Modulation of sheep ruminal urea transport by ammonia and pH

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transport by ammonia and pH. *Am J Physiol Regul Integr Comp
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doi:10.1152/ajpregu.00107.2014.—Ruminal fermentation products
such as short-chain fatty acids (SCFA) and CO_2 acutely stimulate urea
transport across the ruminal epithelium in vivo, whereas ammonia has
inhibitory effects. Uptake and signaling pathways remain obscure.
The ruminal expression of SLC14a1 (UT-B) was studied using poly-
merase chain reaction (PCR). The functional short-term effects of
ammonia on cytosolic pH (pH_i) and ruminal urea transport across
native epithelia were investigated using pH-sensitive microelectrodes
and via flux measurements in Ussing chambers. Two variants (UT-B1
and UT-B2) could be fully sequenced from ovine ruminal cDNA.
Functionally, transport was passive and modulated by luminal pH in
the presence of SCFA and CO_2, rising in response to luminal acidifi-
cation to a peak value at pH 5.8 and dropping with further acidifi-
cation, resulting in a bell-shaped curve. Presence of ammonia reduced
the amplitude, but not the shape of the relationship between urea flux
and pH, so that urea flux remained maximal at pH 5.8. Effects of
ammonia were concentration dependent, with saturation at 5 mmol/l.
Clamping the transepithelial potential altered the inhibitory potential
of ammonia on urea flux. Ammonia depolarized the apical membrane
and acidified pH_i, suggesting that, at physiological pH (< 7), uptake
of NH_4^+ into the cytosol may be a key signaling event regulating
ruminal urea transport. We conclude that transport of urea across the
ruminal epithelium involves proteins subject to rapid modulation by
manipulations that alter pH_i and the cytosolic concentration of NH_4^+.
Implications for epithelial and ruminal homeostasis are discussed.

urea; ammonia; rumen; recycling; urea transporter B; ion-selective
microelectrode

ALTHOUGH THE PROCESS OF RENAL EXCRETION AND RECYCLING by
which urea is concentrated within the kidney medulla has been
studied extensively, not much attention has been paid to
gastrointestinal urea recycling, although more than half of the
hepatically produced urea reenters the gut in humans (5, 20),
with repercussions for intestinal stress, liver, and kidney dis-
ease.

The early studies of Schmidt-Nielsen (53) first demonstrated
that a low-protein diet can shift the secretion of urea away from
the kidney and to the gastrointestinal tract (54). One major
purpose of this arrangement is to provide nitrogen for the
microbial organisms that break down structural carbohydrates
in the fermentative parts of the gut, thus allowing the animal to
profit from the energy contained in dietary fiber even when
protein intake is low. In contrast, nitrogen that is renally
excreted is of no use to the animal while the environment is

contaminated (6). A better understanding of the regulation of
gastrointestinal urea secretion thus appears mandatory, last but
not least since it may lead to novel strategies in the manage-
ment of human disease.

Most of the currently available information concerning the
gastrointestinal recycling of urea has been obtained from the
study of ruminants. In these species, up to 90% of daily
nitrogen intake is secreted into the rumen (26, 32), a large
fermentation chamber lined with a multilayered epithelium that
precedes the glandular stomach compartment of these animals.
In the rumen, urea is broken down to ammonia and utilized for
the synthesis of microbial protein that can be reclaimed in the
small intestine (The term ammonia is used without discrimi-
nation between NH_3 and NH_4^+. Chemical symbols are used
when a specification is required). The rates of urinary urea
excretion and urea recycled to the gastrointestinal tract exhibit
large variations and change reciprocally in response to altera-
tions in the diet (49). Gastrointestinal urea recycling ap-
proaches zero in sheep after 24–36 h of starvation. Because
microbial growth in the rumen ceases due to lack of ferment-
able material, the amount of urea entering the gut declines until
the urea that is formed from protein catabolism is almost
exclusively renally excreted (26, 27). Conversely, when ani-
mals were fed structural carbohydrates with extremely low
quantities of nitrogen, only some 2.3% of the urea entering the
plasma from the liver was excreted via urine, with the remain-
der (97.7%) secreted into the gut (64).

At this point, urea transport across the rumen epithelium is
generally accepted to be mediated by diffusion down the
concentration gradient through transport proteins such as the
urea transporter B (UT-B) (57), with possible involvement of
certain aquaglyceroporin (AQP) family members known to
transport urea (51). Attempts to demonstrate the up- or down-
regulation of candidate genes in response to different dietary
regimes have led to contradictory results (16, 38, 39, 43, 51)
and cannot explain the rapid modulation of ruminal urea
transport by fermentation products such as CO_2 and short-
chain fatty acids (SCFA) (26), an effect that can be observed
minutes after application both in vivo (17, 47, 61) and in vitro
(2). In our study, luminal pH was found to be a decisive
cofactor, with transport being maximal at ~pH 6.2 and dimin-
ishing with increasing and decreasing pH, resulting in a bell-
shaped curve (2).

In contrast to the stimulatory effects of CO_2 and SCFA, the
feeding of high-protein diets inhibits urea recycling to the
rumen (26, 29, 49, 62). Intraruminal production of ammonia
from protein, rather than the protein per se, appears to be a key
factor mediating the inhibitory response, with effects being
maximal at 6 mmol/l ammonia (29, 42). Recent in vitro studies
of sheep and goat rumen have confirmed the modulating effect
of ruminal ammonia, showing that ruminal ammonia concen-

tration in vivo has a negative impact on urea transport rates in vitro in the absence of ammonia (18, 41).

In the present study, we attempted to investigate the direct short-term effects of ammonia on ruminal urea transport in vitro. The following questions were addressed. *1*) Can the inhibitory effect of ammonia on ruminal urea transport be confirmed in vitro? *2*) Since the stimulating effect of CO_2 and SCFA on urea transport depends on the luminal pH (2), are there similar correlations between luminal pH and the inhibitory effects of ammonia on urea transport? *3*) Is it possible to demonstrate interactions between SCFA (stimulation) and ammonia (inhibition) on urea transport? *4*) In what way does ammonia affect intracellular pH (pH_i), and might this play a role in modulating urea transport across the ruminal epithelium?

MATERIALS AND METHODS

Animals and Feeding

Experiments were conducted in accordance with German law for the care and use of experimental animals and performed essentially as described previously (19). The sheep (German dairy) were 9–10 mo old at the time of the experiment, with a range of body weight between 33 and 50 kg. All animals were housed in pens with straw bedding and fed a pure hay diet ad libitum over a period of 6 wk containing [per kg dry matter (DM)]: 144 g of crude protein, 28 g of fat, 277 g of crude fiber, 89 g of ash, 29 g of potassium, 2.2 g of sodium, and 8.5 MJ of metabolizable energy (ME). At the beginning of the experimental period, hay intake was 1,000 g·animal^{-1}·day^{-1} (88% DM) and was offered in two portions at 7 AM and 3 PM, equaling an intake of 7.5 MJ ME, which is slightly above requirements (23). One week before the experiment, the sheep were kept individually in pens on straw to control the feed intake. Part of the experiments was performed with concentrate-fed sheep receiving 400 g of hay and 400 g of concentrate twice a day (7 AM and 3 PM) essentially as described previously (10). The supplemented concentrate contained per kg DM was 176 g of crude protein, 143 g of crude fiber, 33 g of fat, and 104 g of ash. With the latter diet, the energy intake could be increased to 11.2 MJ ME·animal^{-1}·day^{-1}, permitting a growth rate of ca. 200 g/day. The mixed diet (hay + concentrate) was changed stepwise within 4 days and each sheep was fed 2–3 wk before an experiment (19).

Ethical Approval

Animals were stunned by captive bolt and euthanized by exsanguination before tissues were removed for experiments (permit no. T0064/99). All experiments were performed according to German laws for the protection of animals and were approved by the Animal Welfare and Ethics Representative of the Veterinary Faculty/Freie Universität Berlin.

Sequencing of UT-B (SLC14A1) in Sheep

Ovine cDNA was synthesized by reverse transcription of total RNA isolated from a rumen epithelium primary cell culture using the Nucleospin RNA II kit (Macherey & Nagel, Dueren, Germany). Reverse transcription was performed using the iScript cDNA synthesis kit (Bio-Rad, Munich, Germany) containing oligo(dT) primers and random hexamers. PCR primers were designed according to the Bos taurus sequence AY624602, creating four overlapping fragments. PCR was performed using a proofreading polymerase (HotStar high-fidelity polymerase; Qiagen, Hilden, Germany). Fragment lengths were checked by agarose gel electrophoresis and PCR products sent for sequencing in both directions (AGOWA, Berlin, Germany). Results matched the Ovis aries SLC14A1 perfectly with its predicted

1,155 bp (Gene ID 100302356) and were published under the accession no. GQ118969, including all information concerning primers.

Because Stewart et al. (57) published that they had found a longer, alternatively spliced variant in cows (bUT-B2, accession no. AY838799) and Simmons et al. (55) associated the expression level of the longer variant with concentrate feeding, experiments were repeated with total RNA isolated from hay-fed and concentrate fed sheep. Indeed, primers for fragment 1 (5′ end) produced four bands (from 308 to 512 bp) in hay-fed as well as in concentrate-fed sheep. Each of these bands was isolated and sent for sequencing. All four sequences were identical toward the 3′ end. Toward the 5′ end, two out of the four sequences were terminated by a stop codon in any reading frame, one coded for the sequence published previously, and the fourth sequence was in frame with the published sequence but without a stop codon, prolonging the coding sequence potentially by 165 bp beyond the published 5′ exon. Because the translated extension shows 85% identity with the bovine splice variant (AY838799), we conclude that this is a second variant of ovine UT-B and have added it to GenBank (KJ776794).

