Urea Transport in Sheep Rumen Epithelium *in vitro*: Modulation by luminal Ammonia and pH

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Contents

Abbreviations

A Arginase

AS Argininosuccinic acid synthetase

AL Argininosuccinic acid lyase

CA Carboanhydrase

CHO Carbohydrates

CO_2 Carbon dioxide

CP Crude protein

DM Dry matter

G_t Tissue conductance ($mS \cdot cm^{-2}$)

HSCFA Undissociated short chain fatty acids

I_{sc} Short circuit current ($\mu eq \cdot cm^{-2} \cdot h^{-1}$)

J_{ms} Unidirectional ion transport in mucosal to serosal direction ($\mu eq \cdot cm^{-2} \cdot h^{-1}$)

J_{sm} Unidirectional ion transport in serosal to mucosal direction ($\mu eq \cdot cm^{-2} \cdot h^{-1}$)

J_{net} Net-transport of ions ($\mu eq \cdot cm^{-2} \cdot h^{-1}$)

ME Metabolizable energy

N Number of experimental animals

n Number of tissues/epithelia

NHE Na^+/H^+-exchanger

NPN Non-protein-N (N present in DNA, RNA, ammonia, AA, and small peptides)

OTC Ornithine transcarbamylase

PD_a Potential difference of the apical membrane

PD_t Transepithelial potential difference (mV)

pH_i Intracellular pH

PKA Protein kinase A

PKC Protein kinase C

SCFA Short chain fatty acids

SDS Sodium dodecyl sulphate

TK Tyrosine kinase

UER Urea entry rate

UT Urea transporter

UTA Urea transporter A

UTB Urea transporter B

UUE Urinary urea excretion

URG Urea recycled to the gastro-intestinal tract

Chapter 1

General Introduction

1.1 Introduction

The digestive physiology of ruminants exhibits two particular properties which very likely contributed to the successful adaptation to a variety of habitats and explain why ruminants are living in deserts, tundra and classical grassland areas: Firstly, the fermentation of structural carbohydrates as cellulose, hemicellulose and lignin by bacteria, which cannot be digested by enzymes of the ruminant, and the production of fermentation products from these carbohydrates as acetate, propionate and butyrate (short chain fatty acids = SCFA) which covers most of the energy requirement of ruminants. Secondly, recycling of urea into the forestomachs, which is hydrolyzed by ruminal bacteria to CO_2 and ammonia[1]. Ammonia-N is an essential nitrogen precursor for the synthesis of microbial protein. Microbial protein is digested in the small intestine and the absorbed amino acids of microbial protein contribute to more than 70 % of the daily requirement. These two properties – fermentation of carbohydrates which cannot be digested by enzymes of the ruminant – and the re-use of urea-N – an end-product of nitrogen metabolism and normally excreted via urine – for microbial growth rate circumvent the possible bottle necks of nutrition of herbivores in the natural habitat: Shortage of energy and protein.

This interaction between the microbes in the forestomachs and the ruminants is a classical symbiosis: The ruminants are feeding the microbes in the forestomachs and the microbes are feeding the ruminant via SCFA and microbial protein: Benefits for both sides.

Recycling of urea-N and the re-use of ammonia-N for microbial protein synthesis has attracted the attention of physiologists since many decades due to its protein saving mechanisms and led to studies about the synthesis of urea,

1. The term ammonia is used without discrimination between NH_3 and NH_4^+. Chemical symbols are used when a specification is required.

urinary urea excretion via kidneys and particularly urea recycling.

The magnitude of urea synthesis (urea entry rate = UER in g N/d) in ruminants accounts for some 60 – 70 % of daily N-intake (89, 108) and the synthesized urea is either excreted via kidneys in urine (urinary urea excretion: UUE) or recycled to the gastro-intestinal tract (URG), mainly into the rumen. UUE and URG exhibit large variations and change reciprocally (135). For example, URG approaches zero in sheep after 24 - 36 h starvation and hence UER equals UUE. By contrast, at low N-intake 97.7 of UER was recycled and 2.3 % excreted via urine (176).

