200, Kimberly-Clark) clamped into the microelectrode chamber under a microscope yielded better resistances of 5–10 MΩ. In the final stages of the study, a beveller with an impedance meter was purchased (BV-10, Sutter Instrument, Novato, CA) for this purpose; the routine manufacture of functional microelectrodes with resistances >20 MΩ was thereby greatly facilitated.

Electrodes were judged suitable for impalement if they showed a differential response of over 40 mV per pH unit (mV/pH) (pH 7.4 to pH 6.4) between the pH-selective and nonselective barrel immediately before the experiments commenced. This test pulse was used to calculate cytosolic pH via linear regression. The speed of pH change was calculated for each point by subtracting the pH level from the value measured 30 s previously by using Chart for Windows.

Care was taken to choose the sharpest electrodes from the 16 typically available on a given experimental day. Impalements were judged to be stable if the fractional apical resistance remained constant. The resistances of functional microelectrodes were checked after successful completion of the experiment and typically yielded values of over 2 but under 10 MΩ. These resistances are thus clearly lower than desirable, which may explain why the apical potentials measured with pH-sensitive microelectrodes tended to be less negative and less stable than those measured previously with single-barrelled electrodes (43).

*Measurements.* All tissues were allowed to settle in $HCO_3^-$-free solution at pH 7.4 for 10 min before experiments commenced. Tissues were kept in the open-circuit mode but briefly clamped from time to time to measure the $I_{sc}$ ($0.63 \pm 0.11$ μeq·cm$^{-2}$·h$^{-1}$) and tissue conductance ($3.1 \pm 0.5$ mS/cm$^2$). All microelectrodes were rechecked for function by briefly changing the apical perfusion solution to an identical solution buffered to pH 6.4 (Fig. 7) before the start of the actual experiment, yielding an average sensitivity of $53 \pm 4$ mV/pH

Table 3. *Effect of short chain fatty acids and $Na^+/H^+$-exchanger activity on bidirectional urea flux rates across the rumen epithelium in absence of $CO_2/HCO_3^-$*

| Group | $J_{ms}^{urea}$, nmol·cm$^{-2}$·h$^{-1}$ | $J_{sm}^{urea}$, nmol·cm$^{-2}$·h$^{-1}$ | $J_{net}^{urea}$, nmol·cm$^{-2}$·h$^{-1}$ | $I_{sc}$, μeq·cm$^{-2}$·h$^{-1}$ | $G_t$, mS·cm$^{-2}$ | *N/n* |
|---|---|---|---|---|---|---|
| pH 7.4−SCFA | 20.4 ± 1.2* | 18.8 ± 1.5* | 1.5 ± 1.9* | 0.6 ± 0.1* | 2.6 ± 0.1* | 3/9 |
| pH 6.4−SCFA | 21.0 ± 2.1* | 22.8 ± 2.3* | −1.7 ± 1.3* | 0.7 ± 0.1* | 2.7 ± 0.1* | 3/9 |
| pH 6.4+SCFA | 83.6 ± 16.4† | 82.4 ± 12.1† | 1.2 ± 10.3* | 0.6 ± 0.1* | 2.3 ± 0.1* | 3/9 |
| pH 6.4 SCFA + Amiloride | 37.8 ± 7.6‡ | 37.1 ± 5.5‡ | 0.7 ± 4.3* | 0.1 ± 0.1† | 3.3 ± 0.2† | 3/9 |

Values are means ± SE. $N$ = number of animals; $n$ = number of tissues for each treatment. Values in the same column bearing different symbols are significantly different at $P < 0.05$ (Dunnett's $t$-test).

Table 4. *Effects of $CO_2$ and mucosal pH on bidirectional urea flux rates across the rumen epithelium in absence of SCFA*

| Group | $J_{ms}^{urea}$, nmol·cm$^{-2}$·h$^{-1}$ | $J_{sm}^{urea}$, nmol·cm$^{-2}$·h$^{-1}$ | $J_{net}^{urea}$, nmol·cm$^{-2}$·h$^{-1}$ | $I_{sc}$, μeq·cm$^{-2}$·h$^{-1}$ | $G_t$, mS·cm$^{-2}$ | *N*/*n* |
|---|---|---|---|---|---|---|
| pH 7.4, 5% $CO_2$ | 28.2 ± 1.2* | 25.8 ± 1.5* | 2.4 ± 1.9 | 1.1 ± 0.1* | 3.1 ± 0.2 | 3/9 |
| pH 6.4, 5% $CO_2$ | 68.1 ± 2.0† | 63.9 ± 2.3† | 4.1 ± 1.3 | 1.5 ± 0.1* | 2.9 ± 0.2 | 3/9 |
| pH 7.4, 10% $CO_2$ | 43.6 ± 16.4‡ | 39.2 ± 12.0‡ | 4.4 ± 10.3 | 1.5 ± 0.5* | 3.3 ± 0.2 | 3/9 |
| pH 6.4, 10% $CO_2$ | 122.5 ± 7.6§ | 127.2 ± 5.5§ | −4.7 ± 4.3 | 1.9 ± 0.1† | 3.0 ± 0.2 | 3/9 |

Values are means ± SE. *N* = number of animals; *n* = number of tissues for each treatment. Values in the same column bearing different symbols are significantly different at $P < 0.05$ (Dunnett's *t*-test).

unit. The pH response of the pipettes to the change in external solution was rapid, reaching a maximal speed of 1.93 ± 0.05 pH/min.

### *Statistics*

To allow a paired comparison of tissues from the same animal exposed to different treatments, a total of 24 Ussing chambers were available for experiments on each experimental day. All evaluations were carried out by using Sigma Stat (3.0.1) and Sigma Plot program version 8.0 for Windows (SPSS, Chicago, IL). Results are given as means ± SE. Comparisons were performed by using Student's *t*-test, paired or unpaired. Multiple comparisons were statistically evaluated by using one-way ANOVA with the Dunnett *t*-test for pairwise analysis (Ussing chamber data) or the Holm-Sidak pairwise post hoc analysis (microelectrode data); *P* values $< 0.05$ were considered significant. *N* refers to the number of experimental animals, and *n* refers to the number of tissues in the treatment group.