Rumen Epithelium Isolation and Incubation

Again, experiments were essentially performed as described previously (2). Briefly, after stunning and exsanguination, the forestomachs were removed from the abdominal cavity within 2–3 min. A 250-cm^2 piece of rumen wall was taken from the ventral sac, repeatedly cleaned in a warm buffer solution (38°C), and stripped from the muscle layer. The tissues were subsequently transported to the laboratory in a buffer solution that contained (in mmol/l) 115 NaCl, 25 NaHCO$_3$, 0.4 NaH$_2$PO$_4$, 2.4 Na$_2$HPO$_4$, 5 KCl, 5 glucose, 1.2 CaCl$_2$, and 1.2 MgCl$_2$, pH 7.4, at 38.0°C, adjusted to 280 mosmol/l with mannitol and gassed with 95% O$_2$-5% CO$_2$. Epithelia (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 tissue was bathed on each side with 16 ml of buffer solution by using a gas lift system and gassed with 95%O$_2$-5%CO$_2$ at 38°C.

Experimental Solutions for Ussing Chamber Experiments

The standard experimental buffer (which was used on the serosal side throughout) contained (in mmol/l) 115 NaCl, 25 NaHCO$_3$, 0.4 NaH$_2$PO$_4$, 2.4 Na$_2$HPO$_4$, 5 KCl, 5 glucose, 1 urea, 8 MOPS [3-(N-morpholino)propanesulfonic acid (C$_7$H$_{15}$NO$_4$S)], 2.5 glutamine, 1.2 CaCl$_2$, and 1.2 MgCl$_2$ adjusted to pH 7.4 and bubbled with O$_2$. This buffer was modified by equimolar replacement for use on the mucosal side (Tables 1 and 2). Solutions containing NaHCO$_3$ were bubbled with 95%O$_2$-5%CO$_2$. Mannitol was used to adjust the osmolarity of all solutions to 300 mosmol/l (Osmomat 030-D; Gonotec, Berlin, Germany). The urease inhibitor phenyl phosphorodiamidate (0.1 mmol/l; ABCR, Karlsruhe, Germany) was added to all solutions. All reagents were of analytical grade.

Electrical measurements. Electrical measurements were obtained continuously from a computer-controlled voltage clamp device (Mussler, Aachen, Germany). Modified tips filled with KCl-Agar were positioned ~3 mm from each surface of the tissue and connected to Ag-AgCl electrodes for measurement of the transepithelial potential difference (PD$_t$). Similar tips were inserted ~2 cm from the surface of the tissue for the application of current (I$_{sc}$). The tissues were incubated under short-circuit conditions if not specified otherwise. Transepithelial conductance (G$_t$) was calculated by measuring the displacements in the potential difference (ΔPD) caused by the application of a bipolar pulse of 100 μA and 1-s duration.

Flux studies. Unidirectional mucosal-to-serosal (ms) and serosal-to-mucosal (sm) fluxes (J$_{ms}$, J$_{sm}$) of urea were determined on paired tissues from the same rumen under short-circuit conditions. Tissues were paired so that the G$_t$ of each tissue in a pair did not differ by >25%. Net transepithelial fluxes were calculated as

Table 1. *Mucosal buffers (buffers 1–9)*

	Concentration, mmol/l								
Chemicals	Buffer 1	Buffer 2	Buffer 3	Buffer 4	Buffer 5	Buffer 6	Buffer 7	Buffer 8	Buffer 9
NaCl	95	70	70	10	55	55	55	70	70
NaHCO$_3$[a]	0	25	25	25	0	0	0	25	25
NaH$_2$PO$_4$	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Na$_2$HPO$_4$	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.4
KCl	5	5	5	65	5	5	5	5	5
Glucose	5	5	5	5	5	5	5	5	5
MOPS[b]	8	8	8	8	8	8	8	8	8
Urea	1	1	1	1	1	1	1	1	1
Na-gluconate	0	0	0	0	0	80	40	0	0
Na-acetate	25	25	25	25	50	0	25	25	25
Na-propionate	10	10	10	10	20	0	10	10	10
Na-butyrate	5	5	5	5	10	0	5	5	5
Glutamine	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
CaCl$_2$	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
MgCl$_2$	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
CH$_6$ClN[c]	0	0	0	0	0	0	0	0	0
NH$_4$Cl (C E)[d]	0 ┊ 5	0 ┊ 5	0 ┊ 2	0 ┊ 2	0 ┊ 5	0 ┊ 5	0 ┊ 5	0 ┊ 30	X[f]
NMDGCl (C E)[e]	5 ┊ 0	5 ┊ 0	2 ┊ 0	2 ┊ 0	5 ┊ 0	5 ┊ 0	5 ┊ 0	30 ┊ 0	4

[a]Solutions containing no NaHCO$_3$ were bubbled with 100% O$_2$; solutions containing NaHCO$_3$ were bubbled with 95%O$_2$-5%CO$_2$. [b]MOPS [3-(*N*-morpholino)propanesulfonic acid (C$_7$H$_{15}$NO$_4$S)]. [c]Methylammonium chloride. [d,e]As indicated, solutions contained either NH$_4$Cl or NMDGCl [*N*-methyl-D-glucamine chloride (C$_7$H$_{17}$NO$_5$Cl)]; the buffers were used, for example, with either 0 in the control or 5 mmol/l NH$_4$Cl in the experimental group (C E). [f]Luminal ammonia concentrations of 0.1, 0.2, 0.4, 0.6, and 1 mmol/l.

the difference between unidirectional fluxes in opposite directions ($J_{net} = J_{ms} - J_{sm}$).

Experiments commenced after the electrical parameters had stabilized in the open-circuit mode (generally 30–40 min after mounting). At this time the mucosal buffer solution was changed from control to the respective buffer under investigation, and the tissues were short-circuited. [^{14}C] urea was added to the mucosal side of one tissue of each pair and to the serosal side of the other ("hot side"). The first samples (1 ml) were taken from the unlabeled ("cold") side after 20 min of equilibration and then at 20-min intervals for three consecutive flux periods. Each sample was replaced by an equal volume of fresh corresponding buffer solution, and the data were corrected for this dilution. Samples (100 µl) from the labeled bathing solution (hot side)

were taken before the first and after the last flux period for the calculation of the specific radioactivity. [^{14}C] urea (46.25 kBq) was assayed in scintillation liquid (Rotiszint, Roth-Karlsruhe, Germany) by using a β-counter (LKB Wallace-Perkin-Elmer, überlingen, Germany). Flux rates were calculated by relating the radioactivity appearing on the cold side to the specific activity added to the hot side.

Microelectrode Experiments

Electrical measurements. Fresh ruminal epithelium was introduced into a small microelectrode chamber, apical side up, and perfused continuously with solution warmed to 37°C and bubbled with O$_2$, all

Table 2. *Mucosal buffers (buffers 10–14)*

	Concentration, mmol/l														
Chemicals	Buffer 10				Buffer 11				Buffer 12				Buffer 13		Buffer 14
NaCl	60				60				60				70		70
NaHCO$_3$[a]	25				25				25				25		25
NaH$_2$PO$_4$	0.4				0.4				0.4				0.4		0.4
Na$_2$HPO$_4$	2.4				2.4				2.4				2.4		2.4
KCl	5				5				5				5		5
Glucose	5				5				5				5		5
MOPS[b]	8				8				8				8		8
Urea[c]	5 ┊ 10 ┊ 15 ┊ 20				0 ┊ 10 ┊ 15 ┊ 20				1				1		1
Mannitol[c]	15 ┊ 10 ┊ 5 ┊ 0				20 ┊ 10 ┊ 5 ┊ 0				0				0		0
Na-acetate	25				25				25				25		25
Na-propionate	10				10				10				10		10
Na-butyrate	5				5				5				5		5
Glutamine	2.5				2.5				2.5				2.5		2.5
CaCl$_2$	1.2				1.2				1.2				1.2		1.2
MgCl$_2$	1.2				1.2				1.2				1.2		1.2
CH$_6$ClN[d]	0				0				0				0		5
NH$_4$Cl[e]	0 ┊ 0 ┊ 0 ┊ 0				5 ┊ 5 ┊ 5 ┊ 5				0 ┊ 1 ┊ 2.5 ┊ 5 ┊ 15				0 ┊ 2.5 ┊ 5		0
NMDGCl[f]	5 ┊ 5 ┊ 5 ┊ 5				0 ┊ 0 ┊ 0 ┊ 0				15 ┊ 14 ┊ 12.5 ┊ 10 ┊ 0				5 ┊ 2.5 ┊ 0		0

[a]Solutions containing NaHCO$_3$ were bubbled with 95%O$_2$-5%CO$_2$. [b]MOPS. [c]Buffers were used with, for example, either 5 mmol/l urea and the corresponding concentration of 15 mmol/l mannitol or 10 and 10 mmol/l, etc. [d]Methylammonium chloride. [e,f]As indicated in the text, NH$_4$Cl was substituted on an equimolar basis by NMDG (C$_7$H$_{17}$NO$_5$Cl); buffers were used, for example, with 0 NH$_4$Cl and with the corresponding concentration NMDGCl (5, 15, etc.) in the control group and vice versa in the experimental group.