A large number of studies have been performed for a better understanding of mechanisms which are determining these reciprocal alterations of UUE and URG and particularly the transport of urea across the rumen epithelium. Previous and recent reviews have summarized the current knowledge and it is generally accepted that urea transport across the rumen epithelium is mediated by passive diffusion probably using the urea transporter B (UT-B) (162) and modulated by the fermentation products CO_2 and short chain fatty acids (SCFA) (89, 108, 135). Recent in vitro studies have shown that the well known stimulating effect of CO_2 and SCFA on urea transport (42, 132, 133, 167) is influenced by the luminal pH in a bell-shape manner. Maximum transport of urea is observed at some pH 6.2 and is gradually diminished with increasing and decreasing pH. Almost identical and lowest transport rates are noticed at a pH of 7.4 and 5.4 (2).

In addition, it is well known that ruminal ammonia reduces urea recycling (79) and Reynolds and Kristensen (135) showed in a meta-analysis that urea is predominantly recycled to the gut (> 90%) at a low N-intake and recycling of urea is stepwise diminished with increasing crude protein (CP) content of the

diet. At 18 % CP the recycling rate is reduced to 25 % of UER. The link between CP and urea recycling is probably the ruminal ammonia concentration. Kennedy et al. (80) provided convincing evidence from *in vivo* study that an increase of ruminal ammonia concentration reduced urea recycling and ruminal ammonia concentration of > some 6 $mmol \cdot l^{-1}$ ammonia did not further decrease urea recycling (79). The negative effect of ammonia on urea recycling was corroborated by intraruminal infusion of NH_4Cl which caused a significant decrease of urea recycling, increased the urea synthesis and urea excretion via urine (118). In line with these *in vivo* observations is a recent in vitro study with isolated rumen epithelium of goats. A low CP diet caused *in vitro* an enhancement of urea transport across isolated goat rumen epithelium (from blood side to luminal side) (116). This observation was recently confirmed by Doranalli et al. (44) with isolated epithelia from lambs.

Hence, there is no doubt that ammonia modulates urea transport across the rumen epithelium, but the underlying mechanisms of ammonia on urea transport are not known. Therefore the following questions were addressed in the current study:

a) Is urea transport *in vitro* influenced by ammonia as *in vivo*?

b) The stimulating effect of CO_2 and SCFA on urea transport depends on the luminal pH (2). Does the inhibition of urea transport by ammonia depend on the pH too?

c) Is it possible to demonstrate interactions between SCFA (stimulation) and ammonia (inhibition) on urea transport?

d) Is the change of intracellular pH (pH_i) a reliable working hypothesis for change of urea transport by ammonia?

1.2 Review of literature

1.2.1 Ruminants

Domesticated ruminants have had a symbiotic relationship with human beings since the Stone Age. The first livestock species to be domesticated (ruminant or non-ruminant) was the goat, at approximately 8,000 BC in the Fertile Crescent of the Near East (183). Most of the other 8 domesticated ruminant species (sheep, European and Zebu cattle, water buffalo, mithuns, reindeer, yaks, Bali cattle) were brought under human control by 2,500 BC in either the Near East or southern Asia (31). This symbiotic relationship has been maximized by agrarian societies in areas in which arable land is limited, and the harvesting of browse and forage grass by ruminants has increased food resources. Even in some intensely cultivated regions, such as the Gangetic plains of India and Pakistan, ruminants are crucial to the local economy. They were originally domesticated for milk, draft transportation, sacrifice, and barter (31). Data show that the total population size of domestic species is 3.57 billion, nearly 50-fold larger than that of wild ruminants (63). As might be anticipated, cattle, sheep, and goats constitute most (about 95%) of the domestic ruminant population.