## RESULTS

### *Flux Measurements in the Ussing Chamber*

*Effect of SCFA and $CO_2$ on urea flux at pH 7.4.* Urea flux rates in both directions under control conditions (pH 7.4 on both sides of the epithelium; 1 mmol/l urea) ranged from 19 to 35 nmol·cm$^{-2}$·h$^{-1}$ (see Table 1). No significant effect of SCFA on the magnitude of the flux rates could be found. $CO_2$ had a slightly stimulating effect on urea transport (Table 2).

Theoretically, urea flux might occur via simple diffusion through the paracellular pathway, $G_p$, which accounts for some 60% of the transepithelial conductance ($G_t$) in rumen epithelium (43). However, our results did not reveal a significant correlation ($r^2 = 0.09$) between $G_t$ and serosal to mucosal ($J_{sm}$) urea flux (Fig. 1), and no correlation was found between urea and mannitol fluxes as markers for paracellular transport (Fig. 2).

Mucosal to serosal ($J_{ms}$) and $J_{sm}$ urea flux rates were not significantly different and, hence, no net transport was observed supporting a passive transport mechanism. In agreement with this, the $J_{sm}$ showed a linear correlation to the serosal concentration of urea (Fig. 3).

These results suggest that transruminal urea transport is driven by the concentration gradient only and must occur either via a transcellular route or via a paracellular pathway with specificity for urea over mannitol.

*Effect of SCFA on urea flux at pH < 7.4.* The negligible effects of SCFA and $CO_2$ on urea transport at a luminal pH of 7.4 in vitro suggest that addition of these fermentation products alone is not sufficient to induce the increase in urea flux observed postprandially in vivo (65, 87, 90). Because fermentation of carbohydrates in the rumen and production of SCFA and $CO_2$ are accompanied by a decrease in ruminal pH, which is thus typically lower than 7.4, we decided to change the pH of the mucosal solutions accordingly. Whereas a change of pH alone had no impact on urea transport, a reduction of pH from 7.4 to 6.4 in the presence of SCFA resulted in a significant fourfold increase in both $J_{ms}$ and $J_{sm}$ urea flux rates across the rumen epithelium (Table 3 and Fig. 3).

The effect of reducing mucosal pH in the presence of SCFA was so large that we decided to study the effect of lowering luminal pH in more detail, within the pH range from 7.4 to 5.4. The $J_{sm}$ urea flux exhibited a bell-shaped dependency on mucosal pH with a maximum at ~pH 6.2, returning to the prestimulated level at a mucosal pH < 5.8 (Fig. 4).

*Effect of $CO_2/HCO_3^-$ on urea flux.* In the absence of SCFA and at pH 7.4, increasing $CO_2$ tension from 5 to 10% caused a light but significant increase in urea transport (Table 4). In the presence of 5% $CO_2$, a reduction of the pH on the mucosal side from 7.4 to 6.4 significantly ($P < 0.05$) stimulated urea transport and resulted in two- to threefold increases in both $J_{ms}$ and $J_{sm}$ urea flux rates across the rumen epithelium. At pH 6.4, the increase of $CO_2$ from 5 to 10% doubled the urea transport rates (pH 6.4; Table 4). Interestingly, a reduction of the mucosal pH from 7.4 to 6.4 also significantly ($P < 0.05$) enhanced the $J_{ms}$ and net chloride ($J_{net}$) transport across the rumen epithelium (Table 5; see DISCUSSION).

*Effect of a combination of $CO_2/HCO_3^-$ and SCFA on urea flux.* The pH-dependent stimulation of urea flux rates was of similar magnitude in the presence of SCFA (Table 3) or $CO_2$ (5% $CO_2$; Table 4). The combined application of these two fermentation products caused a slight increase of urea flux rates at pH 7.4 (Table 6; see also Tables 1 and 2).

At pH 6.4, the combined application of both fermentation products led to a highly significant increase of urea transport from 34.61 ± 3.78 to 151.68 ± 16.39 nmol·cm$^{-2}$·h$^{-1}$, indicating that the effects of these fermentation products were additive (compare Tables 3, 4, and 6). A decrease of serosal pH

Table 5. *Effect of mucosal pH on bidirectional chloride transport across the rumen epithelium in presence of $CO_2/HCO_3^-$*

| Mucosal pH | $J_{ms}^{Cl}$, nmol·cm$^{-2}$·h$^{-1}$ | $J_{sm}^{Cl}$, nmol·cm$^{-2}$·h$^{-1}$ | $J_{net}^{Cl}$, nmol·cm$^{-2}$·h$^{-1}$ | $I_{sc}$, μeq·cm$^{-2}$·h$^{-1}$ | $G_t$, mS/cm$^2$ | *N*/*n* |
|---|---|---|---|---|---|---|
| pH 7.4 | 3.7 ± 0.6* | 2.3 ± 0.3 | 1.3 ± 0.3* | 1.1 ± 0.1 | 3.3 ± 0.3 | 4/8 |
| pH 6.4 | 5.9 ± 0.6† | 2.6 ± 0.1 | 3.2 ± 0.4† | 1.1 ± 0.1 | 3.2 ± 0.2 | 4/8 |

Values are means ± SE. $J_{ms}^{Cl}$, mucosal-to-serosal chloride flux rate; $J_{sm}^{Cl}$, serosal-to-mucosal chloride flux rate; $J_{net}^{Cl}$, net chloride transport. *N* = number of animals; *n* = number of tissues. Values in the same column bearing different symbols are significantly different at $P < 0.05$ (paired *t*-test).