Table 3. *Effect of luminal ammonia (30 mmol/l) on unidirectional urea flux rates across the rumen epithelium*

NH$_4$Cl, mmol/l	pH	J$_{ms}$, nmol·cm^{-2}·h^{-1}	J$_{sm}$, nmol·cm^{-2}·h^{-1}	J$_{net}$, nmol·cm^{-2}·h^{-1}	I$_{SC}$, μEq·cm^{-2}·h^{-1}	G$_t$ mS/cm^2	*N/n*
0	7.4	22.56 ± 9.00[a]	23.16 ± 8.79[a]	−0.60 ± 6.60	0.72 ± 0.18[a]	2.56 ± 0.51[a]	3/9
30	7.4	22.69 ± 6.30[a]	21.45 ± 4.59[a]	1.24 ± 8.94	1.25 ± 0.54[b]	2.41 ± 0.36[a]	3/9
0	6.4	122.53 ± 32.81[b]	117.66 ± 31.32[b]	4.87 ± 29.86	0.87 ± 0.17[a]	2.18 ± 0.52[a]	4/12
30	6.4	39.78 ± 13.06[c]	47.60 ± 11.81[c]	−7.82 ± 18.95	1.20 ± 0.17[b]	2.17 ± 0.52[a]	4/12

Values are means ± SD. J$_{ms}$, mucosal-to-serosal flux; J$_{sm}$, serosal-to-mucosal flux; J$_{net}$, net flux; I$_{SC}$, short-circuit current; G$_t$, transepithelial conductance; N, no. of sheep; n, no. of tissues. Urea concentration, 1 mmol/l; luminal pH 7.4 and 6.4, *buffer 8*. Values in the same column bearing different superscripted letters are significantly different at $P < 0.05$.

as described previously (2). The pH of the experimental solutions was monitored hourly. Electrophysiological parameters of the epithelium were controlled via voltage clamp (Biomedical Instruments, Munich, Germany) (31). Transepithelial voltage pulses of 10-mV amplitude and 0.5-s duration were used to measure G$_t$, whereas intracellular pH and the apical potential were measured with a two-barreled ion-sensitive microelectrode and an FDA223 Dual Electrometer (World Precision Instruments, Sarasota, FL), all referenced to the mucosal side via a KCl bridge and recorded by using LabChart 7 Pro (version 7.3.3.) for Windows (AD Instruments, Bella Vista, Australia).

Double-barreled microelectrodes were prepared from filamented GC120F 10 and GC150F 15 borosilicate glass tubing (Harvard Apparatus, Kent, UK), adjoined with core cable ends and shrink tubing, baked for 10 min (190°C), and pulled (PMP-107; Microdata Instrument, South Plainfield, NJ). During vapor silanization (dichlorodimethylsilane; Sigma-Aldrich, Taufkirchen, Germany), the smaller capillary was connected to a pressure outlet. After baking at 190°C for 2 h, the larger diameter channel was filled with 3 μl of Hydrogen Ionophore I-Cocktail A (Sigma-Aldrich). This channel was back-filled with a solution containing 100 mmol/l KCl and 20 mmol/l HEPES, pH 7.4, on the morning of the experiment. The reference channel was filled with 0.5 mmol/l KCl. After bevelling to 15–50 MΩ (BV-10; Sutter Instrument, Novato, CA), electrodes were calibrated and used if they showed a differential response of >40 mV/pH unit (pH 7.4 to 6.4) between the pH-selective and nonselective barrel.

Solutions for microelectrode experiments. In all experiments, the serosal side of the tissue was perfused with a standard NaCl solution ("microelectrode standard," *buffer 7*) buffered to pH 7.4 and containing (in mmol/l) 70 NaCl, 0.4 NaH$_2$PO$_4$, 2.4 Na$_2$HPO$_4$, 5 KCl, 40 Na gluconate, 1.2 MgCl$_2$, 1.2 CaCl$_2$, 40 NMDGCl, 5 glucose, 8 MOPS, and 100% O$_2$. This solution was also used to perfuse the apical side of the tissue at the beginning of the experiments. In solutions containing ammonia, NMDGCl was replaced by equimolar amounts of NH$_4$Cl. The osmolarity was adjusted to 300 mosmol/l, using mannitol and the pH of the solution as indicated (TRIS).

Statistics. All evaluations were carried out by using Sigma Plot program version 11.0 for Windows (Systat Software). Results are given as means ± SE. Significance testing was performed between paired values from the same experiment by using Friedman repeated-measures analysis of rank, with the Student-Newman-Keuls (SNK) method used for pairwise multiple comparisons. Where only two columns of values were tested, the data were tested for normality and compared by using the paired Student's t-test. P values of <0.05 were considered significant. N refers to the number of experimental animals, and n refers to the number of tissues.

RESULTS

Sequencing of UT-B in Sheep

UT-B is generally thought to represent the major pathway for the influx of urea into the gastrointestinal tract and has been demonstrated in the rumen repeatedly (13, 38, 55, 57). In analogy to previous findings in the bovine species (55, 57), we have identified two transcriptional variants of UT-B in the

ovine rumen, a short variant (GQ118969) that is in accord with the published gene (Gene ID 100302356) and a second variant (KJ776794) that very likely corresponds to the bovine alternative splice variant (AY838799), as the amino acid sequence of the extension was 85% identical.

Effect of Ammonia on Urea Flux at pH 7.4 and 6.4

To gain a better understanding of the functional regulation of ruminal urea flux, tissues were subsequently studied in Ussing chambers. In the following, only serosal to mucosal urea flux (J$_{sm}$) will be discussed, because this is the physiologically relevant direction of flux. However, fluxes in the opposite direction (J$_{ms}$) were measured routinely in parallel and found to be of comparable magnitude (see Table 3 and Fig. 1). Thus no net flux of urea was observed, and urea flux was entirely passive, which is in agreement with previous observations (2).

Flux Measurements in the Ussing Chamber

Tissues were incubated in a buffer solution that contained SCFA and HCO$_3^-$ and was bubbled with 95%O$_2$-5%CO$_2$. As seen before under these conditions (2), the decrease in luminal pH from 7.4 to 6.4 significantly enhanced urea transport rates in both directions by a factor of five to six, with J$_{sm}$ rising from
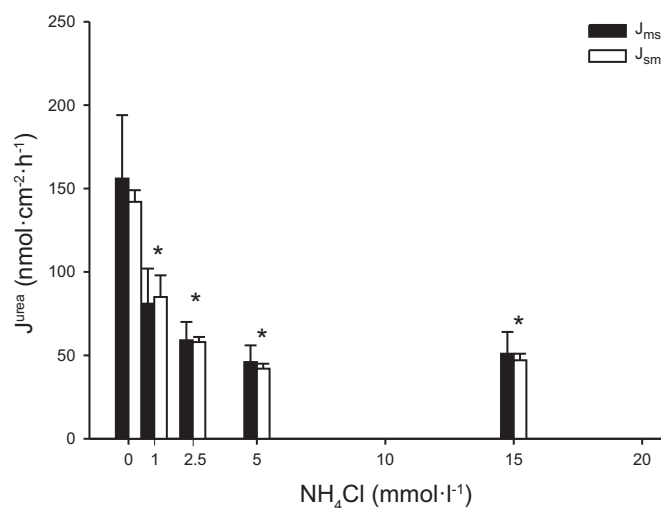

Fig. 1. Effect of increasing the concentration of luminal ammonium on urea flux rates across rumen epithelium of pH 7.4 (urea concentration of 1 mmol/l, means ± SD, luminal pH 6.4, *buffers 12* and *13*). This figure combines results from 2 series of experiments: *1*) 0, 2.5, and 5.0 mmol/l luminal ammonium (3 sheep; 6–9 tissues) and *2*) 0, 1, 2.5, 5.0, and 15 mmol/l (3 sheep; 6–9 tissues). *Unidirectional serosal-to-mucosal (J$_{sm}$) and mucosal-to-serosal (J$_{ms}$) urea significantly different ($P < 0.05$) from control (0 mmol/l). No differences were observed between J$_{sm}$ and J$_{ms}$ urea.
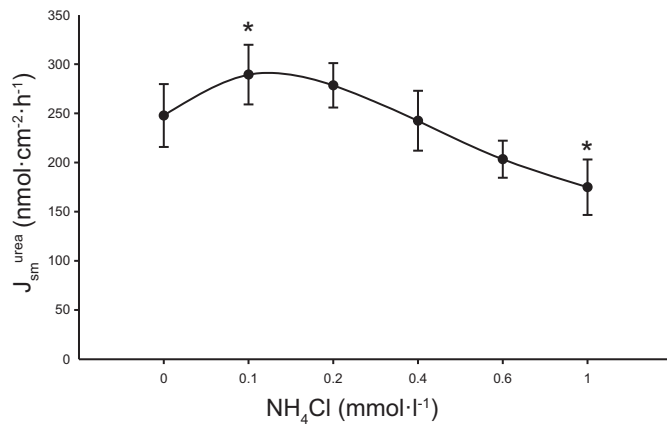
Fig. 2. Effect of low luminal ammonium concentrations on urea flux rates (urea concentration 1 mmol/l, means ± SD, *buffer 9*; luminal pH 6.4; 3 sheep; 7–12 tissues). *J_{sm} urea significantly different ($P < 0.05$) from control (0 mmol/l).
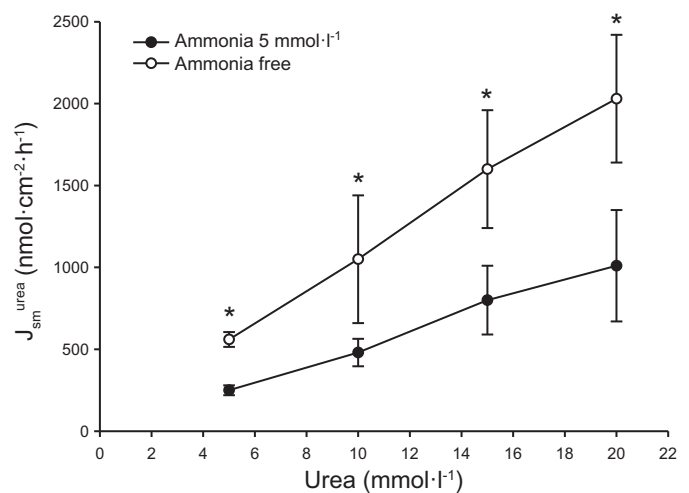

Fig. 3. Correlation between serosal urea concentration and urea transport without ($y = 99.2x + 70$, $r^2 = 0.99$) and with 5 mmol/l luminal ammonia ($y = 52x - 15$, $r^2 = 0.99$); (means ± SD, *buffers 10* and *11*; 3 sheep, 7–9 tissues). *J_{sm} urea significantly different between treatments ($P < 0.05$).