Mammals do not produce enzymes that are able to split special long-chain structural carbohydrates as cellulose. Thus, many animals are not able to convert the energy from plant materials such as cellulose and hemi-cellulose (14). Ruminants have a highly developed and specialized digestive organ, named the “forestomach”, which allows them, by ruminal microbial fermentation, to obtain more energy from fibrous food than most other herbivores or non-ruminant animals (monogastrics, such as humans, dogs, horse and pigs). Ruminants have, with their four-compartmented stomach, namely the rumen, reticulum, omasum, and abomasum (the first three compartments forming the forestomach), a unique digestive system. These

four compartments occupy almost 75% of the abdominal cavity, filling virtually all of the left side and extending significantly into the right side (http://www.vivo.colostate.edu/hbooks/index.html, Digestive System) of the animal. In sheep and goat, the capacity of the forestomach accounts for about 67% of the capacity of whole digestive tract, which allows the storage, mixing, and digestion of the feedstuff (46).

Ruminants enhance the utilization of cellulose carbohydrates by symbiosis with bacteria, protozoa, and fungi in the forestomach (fermentation chamber), which precedes the main site of digestion in digestive tract. The fermentation in the forestomach is much more efficient than that in the hind gut. Large non-ruminant herbivores such as the horse eat similar food as ruminants, but they are unable to use cellulose for energy in such an efficient manner.

1.2.2 Rumen

In mature ruminants, the rumen, being the largest compartment, accounts for 80% of the four-compartment capacity. Its volume, which varies with animal species, is largely related to feed intake. In cattle, it ranges from 60 l to 120 l and, in sheep, between 3 l and 8 l, depending on body weight (131). Rumen volume has been shown to be closely correlated with the amount and quality (% of fiber in dry matter) of food ingested.

The rumen is divided by muscular pillar into dorsal, ventral, caudodorsal, and caudoventral sacs to form a multi-compartmental organ. The large and divisioned rumenal lumen is the first site into which food is taken: it is involved in the storage of bulky fibrous plant foods, in keeping the digesta mixed by reticular-rumen motility, and in providing the proper environment for microbial fermentation. The rumen wall, which is lined with stratified, squamous, keratinized, non-glandular, epithelial cells, is composed of four layers or tunics.

They are, from the lumen surface to the blood side, the stratum corneum, the underlying stratum granulosum, the stratum spinosum, and finally the stratum basale (159). The cells in the outermost layers of the stratum corneum are intensely keratinized. The rumen epithelium is characterized by the presence of finger-like papillae, which are 1.0-1.5 cm in length in cattle (159) and about 2 mm broad and 6 mm long in sheep (43). The rumen papillae significantly enlarge the mucosal surface area.

1.2.3 Rumen environment

The rumen content is a watery and strictly anaerobic vat (redox potential: approximately – 400 mV) with a constant temperature held at about 38-40°C℃ and a pH between 5.5 and 7.0, all of which are essential for effective microbial activity (70). In the rumen, bacteria (10^{10}/ml), protozoa (10^{6}/ml), and fungi (10^{3}/ml) at high density digest plant material. The major end-products of fermentation are: short chain fatty acids (SCFAs), ammonia, CO_2, and methane. The SCFAs are a group containing molecules with one to seven carbon atoms. Acetic acid, propionic acid, and butyric acid are the predominant forms of SCFA (14). They are the major source of energy for ruminants (16, 48, 100). The molar proportions of acetic acid: propionic acid: butyric acid are 70: 20:10 in hay-fed sheep (15, 62) and 55:35:15 in grain-fed sheep (7, 82). Highly fermentable diets increase SCFA production (164) and concentration (126) in the rumen. A starch diet increases propionic acid and decreases the acetic acid proportion.