Table 6. *Effect of pH (mucosal or serosal) on urea flux rates across the rumen epithelium in presence of $CO_2$ and SCFA*

| pH | $J_{ms}^{urea}$, nmol·cm$^{-2}$·h$^{-1}$ | $J_{sm}^{urea}$, nmol·cm$^{-2}$·h$^{-1}$ | $J_{net}^{urea}$, nmol·cm$^{-2}$·h$^{-1}$ | $I_{sc}$, μeq·cm$^{-2}$·h$^{-1}$ | $G_t$, mS/cm$^2$ | *N/n* |
|---|---|---|---|---|---|---|
| Mucosal and serosal pH 7.4 | 34.6 ± 3.7 | 26.1 ± 1.9 | 8.4 ± 3.2 | 1.1 ± 0.1 | 3.1 ± 0.1 | 4/16 |
| Mucosal pH 6.4 | 151.6 ± 16.3* | 155.0 ± 15.6* | −03.3 ± 16.4 | 0.9 ± 0.1 | 2.2±0.1* | 4/16 |
| Serosal pH 6.4 | 38.4 ± 5.0 | 46.4 ± 7.8* | −8.0 ± 6.3* | 0.9 ± 0.1 | 2.8 ± 0.3 | 4/16 |

Values are means ± SE. $N$ = number of animals; $n$ = number of tissues. *$P < 0.05$ (Dunnett's $t$-test) compared with control (pH 7.4).

to 6.4 caused only a slight ($J_{sm}$) or no increase ($J_{ms}$) of urea transport (Table 6). Notably, a reduction of the mucosal pH had no effect on mannitol fluxes (Table 7).

Thus changes of mucosal (extracellular) pH in a buffer solution without SCFA and $CO_2/HCO_3^-$ have no effect on urea transport. The effect of mucosal pH 6.4 on urea transport depends on the presence of the fermentation products $CO_2$ and SCFA, which are known to increase Na transport via $Na^+/H^+$ exchange (NHE) (26), thus indicating a change of intracellular pH ($pH_i$). Therefore, this change of $pH_i$ might modulate urea flux. Further experiments were designed to examine this assumption.

*Effect of amiloride on urea flux at pH 7.4.* The presence of the protein involved in sodium-proton exchange (NHE) in ruminal epithelium is well documented (2, 22, 27, 31, 74), and SCFA are known to stimulate this pH-regulating transport protein in the rumen (25, 76). If urea transport is mediated by changes in cytosolic pH, inhibition of NHE should lead to measurable effects.

At a pH of 7.4, the $J_{sm}$ flux of urea rose with the concentration of amiloride, in agreement with this hypothesis (Fig. 5). In a second step, the effect of the NHE inhibitor amiloride on urea fluxes was studied at pH 6.4 and in the presence of SCFA. Interestingly, at this pH, 1 mmol/l mucosal amiloride significantly reduced urea flux (Table 3).

*Effect of theophylline on the stimulated urea flux by SCFA at pH 7.4.* Theophylline is known to reduce the mucosal to serosal transport of sodium via NHE in the rumen (25). The effects of theophylline (Table 8) on urea transport (pH 7.4) were similar to those of amiloride.

*Effect of phloretin on urea flux.* Phloretin reportedly has inhibitory effects on urea transport when applied both mucosally and serosally (68, 83). In this study, we confirmed the effects of serosal phloretin (1 mmol/l), which reduced urea transport ($J_{sm}$) by almost 50% [from 148.2 ± 15.0 to 75.8 ± 8.8 nmol·cm$^{-2}$·h$^{-1}$, $n = 6$, $N = 2$, $P = 0.002$ (paired Student's $t$-test)].

*Effect of clamping transepithelial potential.* Previous studies of urea transport in the inner medullary collecting duct of rat kidney have demonstrated $Na^+$-linked urea transport (40). Since a number of studies have shown that, despite saturation kinetics, electrogenic cotransporters are responsive to changes in potential, we tried to detect corresponding effects in the ruminal epithelium. However, changes in the $PD_t$ in the Ussing chamber did not significantly influence urea flux rates (Table 9). Likewise, the addition of 1 or 7 mmol/l urea did not alter the electrical parameters of the rumen epithelium ($PD_t$, $G_t$, and $I_{sc}$; data not shown).

Table 7. *Effect of decreasing mucosal pH on the serosal to mucosal urea and mannitol fluxes across the rumen epithelium in presence of SCFA and $CO_2$*

| Mucosal pH | $J_{sm}^{urea}$, nmol·cm$^{-2}$·h$^{-1}$ | $J_{sm}^{mannitol}$, nmol·cm$^{-2}$·h$^{-1}$ | *N/n* |
|---|---|---|---|
| 7.4 | 20.8 ± 1.3 | 4.7 ± 0.3 | 3/18 |
| 6.4 | 135.4* ± 5.1 | 4.6 ± 0.3 | 3/18 |

Values are means ± SE. $N$ = number of animals; $n$ = number of tissues. *$P < 0.05$ (paired $t$-test vs. pH 7.4).

### *Patch-Clamp Measurements*

Likewise, whole cell patch-clamp experiments performed on isolated ruminal epithelial cells did not reveal urea-dependent currents when 30 mmol/l mannitol was exchanged for the same amount of urea at either pH 7.4 or pH 6.4 ($n = 9$) in the presence of sodium (Fig. 6). The influx of $Na^+$ coupled to urea should have led to an increase of inward current coupled to a depolarization of the cells, neither of which could be demonstrated.

In conclusion, these experiments do not support the hypothesis that urea transport of the ruminal epithelium is mediated by an electrogenic transport mechanism.

### *Microelectrode Experiments*

The following experiments were performed to assess directly whether the ruminal epithelium possesses a pH microclimate and whether the pH within the transporting layer of the intact ruminal epithelium is altered by the addition of SCFA at a pH of 6.4.

*Measurement of apical pH.* After a brief pH calibration pulse (see MATERIALS AND METHODS) and a return of the bath

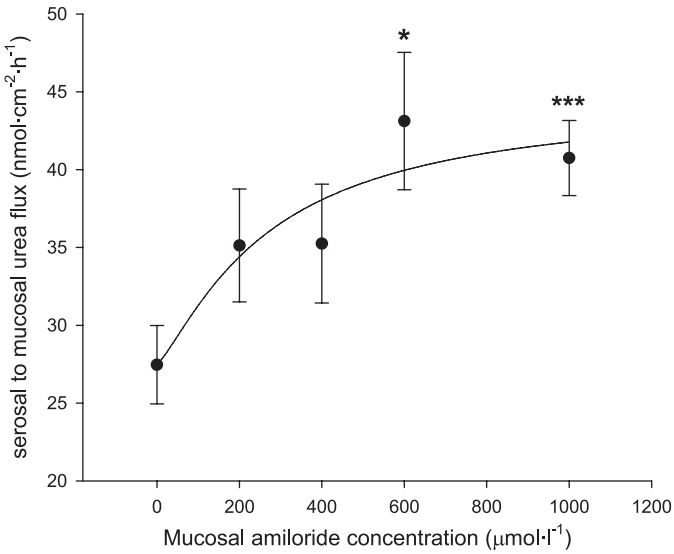


Fig. 5. Effect of increasing amiloride concentration on the mucosal side at pH 7.4 on $J_{sm}$ urea flux across the rumen epithelium of sheep in the presence of SCFA [$N = 3$; $n = 8$, *$P < 0.05$ ; ***$P < 0.001$ vs. 0 mmol/l amiloride (paired $t$-test)].