23.16 ± 8.79 nmol·cm^{-2}·h^{-1} at pH 7.4 to 117.66 ± 31.32 nmol·cm^{-2}·h^{-1} at pH 6.4 (Table 3). The stimulatory effects of lowering luminal pH in the presence of SCFA and CO_2 could also be observed in the additional presence of 30 mmol/l ammonia, with unidirectional transport rate (J_{sm}) rising from 21.49 ± 4.59 nmol·cm^{-2}·h^{-1} at pH 7.4 to 47.60 ± 11.81 nmol·cm^{-2}·h^{-1} at pH 6.4. However, this stimulation was much lower than the corresponding increase in the control group from 23.16 ± 8.79 to 117.66 ± 31.32 nmol·cm^{-2}·h^{-1} (Table 3), indicating that luminal ammonia (30 mmol/l) significantly inhibited J_{sm} urea transport by ~60%. At pH 7.4, urea flux rates were not changed by luminal ammonia (30 mmol/l) (Table 3).

Ruminal ammonia concentration can rise above 30 mmol/l on occasion, but it is usually much lower. Therefore, we wished to determine possible effects of lower and more physiological concentrations of ammonia. In a first series of experiments, we tested 0, 2.5, and 5 mmol/l luminal ammonia. Even at 2.5 mmol/l ammonia, J_{sm} urea was reduced significantly from 142.23 ± 07.48 to 58.22 ± 03.08 nmol·cm^{-2}·h^{-1}; this was further diminished to 42.07 ± 02.75 nmol·cm^{-2}·h^{-1} by 5.0 mmol/l ammonia (Fig. 1).

In a second series of experiments, 0, 1, 5, and 15 mmol/l ammonia were compared. As little as 1 mmol/l ammonia significantly reduced J_{sm} urea by ~40% from 142.23 ± 07.48 to 85.00 ± 12.61 nmol·cm^{-2}·h^{-1}. No significant differences were observed between 5 and 15 mmol/l ammonia (Fig. 1).

The pronounced effect of 1 mmol/l ammonia on urea transport was surprising and led to the conclusion that even lower concentrations might be able to modulate urea transport. Therefore, We used 0 (control), 0.1, 0.2, 0.4, 0.6, and 1 mmol/l ammonia and observed a significant stimulation at extremely low luminal ammonia concentrations (0.1 mmol/l). At an ammonia concentration of >0.2 mmol/l, urea transport was increasingly inhibited (Fig. 2).

In all the foregoing experiments concerning the effects of luminal ammonia, a concentration of 1 mmol/l urea was used. In the subsequent experiments, the serosal urea concentration was increased from 5 to 20 mmol/l. A linear correlation was found between the serosal urea concentration and J_{sm} urea, again supporting passive urea transport (Fig. 3). In the presence of 5 mmol/l luminal ammonia, J_{sm} urea was significantly reduced by roughly 60%, which is in close agreement with the previous experiments with lower concentrations of urea and 5 mmol/l ammonia (Fig. 1).

Ammonia Versus Methylammonia

To further characterize the inhibitory effects of ammonia on urea transport, we tested the ammonium analog methylammonium chloride. Both substrates have a similar pK value, but the oil/water coefficient of methylammonia is much lower than that of ammonia (log P_{ow}: -3.82 and -1.38, respectively), suggesting a limited ability for transport of methylammonium via lipid diffusion. Instead, both substrates compete for transport by the same transporter in a large number of systems. Methylammonium significantly inhibited J_{sm} urea, but to a smaller extent than ammonia ($P = 0.02$; Table 4).

Effect of PD_t on Urea Transport Rates

Ammonia generally occurs in two forms, as NH_3 or NH_4^+. Since the pK of ammonia is ~9.20, NH_4^+ is the predominant form at physiological pH values in the rumen fluid and accounts for 99.9% of total ammonia at pH 6.4. Therefore, luminal NH_4^+ has been suggested to enter the rumen epithelium cell via a cation channel (3, 7), with the luminal uptake of NH_4^+ being driven by the potential differences of the apical membrane (PD_a). Accordingly, a change in PD_t, and hence, of PD_a, had an effect on NH_4^+ transport across the tissue (7).

Table 4. *Effect of luminal methylammonium chloride (CH_6ClN) or ammonia on urea flux rates*

Treatment	J_{sm}, nmol·cm^{-2}·h^{-1}	I_{SC}, µEq·cm^{-2}·h^{-1}	G_t, mS/cm^2	N/n
Control	203.43 ± 47.06[a]	1.85 ± 0.43	3.45 ± 0.97	3/23
NH$_4$Cl, 5 mmol/l	98.97 ± 18.55[b]	1.57 ± 0.29	3.41 ± 0.89	3/20
CH$_6$ClN, 5 mmol/l	155.38 ± 32.66[c]	1.50 ± 0.19	2.74 ± 0.41	3/20

Values are means ± SD. Urea concentration: 1 mmol/l, luminal pH 6.4, *buffers 2* and *14*. Values in the same column bearing different superscripted letters are significantly different at $P < 0.05$.

Table 5. *Effects of PD$_t$ on J$_{sm}$ urea flux rates in the presence of 2 mmol/l luminal ammonia*

NH$_4$Cl, mmol/l	PD$_t$, mV	J$_{sm}$, nmol·cm^{-2}·h^{-1}	I$_{SC}$, μEq·cm^{-2}·h^{-1}	G$_t$, mS/cm^2	N/n
2	0	92.65 ± 19.48[a]	1.98 ± 0.57[a]	3.47 ± 0.92	3/22
2	+25	108.54 ± 27.87[b]	1.33 ± 0.28[b]	3.15 ± 0.88	3/23
2	−25	75.30 ± 13.55[c]	−4.44 ± 1.18[c]	3.43 ± 1.04	3/24

Values are means ± SD; urea concentration: 1 mmol/l, luminal pH 6.4, *buffer 3*. PD$_t$, transepithelial potential difference. Values in the same column bearing different superscripted letters are significantly different at *P* < 0.05.

Table 7. *Effect of luminal K$^+$ on urea flux rates under open-circuit conditions*

NH$_4$Cl, mmol/l	K$^+$	J$_{sm}$, nmol·cm^{-2}·h^{-1}	PD$_t$, mV	G$_t$, mS/cm^2	N/n
0	5	189.7 ± 45.27[a]	13.04 ± 3.40[a]	3.48 ± 0.89[a]	3/24
2	5	86.61 ± 21.84[b]	18.18 ± 4.70[a]	2.48 ± 0.57[a]	3/23
2	65	111.14 ± 19.81[c]	30.8 ± 6.46[b]	4.37 ± 0.81[b]	3/24

Values are means ± SD; urea concentration: 1 mmol/l, luminal pH 6.4, *buffers 3* and *4*. Values in the same column bearing different superscripted letters are significantly different at *P* < 0.05.

A PD-dependent change of NH$_4^+$ transport into the cell might thus indirectly alter urea transport. Therefore, we studied the effect of PD$_t$ on urea transport. In the presence of 2 mmol/l ammonia and at pH 6.4 (99.9% NH$_4^+$), the urea transport rate was significantly enhanced from 92.65 ± 19.48 (short-circuit conditions) to 108.54 ± 27.87 nmol·cm^{-2}·h^{-1} by a PD$_t$ of + 25 mV and, vice versa, was reduced by −25 mV (Table 5). However, in the presence of 5 mmol/l ammonia at the lumen side, corresponding variations of PD$_t$ did not change J$_{sm}$ urea significantly (*P* = 0.14; Table 6).

K$^+$ is a major cation in the ruminal fluid, and as is well established, K$^+$ changes PD$_a$ and PD$_t$ (31, 33, 40). In the presence of 2 mmol/l ammonia, we tested the effects of luminal K$^+$ on urea flux rates by raising luminal K$^+$ from 5 to 65 mM and observed a significant increase in J$_{sm}$ urea (*P* = 0.025) under open-circuit conditions (Table 7). However, no effect of K$^+$ was observed (*P* = 0.128) under short-circuit conditions (Table 8), suggesting that effects might indeed be mediated by depolarization of the apical membrane with reduced influx of NH$_4^+$, possibly via a cation channel in the luminal membrane (1, 35).

Accordingly, three cation channel inhibitors, tetraethylammonium chloride (TEACl; 10 mmol/l), quinidine (1 mmol/l), and verapamil (0.25 and 1 mmol/l), were added to the mucosal side of the ruminal epithelium in the presence of 5 mM ammonia. Neither TEACl nor quinidine changed urea transport rates (data not shown). Luminal verapamil (1 mmol/l) significantly diminished the effect of ammonia (*P* = 0.025; Table 9). In the absence of ammonia, any effects of verapamil were not significant.

Modulation of Urea Transport by SCFA

As is well established, SCFA stimulate urea transport in vivo (2, 17, 42, 61) and in vitro (2). By contrast, the results of the present study and previous in vivo experiments (30) have clearly shown an inhibitory effect of ammonia on urea transport so that both fermentation products modulate urea transport

in an opposite manner. The interaction between both substrates was tested by increasing luminal SCFA concentration (0, 40, and 80 mmol/l) without and with a luminal ammonia concentration of 5 mmol/l at all SCFA concentrations. At a luminal pH of 6.4, the flux rates of J$_{sm}$ urea increased linearly and significantly with increasing SCFA concentration in both groups (control and luminal ammonia) (Fig. 4). However, the increment of J$_{sm}$ urea was significantly lower (*P* = 0.015 and 0.011) in the ammonia group (at both 40 and 80 mmol/l SCFA), and an increase in SCFA concentrations did not change the relative inhibition of urea transport by ammonia, which was roughly 63% at both 40 and 80 mmol/l SCFA.

Modulation of Urea Transport by Luminal pH.