The SCFAs are generated primarily from fiber feeds containing cellulose and hemicellulose and grains rich in starch, with a lower proportion of SCFAs being produced from dietary protein, pentoses of nucleic acids, and glycerol of glycerophospholipids. In the rumen, about 30%-50% of the cellulose and hemicellulose is digested by the microbial population. Sixty percent or more of

the starch is degraded, and most sugars are digested 100% within the rumen. In general, the total amount of SCFA concentration in rumen is between 60 and 150 $mmol \cdot l^{-1}$ (14), but this varies considerably with diet intake. The ruminal SCFA concentration is higher on grain than forage intake. When animals graze on fresh grass or when fed starch-rich diets, exceptionally high values can be reached, e.g., 200 $mmol \cdot l^{-1}$ (14).

The rumen SCFA concentrations vary not only with respect to the composition of the diet, but also with respect to the time after feeding (14, 146, 165). The maximal concentrations of SCFAs usually occur 2-4 h after feeding (60, 96). Increasing feeding frequency results in a more stable ruminal environment (158). Provision of a fixed ration of hay and high-cereal concentrates to lactating cows in equal portions 24 times per day reduces the diurnal variation in ruminal SCFA concentrations and pH compared with two daily feeds (164).

The SCFAs, with a pK of ≤ 4.8, acidifies the rumen content by the release of protons. The ruminal pH is in the range of 5.5 – 7.0, varying with dietary composition and feeding strategies (14). In sheep challenged by different rumen fermentable diets, the mean ruminal pH is lower for wheat (readily fermentable starch, 4.85) than for corn (slowly fermentable starch, 5.61) and beet pulp (easily digestible fiber, 6.09) (96). Shabi et al. (146) have observed a reduced diurnal variation in ruminal pH in dairy cows fed four times per day as compared with those fed twice per day. Rumen pH is one of the most important factors influencing ruminal microflora and fermentation, rumen epithelial functions, and in turn, animal health and production. In a feeding trial with sheep, an intake of a diet with a concentrate : forage ratio of 50:50 ad libitum results in a time duration of a ruminal pH of < 5.6 for more than 7 h/d, thereby running the risk of subacute ruminal acidosis (34). The increase in readily fermentable carbohydrates in the diet initially leads to an increased growth rate

of most rumen bacteria (117) but consequently causes the accumulation of SCFAs and the reduction of ruminal pH (49, 59). Rumen cellulolysis is totally inhibited at a pH value of < 6.0. On the other hand, an increase of rumen starch fermentation decreases feed intake in sheep (125); this is probably associated with the low rumenal pH. The inhibition of feed intake by low pH is assumed to be mediated by the excitation of acid-sensitive receptors that lie in the rumen wall (37), located at the basement membrane of the forestomach epithelium (64, 65).

To provide a suitable and stable ruminal environment, ruminants have developed buffer system to move protons from the rumen; this system is constituted by two predominant mechanisms: the well-known salivary buffers (i.e., bicarbonate, phosphate) for ruminal proton removal, and SCFA absorption across the ruminal epithelium (9). Ruminants produce large amounts of saliva: 100 to 200 l per day for an adult cow (13, 46) and about 6 -16 l for a sheep (77). Ruminant saliva is particularly rich in $NaHCO_3$ and Na_2HPO_4 buffer, constituting a powerful buffering capacity. During eating or rumination, salivary secretion increases about fourfold in volume. In addition, the continuous flow of parotid saliva in the ruminant provides a constant inflow of buffer for the rapid neutralization of SCFA produced by continuous fermentation in the rumen (32, 47, 78). The second mechanism of rumen pH regulation is SCFA absorption across the ruminal epithelium in an undissociated form. During the last two decades, several protein-dependent SCFA absorption mechanisms have been studied. These include transport in the dissociated form via apical acetate-bicarbonate exchange, the nitrate-sensitive bicarbonate-independent transport mechanism, and the lipophilic diffusion of undissociated SCFA (55).