Table 8. *Effect of theophylline (10 mmol/l) on urea flux rates across the rumen epithelium in presence of SCFA (pH 7.4 without $HCO_3^-$)*

| | $J_{ms}^{urea}$, nmol·cm$^{-2}$·h$^{-1}$ | $J_{sm}^{urea}$, nmol·cm$^{-2}$·h$^{-1}$ | $J_{net}^{urea}$, nmol·cm$^{-2}$·h$^{-1}$ | $I_{sc}$, μeq·cm$^{-2}$·h$^{-1}$ | $G_t$, mS/cm$^2$ | *N/n* |
|---|---|---|---|---|---|---|
| 0 mM Theophylline | 23.1 ± 1.9 | 22.1 ± 2.0 | 0.9 ± 1.5 | 1.3 ± 0.1 | 3.1 ± 0.1 | 3/15 |
| 10 mM Theophylline | 45.1 ± 2.9* | 49.5 ± 4.5* | −4.4 ± 3.5 | 0.8 ± 0.1* | 3.1 ± 0.1 | 3/16 |

Values are means ± SE. $N$ = number of animals; $n$ = number of tissues. *$P < 0.05$ (unpaired $t$-test vs. 0 mM Theophylline).

perfusion to the original solution, the pipette was lowered onto the epithelium with a micromanipulator. A drop in pH from 7.4 to 7.2 ± 0.5 ($P = 0.05$, $n = 7$, Holm-Sidak) signaled the proximity of the transporting layer of cells (Fig. 7). At this point of the measurement, the potential of the reference electrode did not change notably and the response to transepithelial voltage pulses remained negligible. The initial pH response probably reflected the entry of the microelectrode into the stratum corneum, which is thought to form a pH microclimate (compare Ref. 45).

*Measurement of cytosolic pH.* Impalement of the transporting syncytium (32) was considered to have occurred when the apical potential dropped and the response of the electrode to a transepithelial 10-mV voltage pulse increased [from 0% to 50 ± 10% (Fig. 7)]. Following impalement, the cytosolic pH was 7.08 ± 0.09 [$n = 7$, $P = 0.002$ vs. pH of bulk solution (7.4) and $P = 0.06$ vs. pH microclimate, Holm-Sidak]. Cytosolic pH remained relatively stable with a mean drift at −0.03 ± 0.02 pH/min.

When mucosal perfusion was switched to a solution with a pH of 6.4, cytosolic pH showed a slow decline (−0.05 ± 0.02 pH/min), reaching 6.8 ± 0.2 ($n = 7$, $P = 0.004$ vs. original level, Holm-Sidak) (Figs. 7, 8, and 9). Following the addition of SCFA (40 mmol/l) to both sides of the epithelium, the rate of acidification increased markedly (to −0.10 ± 0.01 pH/min, $P = 0.02$ vs. rate of acidification at pH 6.4 before addition of SCFA) with cytosolic pH dropping to 6.60 ± 0.15 ($n = 5$). Return to a pH of 7.4 resulted in a recovery of $pH_i$ to 6.9 ± 0.1 [+ 0.17 ± 0.08 pH/min, $P = 0.005$ vs. previous (Holm-Sidak)].

These data thus confirm the presence of an apical microclimate above the transporting layer of cells (24, 45, 75, 93). Lowering the mucosal pH has slow acidifying effects on the cytosolic pH. The rate of acidification increases significantly when SCFA are added to the solution.

## DISCUSSION

This study represents the first clear demonstration of a pH-dependent short-term regulation of a mammalian urea transporter (6, 81), with possible consequences for in vivo situations. Moreover, the data presented here significantly enhance our understanding of ruminal urea transport by the demonstration of a number of new findings. *1*) We demonstrate that ruminal urea transport cannot be explained by paracellular diffusion and thus enhance the argument for a transcellular passage via a specific protein such as UT-B (49–51, 68, 69, 80, 83). *2*) Effects of SCFA and $CO_2$ on urea transport are absent or of quantitatively minor importance at pH 7.4. *3*) In the presence of SCFA or $CO_2$ and within the pH range of 6.00–7.00, urea transport rates are increased by lowering mucosal pH. *4*) At a mucosal pH of <6.00, the addition of SCFA inhibits urea transport. *5*) Transruminal urea transport is stimulated by amiloride at a pH of 7.4, whereas at a pH of 6.4 amiloride has inhibitory effects.

In addition, the data confirm the presence of an apical pH microclimate and directly demonstrate the acidifying effects of the application of SCFA on the $pH_i$ of the underlying transporting epithelium.

### *Ruminal Urea Transport Is Passive and Sensitive to Phloretin*

The transport of urea across the rumen epithelium is generally accepted to be driven by the concentration gradient between blood plasma and ruminal fluid with permeability being strongly limited by the stratum corneum (38, 89). The absence of significant urea net transport and the close correlation between the serosal urea concentration and $J_{sm}$ urea are in agreement with this assumption (Fig. 3). In our study, no correlation could be seen between $J_{sm}$ urea and the $G_t$ of the isolated rumen epithelium (Fig. 1) or mannitol fluxes (Fig. 2). A transcellular pathway for urea transport is supported by the pH-dependent increase of urea flux without any effect on mannitol fluxes (Table 7) and by the inhibition of urea transport by phloretin in this study and in others (68, 69, 83). Our hypothesis of a transcellular pathway agrees well with the urea transporters (UT-B) demonstrated in the plasma membranes of all cell layers of the bovine rumen epithelium, with the exception of the stratum corneum (83). However, we were unable to confirm the close correlation between mannitol and urea fluxes found in the same study (83). Both edge damage and species-dependent differences may have contributed to what would appear to be a larger paracellular "leak" of the bovine tissues studied by Stewart et al. (83), but such speculation is problematic, since the authors do not report values for $G_t$.