Earlier work from our laboratory has demonstrated that in the presence of SCFA, a bell-shaped dependency exists between luminal pH and urea flux (2). To test whether this effect could also be observed in the presence of ammonia, we determined urea flux rates (J$_{sm}$) at decreasing luminal pH from 7.4 to 5.4 on J$_{sm}$ urea in the presence and absence of luminal ammonia (5 mmol/l). Otherwise, the buffer solutions were identical to those used previously, contained SCFA, and were bubbled with 95% O$_2$-5% CO$_2$. As in our previous experiments, the luminal pH clearly modulated urea transport, resulting in a bell-shaped alteration of J$_{sm}$ urea (Fig. 5), with J$_{sm}$ urea progressively increasing with the decrease of luminal pH from 7.4 to 5.8. The peak of flux rates was observed at pH 5.8. Lower pH values reduced J$_{sm}$ urea. The fluxes of J$_{sm}$ urea differed significantly between the two groups at pH 6.6 (*P* = 0.046), 6.2 (*P* = 0.041), 5.8 (*P* = 0.032), and 5.4 (*P* = 0.043). Whereas the addition of ammonia shifted the curve toward lower values, the shape of the curve was not altered significantly. In particular, the peak remained at a pH of 5.8.

There is a growing body of evidence that the feeding regime modulates urea transport (18, 41) and the expression of UT-B (55). Therefore, we repeated the experiment with decreasing luminal pH with tissues from concentrate-fed animals. A sim-

Table 6. *Effects of PD$_t$ on urea flux rates in the presence of 5 mmol/l luminal ammonia*

NH$_4$Cl, mmol/l	PD$_t$, mV	J$_{sm}$, nmol·cm^{-2}·h^{-1}	I$_{SC}$, μEq·cm^{-2}·h^{-1}	G$_t$, mS/cm^2	N/n
5	0	75.27 ± 11.29	2.05 ± 0.35[a]	3.72 ± 0.98	3/20
5	+25	78.86 ± 12.79	1.01 ± 0.80[b]	3.49 ± 0.84	3/22
5	−25	64.12 ± 15.02	−6.29 ± 1.40[c]	4.24 ± 0.99	3/22

Value are means ± SD; urea concentration: 1 mmol/l, luminal pH 6.4, *buffer 2*. Values in the same column bearing different superscripted letters are significantly different at *P* < 0.05.

Table 8. *Effect of luminal K$^+$ concentrations on urea flux rates under short-circuit conditions*

NH$_4$Cl, mmol/l	K$^+$	J$_{sm}$, nmol·cm^{-2}·h^{-1}	I$_{SC}$, μEq·cm^{-2}·h^{-1}	G$_t$, mS/cm^2	N/n
0	5	191.02 ± 46.57[a]	1.02 ± 0.16[a]	2.75 ± 0.55[a]	3/24
2	5	99.20 ± 21.87[b]	1.20 ± 0.20[a]	2.18 ± 0.17[a]	3/23
2	65	104.51 ± 32.59[b]	4.53 ± 0.61[b]	3.89 ± 0.59[b]	3/24

Values are means ± SD; urea concentration: 1 mmol/l, luminal pH 6.4, *buffers 3* and *4*. Values in the same column bearing different superscripted letters are significantly different at *P* < 0.05.

Table 9. *Effect of 1 mmol/l luminal verapamil on urea flux rates*

NH_4Cl, mmol/l	Verapamil	J_{sm}, $nmol \cdot cm^{-2} \cdot h^{-1}$	I_{SC}, $\mu Eq \cdot cm^{-2} \cdot h^{-1}$	G_t, mS/cm^2	N/n
0	0	185.17 ± 32.93^a	0.97 ± 0.26^a	4.00 ± 1.10	2/12
0	1	155.69 ± 20.90^a	0.76 ± 0.16^b	4.24 ± 1.20	2/11
5	0	90.47 ± 16.00^b	1.09 ± 0.22^a	3.72 ± 0.76	2/12
5	1	131.64 ± 29.11^c	0.72 ± 0.18^b	5.10 ± 1.27	2/12

Values are means $\pm$ SD; urea concentration: 1 mmol/l, luminal pH 6.4, *buffer 2*. Values in the same column bearing different superscripted letters are significantly different at $P < 0.05$.

ilar bell-shaped curve was obtained (see Fig. 6), but the peak had shifted from pH 5.8 to 6.2.

Microelectrode Experiments

If ammonia enters the tissue primarily as NH_3, the intracellular pH should change toward more alkaline values, whereas entry primarily in the ionic form as NH_4^+ should result in changes in apical potential and a decrease in pH. For this reason, double-barreled pH-sensitive microelectrodes were used to investigate the response of cells within the intact ruminal epithelium upon exposure to luminal ammonia (see Figs. 7 and 8). The electrophysiological parameters of the tissue in toto were monitored simultaneously.

An alkalinization of the intracellular pH could not be observed. At luminal pH 7.4, the addition of ammonia had only a nonsignificant effect on intracellular pH, which dropped slightly from 6.76 ± 0.16 without ammonia to 6.68 ± 0.20 with ammonia ($N = 3$, $n = 5$, $P > 0.05$; Figs. 8 and 9). Conversely, at luminal pH 6.4, the addition of 40 mmol/l luminal ammonia reduced intracellular pH significantly from pH 6.44 ± 0.13 to 6.19 ± 0.09 ($N = 3$, $n = 5$, $P < 0.05$, SNK test).

The PD_a was depolarized by luminal ammonia at both pH 7.4 (from -24 ± 2 to -15 ± 6 mV, $N/n = 3/5$, $P < 0.05$; Fig. 9) and pH 6.4 (from -17 ± 3 to -9.4 ± 1 mV, $N/n = 3/5$, $P < 0.05$). The ammonia-induced depolarization (of 8 ± 2 mV at pH 6.4 and 10 ± 2 mV at pH 7.4) did not depend significantly on luminal pH.

Fig. 5. Effect of luminal ammonia and luminal pH on urea flux rates (urea concentration 1 mmol/l, means $\pm$ SD, *buffer 1*; 4 sheep, 6–8 tissues). *J_{sm} urea significantly different ($P < 0.05$) between treatments.

As in a previous Ussing chamber study (3), luminal ammonia had a positive effect on the tissue conductance and increased the short-circuit current at luminal pH 7.4 and 6.4. PD_t rose by an average of 3.7 ± 0.6 mV at pH 7.4, with similar effects being seen at pH 6.4 (3.7 ± 0.3 mV; serosal versus mucosal side, open-circuit mode). Interestingly, the apical depolarization attributable to ammonia was slightly larger than the difference induced in the PD_t, suggesting that the apical permeability of the epithelium to NH_4^+ was higher than that of the basolateral membrane.

Smaller, clearly visible effects emerged when ammonia was added luminally in a concentration of 10 mmol/l at pH 6.4, with the pH_i dropping by 0.10 ± 0.06 pH units, the PD_a rising by 1.24 ± 0.03 mV, and the PD_t rising by 0.53 ± 0.31 mV. Changes were reversible after washout. The data did not test for significance at $N/n = 3/3$. When given at concentrations of <5 mmol/l, the effects of ammonia on both cytosolic pH and PD_a were too small to be detected with the present method.

DISCUSSION

The present study was performed to improve our understanding of the mechanisms that lead to changes in urea flux across the ruminal epithelium in response to changes in dietary
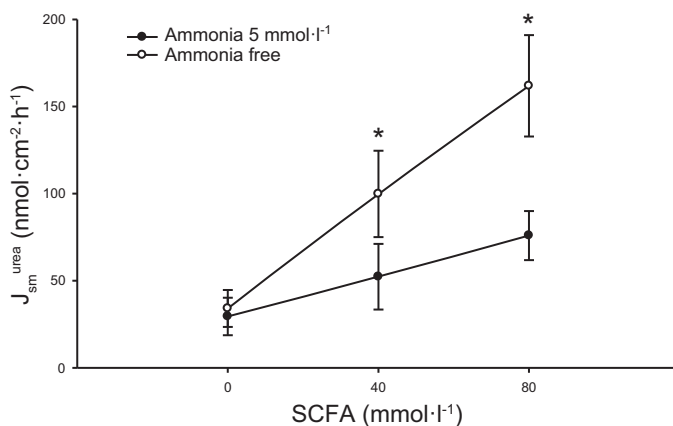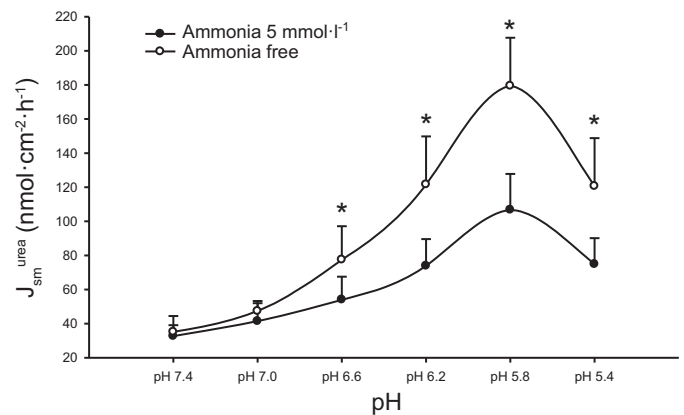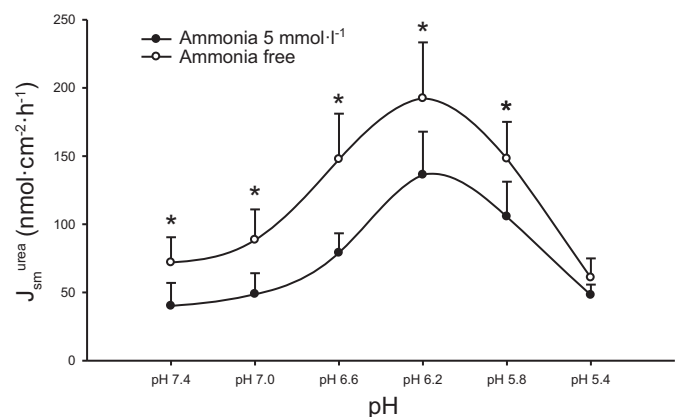

Fig. 4. Effects of luminal short-chain fatty acid (SCFA) concentrations on urea flux rates without ($y = 1.60x + 34.69$, $r^2 = 0.99$) and with 5 mmol/l luminal ammonia ($y = 0.58x + 29.34$, $r^2 = 0.99$; urea concentration: 1 mmol/l, means $\pm$ SD, *buffers 5-7*, luminal pH 6.4; 4 sheep, 13–16 tissues). *J_{sm} urea significantly different between treatments ($P < 0.05$).