1.2.4 Rumen fermentation: Carbohydrates

The carbohydrate fraction of the herbivorous diet can be roughly divided into 1) cellulose, associated with a variable amount of lignin, depending on the nature of the diet; 2) hemicellulose and pentosans; 3) polysaccharides, e.g., starch and fructosan; 4) simple sugars such as glucose and sucrose (45). In ruminants, the major sources of carbohydrates are cellulose/hemicellulose and grains rich in starch, which are mainly fermented by microorganisms in the rumen. Some 5% to 20% of consumed dietary carbohydrates (starch) are digested in the small intestine (74). SCFA production varies between 5.4 mol/kg (digestible organic matter intake) in sheep consuming various diets and 79 mol/d in cows consuming lucerne or maize silage (15, 91-93, 163, 175). The dietary composition (starch and cellulose) influences the proportions of the fermentation products. Dietary cellulose is fermented to SCFAs, most of which is acetic acid (138). The acetic acid concentrations are 34-59 or 36-65 $mmol \cdot l^{-1}$ in the rumen fluid of sheep after an intake of hay or lucerne, respectively, which (acetate) accounts for about 69% of total SCFA (62). Cows fed a high-concentrate diet have a lower ruminal pH, lower ruminal acetate and butyrate concentrations and a higher propionate concentration (81). The intake of carbohydrate (starch)-rich diets increases propionic acid and butyric acid production (see figure 1).

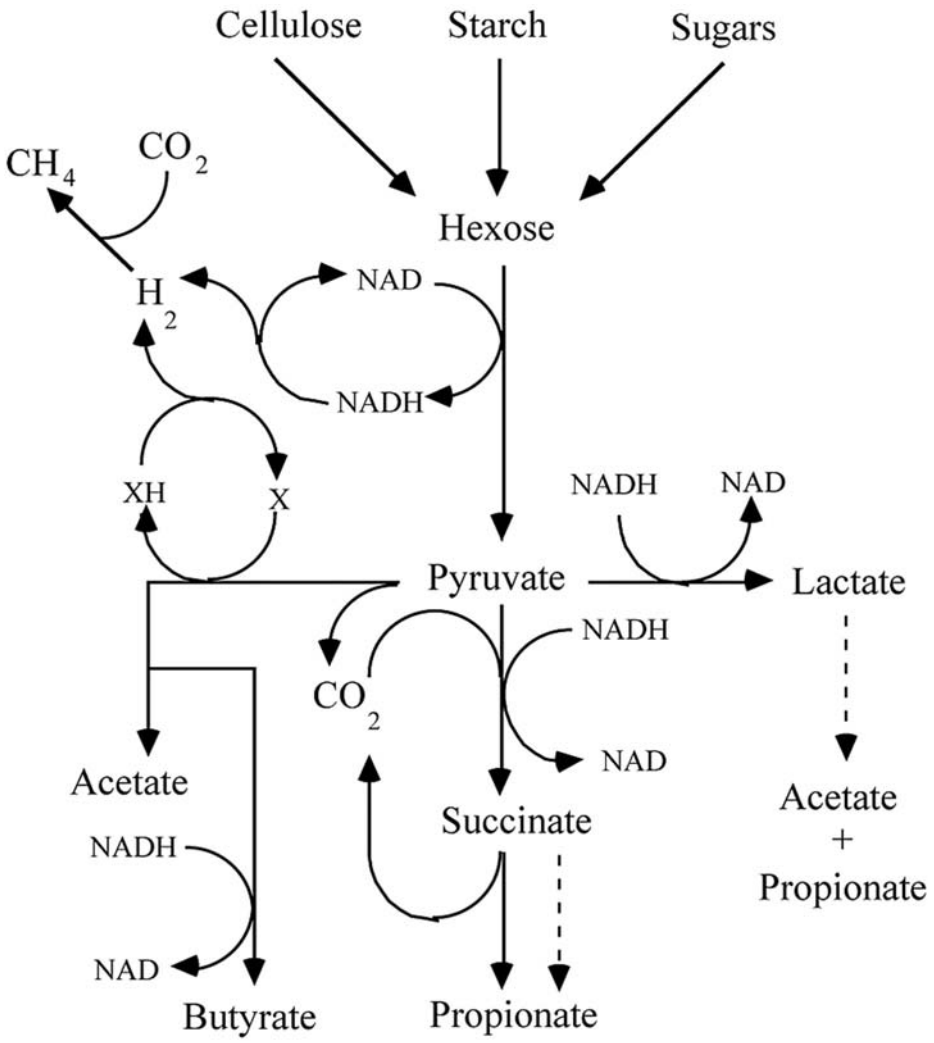


Figure 1: Scheme of rumen fermentation of carbohydrates to short chain fatty acids (138).