Table 9. *Effect of transepithelial potential difference on bidirectional urea flux rates across the rumen epithelium at a mucosal pH of 6.4 in presence of SCFA and $CO_2/HCO_3^-$*

| Transepithelial Potential | $J_{sm}^{urea}$, nmol·cm$^{-2}$·h$^{-1}$ | $J_{ms}^{urea}$, nmol·cm$^{-2}$·h$^{-1}$ | $J_{net}^{urea}$, nmol·cm$^{-2}$·h$^{-1}$ | $I_{sc}$, μeq·cm$^{-2}$·h$^{-1}$ | $G_t$, mS/cm$^2$ | *N/n* |
|---|---|---|---|---|---|---|
| 0 mV | 134.2 ± 11.8 | 122.1 ± 8.4 | 12.1 ± 12.4 | 1.1 ± 0.1 | 2.9 ± 0.2 | 2/6 |
| +25 mV | 125.3 ± 12.9 | 130.0 ± 11.0 | −4.6 ± 8.4 | 0.5 ± 0.1* | 2.7 ± 0.1 | 2/6 |
| −25 mV | 132.2 ± 7.5 | 116.2 ± 7.7 | 15.9 ± 7.7 | 3.0 ± 0.1* | 2.7 ± 0.1 | 2/6 |

Values are means ± SE. $N$ = number of animals; $n$ = number of tissues. *$P < 0.05$ (Dunnett's $t$-test) vs. control (0 mV).

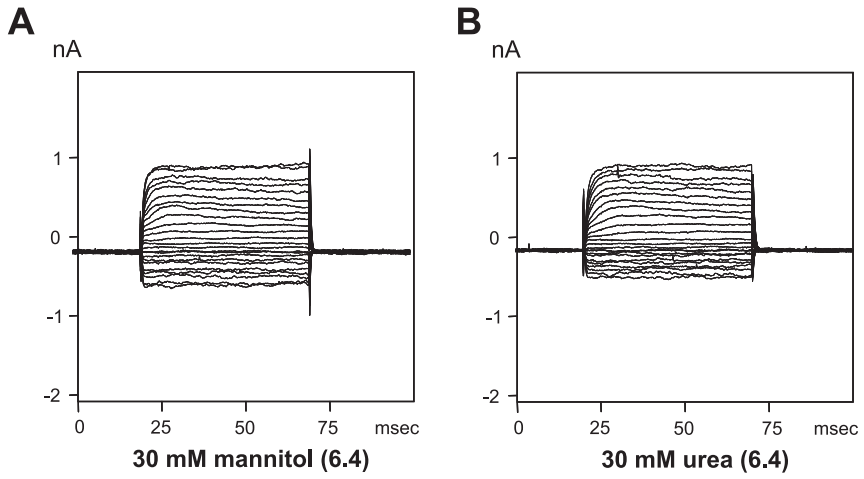


Fig. 6. Original traces of a ruminal epithelial cell filled with a physiological K-gluconate solution and exposed to Ringer's solution containing 30 mmol/l of mannitol (*A*) and 30 mmol/l urea (*B*). No effects could be detected.

### *Luminal pH Is a Decisive Cofactor for the Effects of SCFA/$CO_2$ on Urea Transport*

Given the well-documented and major impact of fermentation on urea transport across the rumen in vivo, we were initially surprised to find only insignificant (SCFA) or small ($CO_2$) effects of these fermentation products on urea transport across the isolated epithelium under standard conditions in the Ussing chamber. However, a pH of 7.4 is unusually high for in vivo conditions and is more typical of fasting animals in which the rate of fermentation is slow. In these situations, the backflow of urea into the gut approaches zero, despite an increase in the level of urea in plasma (35). Conversely, the highest rates of urea influx into the rumen are observed postprandially, when fermentational processes lead to a drop in ruminal pH (63, 64). However, in this study, the lowering of luminal pH alone merely had minor effects on urea flux (Table 3). Only after the physiological situation was simulated more completely by the addition of $CO_2$ at a pH of 6.4 were we able to see large stimulatory effects on urea flux (Tables 3, 4, and 6), corresponding to the well-documented effects observed in vivo (35, 65, 87, 90). Similar effects were seen with SCFA. Interestingly, a stepwise reduction of pH in the presence of SCFA (without $CO_2$) from 7.4 to 5.4 led to a bell-shaped modification of urea transport (Fig. 4). Transport rates were low at pH 7.4 and pH 7.0, rising to maximal levels at around pH 6.2, followed by a steep drop to the original level when pH was lowered to $<$5.8. This bell-shaped dependency may be one of many possible reasons why in vivo studies of the stimulation of urea transport by SCFA have produced conflicting results (65, 87, 90).

Physiologically, the meaning of this finding is evident: in fasting animals, the influx of urea with subsequent hydrolysis by ruminal urease (9, 63) leads to an increase in ruminal ammonia concentration. Free ammonia, however, is rapidly absorbed back into the portal system (1) and must again be detoxified in the liver. Thus a reduction of urea recycling (35) can be seen as an adaptational response that is beneficial to the fasting animal. Conversely, postprandial stimulation of urea transport into the rumen by fermentation products, as demonstrated in vivo in previous investigations (63, 65–67, 87, 90) and in vitro in this study, ensures that the influx of urea into the rumen is tightly linked to the nitrogen requirements of a growing microbial population.