Fig. 6. Effect of luminal ammonia and luminal pH on urea flux rates in tissues of concentrate-fed sheep (urea concentration 1 mmol/l, means $\pm$ SD, *buffer 1*; 2 sheep, 6–8 tissues). *J_{sm} urea significantly different ($P < 0.05$) between treatments.
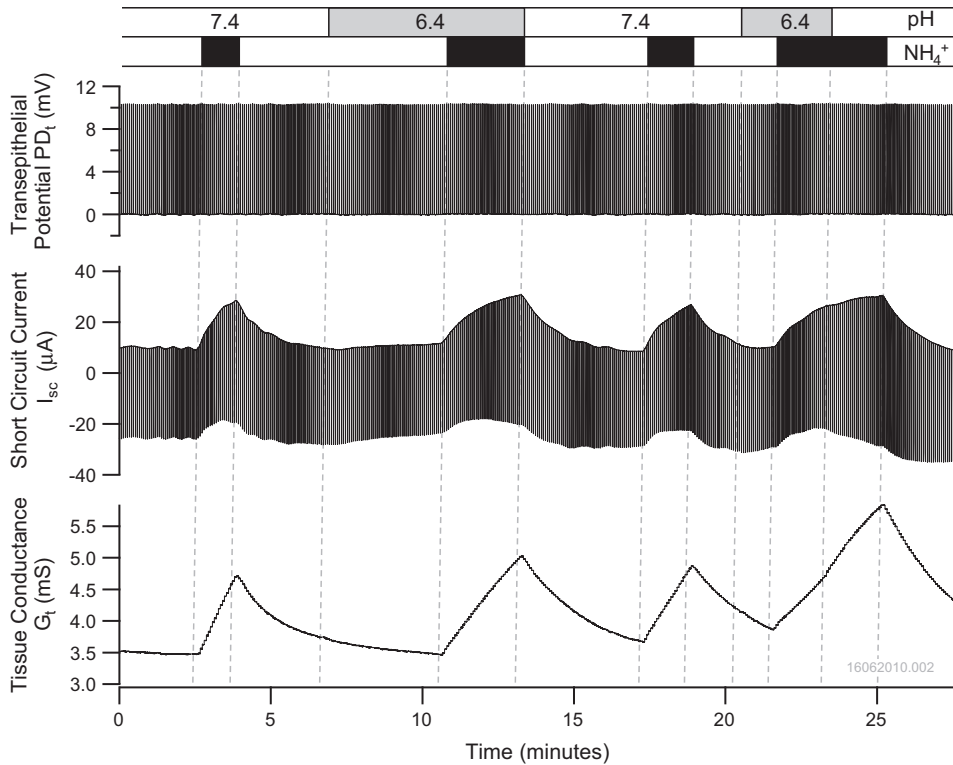
Fig. 7. Original recording showing the response of a preparation of ruminal epithelium to acute changes in luminal mucosal pH and to the addition of ammonia (40 mmol/l). The *top* trace shows the transepithelial potential (PD_t), which was alternately clamped to 0 or 10 mV, the *middle* trace the corresponding short-circuit current (I_{sc}), and the *bottom* trace the transepithelial conductance ($G_t = I_{sc}/PD_t$), which clearly rises in response to changes in luminal ammonia but not to pH.

protein intake (49). As is well-known in ruminants in vivo, SCFA and CO_2 stimulate urea transport (61), whereas ammonia has an inhibitory effect (26, 29). The functional significance appears to be simple, to synchronize the delivery of ruminal nitrogen with available carbohydrates while preventing ruminal hyperammonia. However, the molecular mecha-

nisms behind the effect on urea transport are not clear despite some progress in understanding the modulation of ruminal urea transport.

At the outset of the deliberations that led to this study, we discussed two principal hypotheses. Given the inverse effects of weak acids and weak bases on urea transport, it is tempting
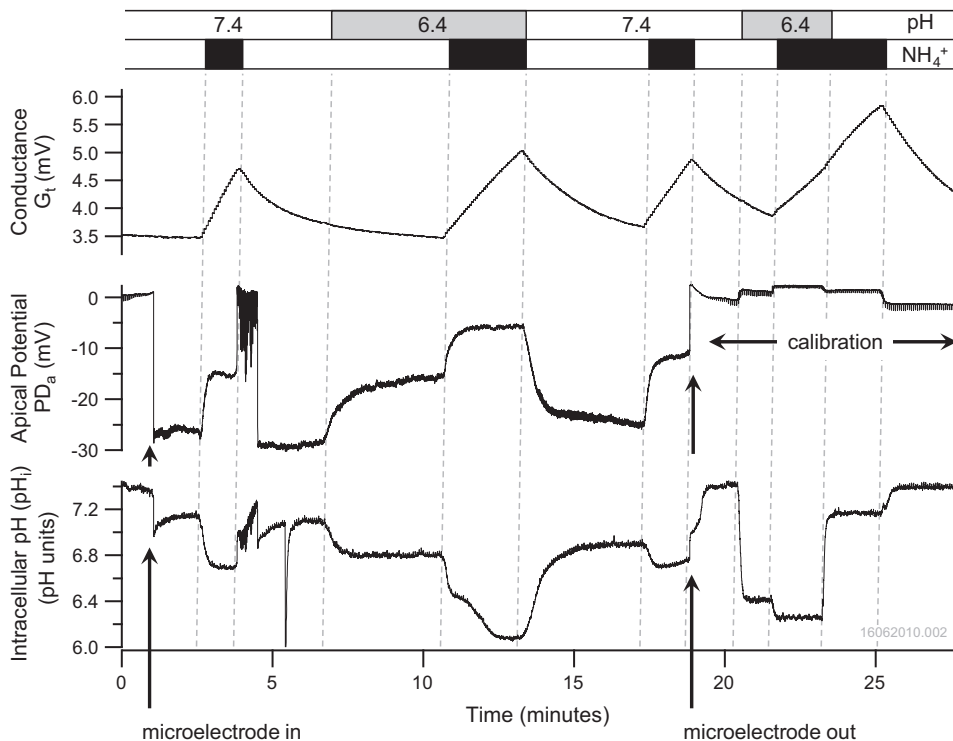

Fig. 8. Effect of 40 mmol/l luminal ammonia on the apical potential (PD_a) and the intracellular pH (pH_i). Same preparation as in Fig. 7, but showing the response of an impaled ion-selective microelectrode to changes in mucosal pH and the addition of ammonia (40 mmol/l). The arrows designate the beginning and the end of the impalement, after which the electrode is recalibrated. The presence of ammonia leads to both an apical depolarization and an acidification of the epithelium, suggesting influx of NH_4^+. The transepithelial parameters PD_t, I_{sc}, and G_t were monitored in parallel (see Fig. 7).
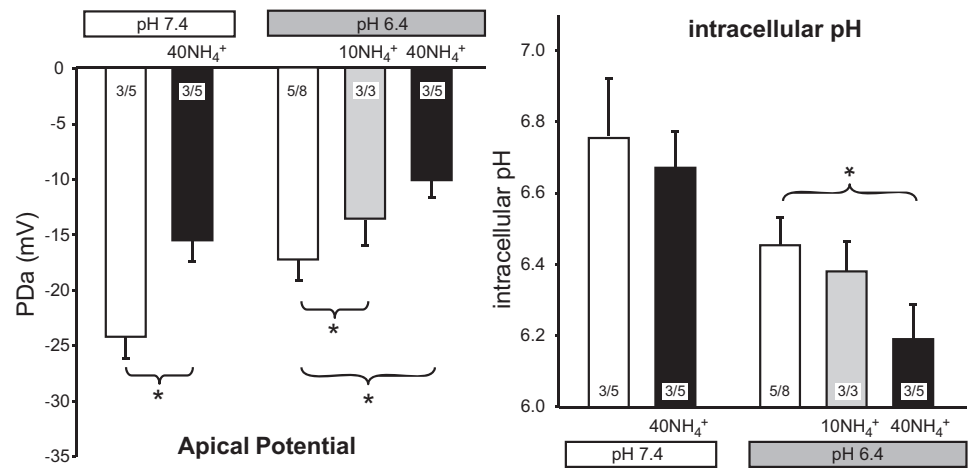
Fig. 9. Concentration-dependent effects of ammonia on PD_a and pH_i [nos. in the columns: N/n (no. of animals/no. of tissues)]. *$P < 0.05$.

to speculate that the effects are mediated by the inverse changes in cytosolic pH that should follow a diffusive uptake of these fermentation products into the cytosol.

Alternately, urea and ammonia might compete for uptake via a common pathway. In the course of this study, a more complex picture has emerged, suggesting that the transport of urea into the rumen may be gated both by intracellular pH and by the intracellular ammonia concentration.

Ruminal Urea Transport is Mediated by Transport Proteins

The data of this study provide further clear evidence against the classical diffusion model, according to which urea transport is mediated by "lipid diffusion" modulated via regulation of blood flow through the organ. It may be mentioned in passing that a careful analysis of in vivo data must also lead to a rejection of the lipid diffusion hypothesis (26). Stimulation of ruminal blood flow should equally affect the absorption rates of all transported substrates and not just a select few, which is in contrast to the observation that various manipulations alter the ratio between the passage rates of ammonia, urea, and SCFA across the ruminal wall in vivo (48).

UT-B is generally considered to be a highly likely candidate for mediating urea transport across gastrointestinal tissues. Thus, a strong correlation can be found between the expression of UT-B1 and transepithelial transport of urea across the entire length of the rat gastrointestinal tract (12). In a carefully done study of the bovine species (55), an upregulation of the longer variant of SLC14A1, UT-B2 was found in response to a concentrate-rich diet on both the mRNA and protein levels. Although a quantification was beyond the scope of this study, we confirm the expression of both UT-B1 and UT-B2 by the rumen of hay- and concentrate-fed sheep.

Uptake of NH_4^+ Into the Epithelium

Ammonia transport in vivo does not drop with pH, as is predicted from the Henderson-Hasselbalch equation, and remains robust at low pH levels, leading to high losses of ammonia from the rumen (9, 28). In vitro studies suggest that, at low pH, NH_4^+ diffuses primarily through a cation channel (3, 7, 35, 59). The microelectrode experiments in the present study support this hypothesis, since a significant depolarization of the apical potential could be measured in response to ammonia both at pH 7.4 and at pH 6.4.

At pH 6.4, we observed a significant intracellular acidification (Figs. 8 and 9). Most likely, a certain fraction of the ammonia entering the cytosol as NH_4^+ leaves basolaterally as NH_3, thereby leaving behind a proton. This model is supported by data from a previous study of the ruminal epithelium showing that, at pH 6.4, ammonia (NH_4^+ uptake) stimulates proton extrusion via Na^+/H^+ exchange (3). At pH 7.4, changes in intracellular pH did not emerge as statistically significant. The depolarization of PD_a in response to ammonia and the lack of an alkalinization suggest that, even at this pH, substantial amounts of ammonia enter the cytosolic space as NH_4^+, with acidification prevented by concomitant influx of NH_3. The change in PD_t at this pH suggests that efflux of ammonia can also occur in the form of NH_4^+, most likely through basolateral potassium channels. Basolateral efflux of NH_4^+ should be higher than suggested by the electrophysiological parameters due to a compensation of the charge by SCFA anions leaving the epithelium through an anion channel (8, 21, 60) (Fig. 11).

Dose-Response Curves

Urea transport is highly sensitive to luminal ammonia; 1 mmol/l reduces J_{sm} urea by roughly 40%. The data show a half-maximal inhibition of urea transport by 1.1 mmol/l ammonia, with no further inhibition of urea transport observed at >5 mmol/l ammonia. A similar value of 6 mmol/l ammonia was reported by Kennedy (29), who studied urea recycling at various ruminal ammonia concentrations in vivo. Maximum microbial growth is observed at ~3.6 mmol/l (52). The inhibition of urea recycling by 5 mmol/l would thus appear to be optimal for the maintenance of conditions suitable for microbial growth, and in vitro and in vivo data are in excellent agreement.

Effect of Luminal pH

The effect of pH on the inhibition of urea transport by ammonia was studied in more detail by a stepwise decrease of pH from 7.4 to 5.4 in the presence of mucosal SCFA. A bell-shaped dependency between the magnitude of urea transport and pH was observed, as described previously (2). In the absence of diffusible weak acids such as SCFA, changes in the pH of the luminal solution do not result in changes in urea

transport (1, 2), so a signaling cascade coupled to changes in intracellular rather than luminal pH appears to be a likely model for explaining the overall form of the curve.

In the presence of ammonia, a similar bell-shaped curve emerged, but with transport rates significantly lower (Fig. 5). Notably, the pH maximum of the curves in the presence and absence of ammonia was roughly the same (pH 5.8). If the inhibitory effects of ammonia were mediated by protons or a shift in cytosolic pH toward more acidic values (as observed), the maximum urea flux should have been observable at a higher level of mucosal pH. Conversely, alkalinization of the cytosolic compartment by ammonia should have led to a shift of the curve in the opposite direction. Neither was the case, and therefore, it would appear that signaling other than cytosolic pH is needed to mediate the inhibitory effects of ammonia on urea transport.

Influx of NH$_3$ and Urea Transport

Since the pK value of ammonia is 9.20, a reduction in the pH should alter the concentration of ammonia (NH$_3$) from a little more than 1% at pH 7.4 to less than 0.2% at pH 6.4, when ammonia is present almost completely in the protonated form as NH$_4^+$. If the inhibitory effects of ammonia on urea transport are attributable to competition within the pore of a transporter with affinity for both urea and NH$_3$ (22), it should be possible to observe a reduction of the inhibitory effects of ammonia by reducing the luminal pH and thus the concentration of NH$_3$. In marked contrast to this hypothesis, no significant effects of ammonia on urea transport could be found at a pH of 7.4, even at 30 mmol/l ammonia. Conversely, the addition of ammonia halved urea transport at a luminal pH of 6.4 (see Table 3). A note of caution is advisable, however, since urea fluxes were very small to begin with at pH 7.4, making it difficult to detect potential small effects.

Influx of NH$_4^+$ and Urea Transport

In a series of further experiments, we could show that a change in the driving force for NH$_4^+$ uptake into the cell changed urea transport significantly. This could be achieved either by depolarizing the apical membrane with luminal potassium or by clamping the PD$_t$, with an impact on the apical potential (31).

The PD$_a$ has been shown to change with the PD$_t$ according to PD$_a$ = 0.66 PD$_t$ − 47.7 mV (31). Using this relationship, we could plot the measured urea transport against the (calculated) PD$_a$ at a luminal concentration of 2 mmol/l luminal ammonia (Fig. 10).

The mean of the urea transport of all control groups without ammonia in the study is 168.75 nmol·cm^{-2}·h^{-1}, which therefore represents the uninhibited flux rate of urea across the epithelium. According to the plot in Fig. 10, a PD$_a$ value of +28.33 mV is necessary to restore flux rate to the original level and eliminate the inhibitory effects of 2 mmol/l luminal ammonia.

Effects of Intracelluar NH$_4^+$ on Urea Transport?

If it is assumed that NH$_4^+$ is in equilibrium across the membrane, a rough estimate of the intracellular ammonia concentration at each level of PD$_a$ is possible using the Nernst equation. At PD$_t$ = 0 mV, PD$_a$ is approximately −47 mV, and
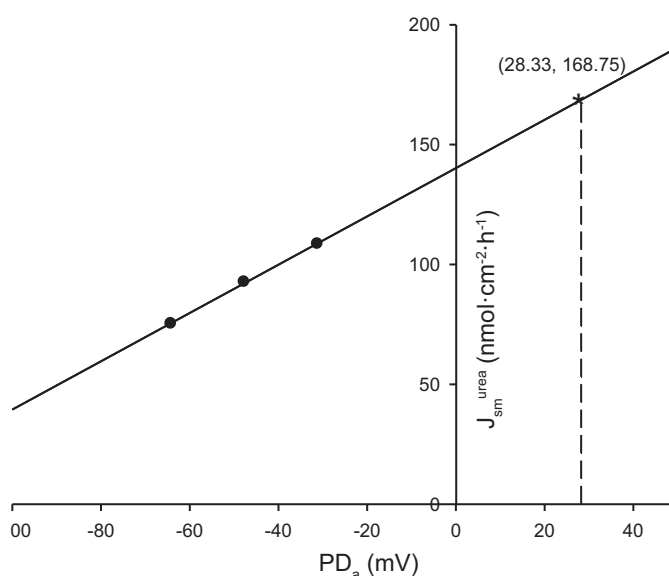

Fig. 10. Effects of the potential difference of the apical membrane PD$_a$ on urea fluxes rate in the presence of luminal 2 mmol/l ammonia [urea concentration 1 mmol/l (data from Table 6), $y = 1.0073x + 140.21$, $y = J_{sm}$ urea, $x = $ PD$_a$, $r^2 = 0.99$]. PD$_a$ is calculated from the equation PD$_a$ = 0.66PD$_t$ − 47.7 mV (31). The mean of J$_{sm}$ urea of all control groups in this study is 168.75 nmol·cm^{-2}·h^{-1}, which was inserted in equation $y = 1.0073x + 140.21$ for the calculation of PD$_a$ at that flux rate. The calculated PD$_a$ of +28.33 mV appears to abolish the effect of 2 mmol/l ammonia (see text for details).

the estimated intracellular concentration of NH$_4^+$ according to this approach is ~6 mmol/l at an extracellular concentration of 2 mmol/l ammonia. A marked reduction of urea transport could be observed. For a PD$_a$ value of +28.33 mV, an intracellular equilibrium concentration of NH$_4^+$ of ~0.7 mmol/l follows. At this level, no effect of ammonia on urea transport is to be expected, according to Fig. 10.

In the manner described above, and always assuming equilibrium conditions, we could also calculate the luminal concentration of NH$_4^+$ (0.1 mmol/l), at which the intracellular concentration drops below 0.6 mmol/l at a PD$_t$ of 0 mV. From what was outlined above, luminal concentrations of ammonia below this margin should not disturb urea transport. Perhaps somewhat surprisingly, a small but statistically significant stimulation of urea transport was found at 0.1 mmol/l NH$_4^+$. The possibility that urea transport is stimulated by extremely small amounts of NH$_4^+$ is interesting, because it means that a low ruminal ammonia concentration, which limits microbial growth, stimulates urea recycling and hence the availability of ammonia for the microbes.

Effects of Protons and Ammonia on Urea Transport: Acid Sensing or Channel Gating?