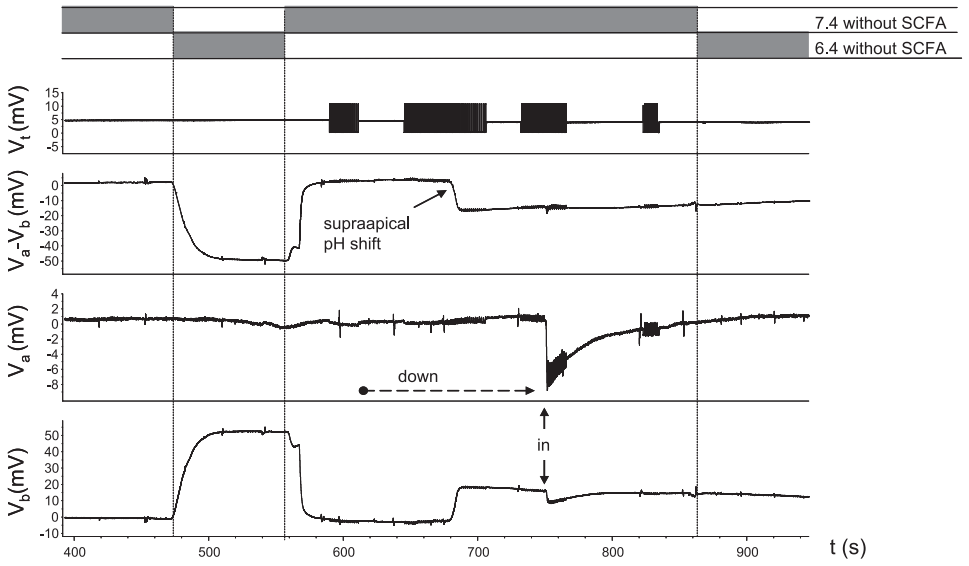


Fig. 7. Original recording showing the transepithelial potential ($V_t$), the potential of the pH-insensitive electrode ($V_a$), the pH-sensitive electrode ($V_b$), and the difference between the 2 ($V_a-V_b$) over time. For calibration, the double-barrelled electrode was positioned in the bath well above the tissue and the mucosal bath solution was switched from pH 7.4 to pH 6.4 and back again, resulting in a marked change in the potential of $V_b$, but not of $V_a$. The electrode was moved downward slowly. A pH shift toward a more acidic value ("supra-apical pH shift") could be seen before the electrode entered the epithelium (in). Note the drop in the potential of $V_a$ and $V_b$ and the rise in the fractional apical resistance as measured by the response of the electrode to transepithelial voltage pulses. *t*, Time.

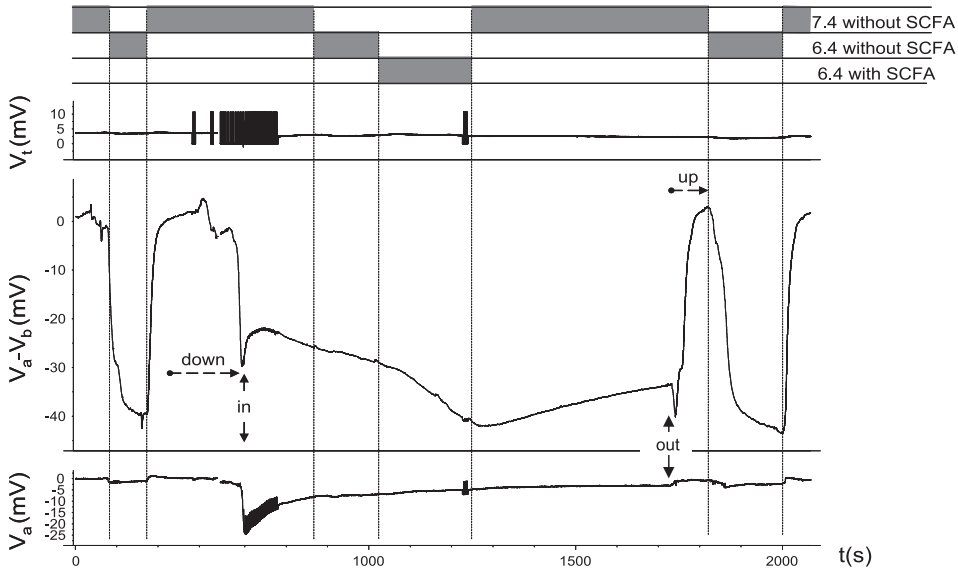


Fig. 8. Original recording showing the response of an impaled microelectrode to a change in mucosal pH to 6.4 and the addition of SCFA at pH 6.4, followed by recovery after return to a SCFA-free solution at pH 7.4. The first arrow designates the beginning of the impalement (in) corresponding to a shift in the potential of both electrodes, whereas the second arrow (out) reflects the end of the impalement (brought about by elevating the electrode). Calibration of the electrode was performed before and after impalement. The measurement was performed in the open circuit mode but the transepithelial potential was clamped briefly (0.3 s) at various points to measure the epithelial conductance (2.9 mS/cm$^2$) and fractional apical resistance (~70%).

Likewise, the inhibition of urea transport at a luminal pH of <6.0 may be of significant practical importance. Thus the attempt to raise urea recycling by increasing intraruminal fermentation with the production of $CO_2$ and SCFA by feeding highly fermentable carbohydrates can result in failure (30), which is to be expected since ruminal pH frequently drops to values below 5.7 under these conditions. Feeding conditions with low ruminal pH values can therefore be expected to reduce the use of endogenous urea-N for the synthesis of microbial protein on the one hand, whereas, on the other hand, urea excretion via urine is increased with an adverse environmental impact.

### *Effect of Theophylline and Amiloride on Urea Transport*

The stimulatory effect of theophylline (Table 8) might lead to the false conclusion that the signaling cascade leading to the activation of urea flux into the rumen is linked to a cAMP-dependent pathway (10). However, UT-A does not appear to be expressed by the ruminal epithelium (83), whereas transporters from the UT-B group typically do not show cAMP-mediated phosphorylation (6, 81, 83). Instead, we have shown that amiloride has similar effects on urea transport as theophylline (Fig. 5). Both agents have been demonstrated to suppress electroneutral sodium transport via NHE3 (25, 56, 94). Conversely, at pH 6.4, amiloride inhibits urea transport. We suggest that all the effects observed in this study are mediated by changes in cytosolic pH (compare Fig. 4).

### *Na and Urea Transport*

The possibility of an active transport of urea was discussed in the past (16, 28, 29), although this possibility was ruled out in later studies that failed to demonstrate saturation kinetics (38, 89). $Na^+$-linked urea transporters have been identified in a number of mostly amphibian epithelia (70), mediating both $Na^+$-urea cotransport and $Na^+$-urea antiport. Any $Na^+$-linked urea transport should be uncovered by changing the driving forces of luminal $Na^+$ uptake in the Ussing chamber. The

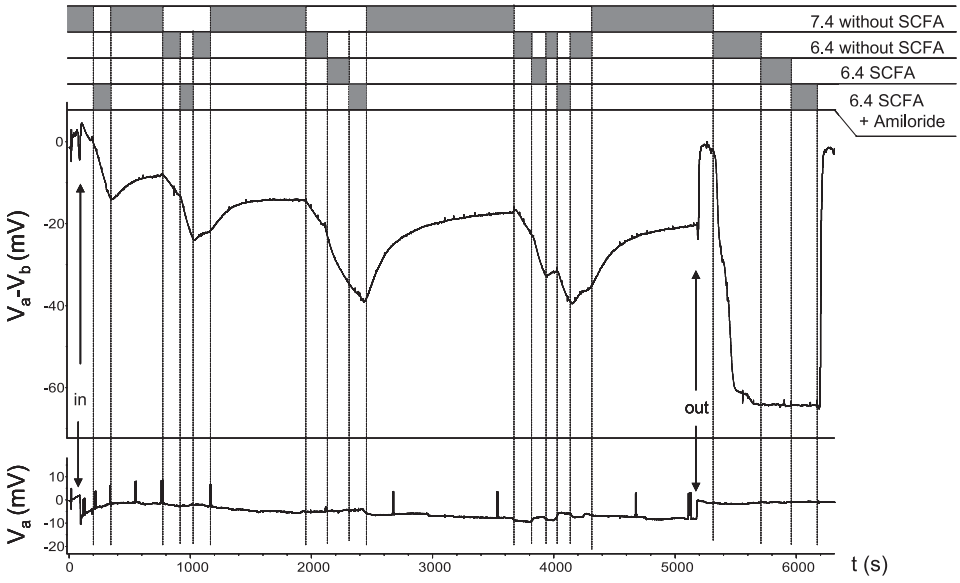


Fig. 9. Original recording showing the potential of an impaled double-barrelled microelectrode over time and in response to various experimental solutions, followed by an extracellular calibration (after out). Note that addition of SCFA resulted in a much stronger acidification than a change in pH alone. After removal of SCFA, recovery of intracellular pH was observed even when extracellular pH remained at 6.4. As before, the transepithelial potential was occasionally clamped to measure the fractional apical resistance (~80%) and tissue conductance (2.7 mS/cm$^2$).

change of transepithelial potential difference (±25 mV), which alters the potential difference of the apical membrane by ±15 mV (43), does not influence urea transport and argues against $Na^+$-urea cotransport (Table 9).

Whereas the evidence argues against the direct coupling of $Na^+$ and urea transport, both transport rates appear to be regulated in a similar fashion. The experimental conditions that caused the increase of urea transport in this study (Tables 3, 4, and 6; Fig. 4) are similar to those in corresponding studies in which the stimulatory effect of these fermentation products on amiloride-sensitive $Na^+$ absorption via NHE has been characterized (24, 26, 53, 54, 77). As in monogastric animals in which $Na^+$ uptake also increases in response to a change in the luminal end products of digestion (20), the underlying transporter is NHE3, an extremely well-characterized transport protein that utilizes the energy from the influx of $Na^+$ to drive the efflux of $H^+$ on a 1:1 basis, in allosterically regulated manner so that the extrusion of protons stops when the cytosolic pH reaches the set point optimal for cellular function (3, 20, 91).

### *Effect of SCFA and $CO_2$ on the Transport of Urea*

$CO_2$ caused a small concentration-dependent and significant increase of urea transport at pH 7.4 and a marked enhancement at pH 6.4 (Table 4), with a simultaneous increase in $J_{ms}$ and $J_{net}$ chloride flux rates (Table 5). Since the pressure of $CO_2$ was constant in all of these experiments, the lowering of mucosal pH must have caused a decrease in luminal $HCO_3^-$ concentration, which promotes $HCO_3^-$ (base) extrusion and $Cl^-$ uptake. According to this classical model, this should be followed by the dissociation of cytosolic $CO_2$ with acidification and stimulation of NHE (12, 54) and of the urea transporter of this study. In agreement with this hypothesis, the inhibition of carboanhydrase by acetazolamide (21) or the replacement of $CO_2$ reduces electroneutral Na transport (12, 54). A similar coupled stimulation of both $Na^+$ (24, 26, 53, 54, 77) and urea transport follows exposure of the tissue to SCFA. It thus appears likely that the pH-dependent effects of amiloride, theophylline, $CO_2$, and SCFA on both sodium (24, 26, 53, 54, 77) and urea transport are mediated by the same central event: a change in cytosolic pH.

### *Effect of SCFA on the Intraepithelial pH of the Intact Ruminal Epithelium*

Some counterarguments can be raised against the hypothesis that cytosolic pH is altered after exposure of the epithelium to SCFA. Both diffusion of the protonated form (H-SCFA) (93) and uptake via $SCFA^-/HCO_3^-$ exchange with dissociation of $CO_2$ (5, 24, 78) should lead to an acidification of the cell (78), as shown in isolated cells of the ruminal epithelium in vitro (8). However, if, in intact tissue, the influx of SCFA is mediated by an anion exchanger (5) physically linked to NHE3 via scaffolding proteins (20) or if an influx of $SCFA^-$ anions occurs primarily via an apical anion channel similar to that mediating the basolateral efflux of acetate (85), cytosolic pH should remain constant or show a slight increase. For this reason, we attempted to measure the cytosolic pH of the intact ruminal epithelium under the same conditions as those applied in the Ussing chamber directly by using pH-sensitive microelectrodes.