Previous studies about modulation of urea transport across the rumen epithelium suggested that the stimulating effect of SCFA and CO$_2$ is mediated via changes in pH$_i$ (2). According to one classical hypothesis, acid-sensitive receptors exist within the ruminal epithelium (14, 15, 24, 25) and appear to reduce rumen motility during bouts of acidosis. However, the data of this study, which was performed under tightly controlled conditions using ruminal epithelium stripped of all underlying layers, suggest more direct effects. Since UT-B is

basically a channel protein, the transport rate can change within nanoseconds of binding a suitable ligand (36). Thus, the neurotoxic effects of ammonia are thought to be linked to changes in the gating of ion channels in the central nervous system (44). A similar modulation of gating behavior may underlie the inhibitory effects of ammonia on ruminal urea transport.

The gating of bacterial urea channels by protons is classical at this point and enables *Helicobacter pylori* to maintain its internal pH within the extremely acidic environment of the mammalian glandular stomach. The opening of the urea channel pore depends upon the formation of hydrogen bonds by periplasmic residues that in turn produce conformational changes of the transmembrane domain (4, 63). The influx of urea into the periplasmic compartment follows, where it is degraded by urease to form buffering NH_3. Likewise, the permeability of erythrocytes to glycerol has

long been known to depend on extracellular pH, with a bell-shaped dependency that closely resembles the curve found in the present study for urea (11, 56, 66) and reflecting protonation of aquaporin 3 (AQP3) (65). As the pH drops, increasing numbers of protons bind to amino acids within the pore region, thus altering the local charge distribution so that the selectivity shifts from water to glycerol, with permeability for the latter being highest at a pH of ~6.4 and reducing again at lower pH (66). AQP3 conducts urea (37) and has been identified recently in the rumen of cows (51), making it an interesting candidate. Possibly, both UT-B and AQP3 are involved in ruminal transport of urea. In this context, it should be mentioned that results of a parallel study of pig cecum suggest that in this organ urea transport is not regulated in the manner found in the rumen (58). A differential pattern of expression and/or regulation of urea-transporing proteins thus appears likely.
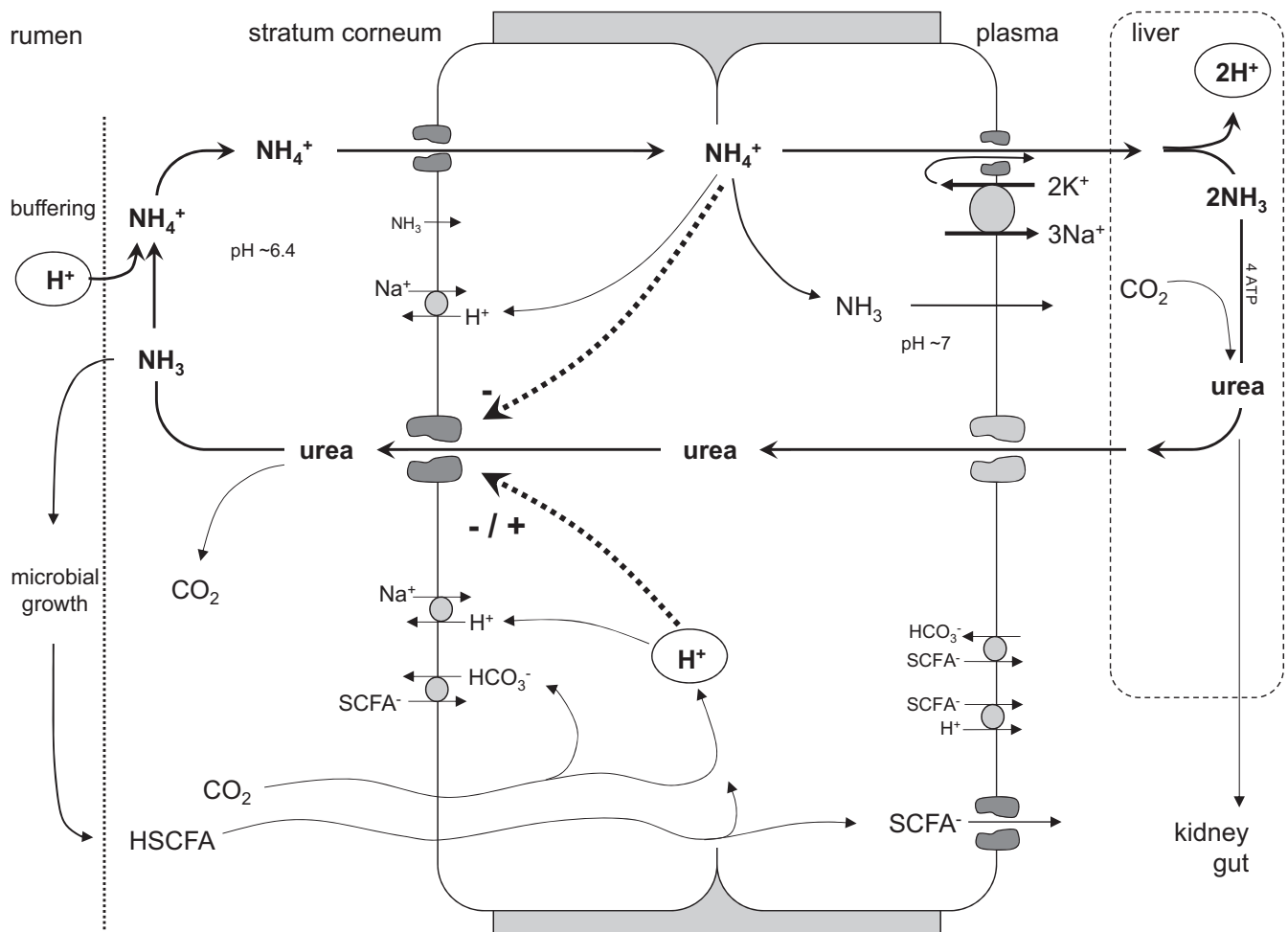

Fig. 11. Model of ruminal urea transport. The multilayered epithelium represents 1 compartment, with transport from layer to layer mediated by either gap junctions or urea transporters (47). Urea is taken up across the basolateral and released across the apical membrane by facilitated diffusion through transport proteins, with 1 or both most likely corresponding to urea transport B(UT-B)1 or UT-B2 (50, 57). The activity of this transport is acutely regulated and stimulated at a luminal pH of 6.4 in the presence of CO_2 and SCFA, indicating an effect of pH_i (2). The negative effect of ammonia is restricted to the uptake as NH_4^+ via a cation channel (3, 7, 34). Depolarization of PD_a and a decrease in pH_i by luminal ammonia support channel mediated uptake of NH_4^+. However, the mechanism behind the inhibitory effects of NH_4^+ on urea transport is not clear. The high intracellular NH_4^+ concentrations at equilibrium (see text) even at low luminal concentrations of ammonia hint at a possible direct interaction of intracellular NH_4^+ with the urea transporter, resulting in lower urea transport rates. Also shown are the interactions between NH_4^+ and the apical NHE (3, 46) and the stimulatory effect of SCFA on the absorption of NH_4^+ (8, 46), which is most likely linked to the efflux of SCFA anions through a basolateral anion channel (21, 60). After absorption into portal blood, NH_4^+ is reconverted to urea in the liver, which can again be secreted into the rumen and degraded to ammonia. Since 2 protons are removed from the rumen for every urea secreted, the cycle may contribute to the buffering of the epithelial microclimate.

Implications for Ruminal and Epithelial Homeostasis

The findings of this study confirm the bell-shaped dependency between pH and urea flux into the rumen in the presence of CO_2 and SCFA (2) and are possibly linked to an uptake of protons by the epithelium (Fig. 11). We further show that at the pH found physiologically in the rumen ($\sim$6.4), absorption of ammonia occurs in the form of NH_4^+ through a transport protein, coupled to an acidification of the epithelium. Finally, we demonstrate that NH_4^+ inhibits urea transport in a manner that appears to be linked to the uptake of the charged moiety (NH_4^+) into the epithelium.

As discussed previously (1–3), the acute regulation of urea transport by the fermentational products SCFA, CO_2, and NH_4^+ should ensure that urea transport is synchronized with the nitrogen requirements of the microbial populations within the rumen, rising with fermentational activity but falling when nitrogen levels exceed microbial requirements or when fermentational activity becomes excessive. However, the secretion of urea into the rumen may have a further function, namely to buffer the microclimate in the vicinity of the ruminal wall. Intriguingly, the energy for this process does not have to be supplied by the epithelium but is derived from the conversion of ammonia to urea in the liver (Fig. 11). Note that hydrolysis of as little as 50 μmol/l of urea is sufficient to buffer 10^{-4} mol/l protons or to restore a pH of 4 to a pH of 7 in an otherwise unbuffered system.

Present feeding strategies in high-producing dairy cows lead to low ruminal pH and high ruminal NH_4^+ concentrations. If the data of this in vitro study are applicable, low ruminal pH will not lead to the desired reduction in ammonia flux from the rumen because NH_4^+, and not NH_3, is the major species of ammonia absorbed. The NH_4^+ will continue to be leave the rumen via a cation channel (3, 34), thereby increasing N excretion into the environment via the kidney (5). Another aspect should be considered; both the reduction of urea influx by high ruminal ammonia (with reduced buffering of the microclimate) and epithelial uptake of ammonia in the form of NH_4^+ should contribute to the acid load of the epithelium, with possible detrimental consequences when epithelial homeostasis is challenged by ruminal acidosis (14, 45). A better understanding of the function and the regulation of proteins that mediate the transport of nitrogen across epithelia thus appears mandatory.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Z.L., F.S., C.D., J.R., H.B., and K.A. performed experiments; Z.L., F.S., C.D., J.R., H.B., and H.M. analyzed data; Z.L., F.S., C.D., H.B., K.A., and H.M. interpreted results of experiments; Z.L., F.S., and J.R. prepared figures; Z.L., F.S., C.D., J.R., H.B., K.A., J.R.A., and H.M. approved final version of manuscript; F.S. and H.M. conception and design of research; F.S. drafted manuscript; F.S., C.D., J.R.A., and H.M. edited and revised manuscript.

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