The microelectrode experiments show surprisingly strong responses of the tissue to a lowering of mucosal pH, even before the addition of SCFA. This may in part reflect the fact that the experiments were performed under the same standard conditions as those used in the flux experiments, with SCFA present bilaterally. It should also be noted that cell potential tended to decrease rapidly with time. This suggests that some damage to the tissue may have occurred during impalement by the microelectrode and that pH effects might be less pronounced in the Ussing chamber or in vivo. Despite this caveat, the microelectrode experiments show that, at a constant apical pH of 6.4, the addition of SCFA to the solution has an obviously acidifying effect on the $pH_i$ of the transporting cell layer (compare Figs. 8 and 9). Accumulation of protons within the cytosol thus clearly occurs more rapidly than the influx of $SCFA^-$ anions via a channel or via a major unspecific leak pathway. Whereas it appears likely that the pH regulatory response (24, 26, 53, 54, 77) is more efficient under in vivo conditions, an influx of SCFA into the transporting syncytium of the ruminal epithelium (32) is evidently coupled to an increase in intracellular protons.

We consider it worth mentioning that the microelectrode data also support the presence of a pH microclimate over the transporting layer of cells (see Fig. 7). This finding is in agreement with a recent fluorescence study (45) and previous suggestions (12, 24). Most likely, the thick stratum corneum of the ruminal epithelium (32) creates an unstirred layer with a pH distinct from the bulk of the ruminal solution; this might play a role in the diffusional uptake of H-SCFA (24, 45, 75, 93).

### *A Role for Cytosolic pH in Regulating Ruminal Urea Transport?*

To the knowledge of the authors, acute pH-dependent effects on mammalian urea transporters have not previously been reported (7, 70, 81, 95), although a number of such transporters have been identified in bacteria or yeasts (7).

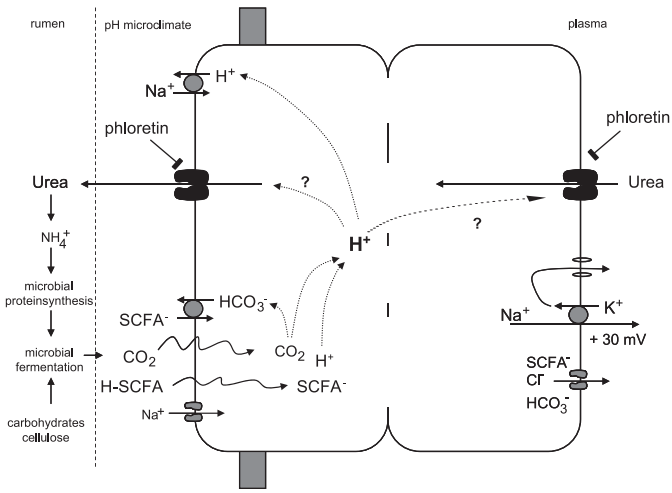


Fig. 10. Model of ruminal urea transport. Endogenous secretion of urea for microbial protein synthesis occurs transcellularly across the multilayered ruminal epithelium, crossing both the basolateral and apical membranes via phloretin-sensitive proteins that may belong to the urea transporter-B group. Secretion of urea into the rumen is highest when $CO_2$ and SCFA are present and when the rumen pH is within a physiological range ~6.2, suggesting regulatory effects on the urea-transporting proteins. This mechanism might synchronize the growth of ruminal microbial populations with the availability of nutrients and the requirements of ruminal homeostasis (for details, see text).

The present study suggests that a change in the cytosolic concentration of protons is a central signaling event that regulates the permeability of the rumen to urea via UT-B or another permeable urea-carrying protein as an immediate response to an increase in ruminal fermentation. In the simplest case, the transporter might be activated by intracellular protons through the formation of hydrogen bonds that induce conformational changes, as reported for bacterial urea transporters (7). A pH optimum may be necessary to protonate certain binding sites within the UT-B pore, configuring it for the passage of the urea molecule. An analogous situation has been reported for the CLC-2 chloride channel (57). The open probability of this channel is low at high values of pH but increases after acidification by protonation of a glutamine residue within the channel pore. When the pH decreases further to values below 7.0, the protonation of an extracellularly located histidine residue (with a near neutral pK value) closes the channel. This results in a biphasic activation-inactivation curve around the physiological pH. Similar pH-dependent effects are reported for the permeability of native chloride channels (4) and bacterial toxins (37). UT-B in the rumen could well be regulated in a similar fashion. However, we do not exclude the possibility that signaling may be far more complex and involve currently unknown pathways, such as sensors for acid or volume (15, 47).

### *Functional Significance*

From the perspective of ruminal physiology, some speculation about the significance of these findings is possible: since the natural diet of the ruminant tends to be low in protein, increased rates of fermentation with rising bacterial populations require an additional influx of urea-nitrogen into the rumen for protein synthesis. Conversely, in situations in which ruminal pH (and in consequence, also cellular pH) drops dangerously low, a decrease in urea influx into the rumen may lower the rate of bacterial growth and, thus, slow down fermentational processes with the release of acidic equivalents. Disturbance of this delicate balance might be one of the reasons that feeding regimes with the imposition of nutritional synchrony of protein and energy have not consistently generated the expected increases in animal productivity (14, 33). Interestingly, UT-B$^{-/-}$ mice mutants display lower weight gain (95), suggesting that the fine-tuning of the urea flux into the gastrointestinal tract may also play a role in monogastric species.

A determination of whether the urea transporters of the erythrocyte (6) or of the descending limb of the vasa recta of the kidney (95) are regulated in the manner described here would be most interesting; this could be of significant importance given the pH drop along the renal vasa recta in conjunction with high levels of $CO_2$ (42). The current consensus appears to be that UT-B is not regulated by short-term effects (6, 81), but we have been unable to find studies of UT-B regulation carried out under conditions that resemble those of this study.

### *Model for the Regulation of Ruminal Urea Transport*

In conclusion, urea is transported across the rumen epithelium passively via phloretin-sensitive proteins that may well correspond to UT-B (Fig. 10). The well-documented positive effects of fermentation products on urea transport in vivo can be also be shown in vitro. The effects are predominantly pH dependent and only apply to the range of luminal pH from 7.0 to 6.2. At extremely high levels of intake, readily fermentable carbohydrates may therefore lead to a reduction in urea recycling to the rumen and increase the amount of urea excreted with the urine, while simultaneously elevating the amount of protein necessary to meet the nutritional requirements of the animal. A better understanding of the factors that increase and decrease the gastrointestinal recycling of urea in vitro and in vivo thus appears to be of central importance in reducing production costs and the release of nitrogen into the environment.

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## DISCLOSURES

No conflicts of interest are declared by the author(s).

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