In general, an increase in the dietary supply of fermentable grain starch is associated with an increased production of SCFA, increased production of microbial protein, decreased fiber digestion, decreased ammonia concentrations, and decreased acetate: propionate ratio (112, 120-122, 127). However, an excessive consumption of readily fermentable carbohydrates induces a reduction in ruminal pH (about 5.5 to 5) that affects the ruminal ecosystem (83). Ruminants and ruminal microorganisms are widely accepted to have a symbiotic relationship that facilitates fiber digestion. However, domestic ruminants in developed countries are often fed an abundance of

grain and little fiber. When ruminants are fed fiber-deficient rations, physiological mechanisms of homeostasis are disrupted, ruminal pH declines, microbial ecology is altered, and the animal becomes more susceptible to metabolic disorders and, in some cases, infectious diseases (138).

1.2.5 Rumen nitrogen metabolism

Nitrogen materials (from the diet or muco-proteins in saliva or sloughed epithelial cells) are composed of protein and non-protein-N (NPN: N present in DNA, RNA, ammonia, AA, and small peptides) (11), which enter the digesta in the rumen. Protein comprises most of the dietary nitrogen (94) and is degraded by ruminants in two phases: 1) by microbial action in the reticulo-rumen; 2) by post-ruminal digestion that occurs principally in the small intestine (85, 140). Many strains and species of ruminal ciliated protozoa, bacteria, and anaerobic fungi have been found to be proteolytic; they contain a variety of types of proteolytic enzymes (170). Proteolytic activity and the microbial species responsible for that activity are highly diet-dependent (69, 119, 150).

The first step in protein degradation in the rumen is the hydrolysis of proteins by proteinases to peptides and amino acids, which are either utilized directly by the microflora for microbial protein syntheses or degraded further by peptidases and deaminating enzymes to SCFAs and ammonia (11, 23, 27, 129, 130). Peptides and amino acids are intermediates in the conversion of protein to ammonia (94, 170, 171). Some peptides can resist fermentation for a sufficient time to pass out of the rumen with the digesta (28). Absorption of both peptides and amino acids from the rumen is of minor importance and not of nutritional significance (90, 111). Peptides and amino acids arising from proteolysis are parlty taken up by the microorganisms (166). The fate of absorbed peptides and AA once inside the microbial cell will depend on the availability of energy [carbohydrates (CHO)]. If energy is available, AA will be

transaminated or used directly for microbial protein synthesis. However, if energy is limiting, AA will be desaminated, and their carbon skeleton will be fermented into SCFA (11).

The concentration of amino acids in the rumen is low because of rapid metabolism, and concentrations of 0.3-1.5 mg/100 ml, (6, 99, 179, 180) have been determined. As mentioned above, NPN is composed of N present in DNA, RNA, ammonia, AA, and small peptides (11). N-containing compounds leave the rumen via outflow into the omasum as ammonium (NH_4^+), AA, peptides, and undegradable protein. The only significant absorption of N is the transport of ammonia across the rumen wall; this occurs for both forms of ammonia, NH_3 and NH_4^+, depending on the ruminal pH and according to the Henderson-Hasselbalch equation. At a physiological pH (< 7.0), less than 1 % of ammonia is NH_3 and > 99 % NH_4^+ (1). When dietary protein is broken down in excess of the ammonia requirement for microbial growth, ammonia is absorbed and is metabolized to urea in the liver via the ornithine cycle (Figure 2).

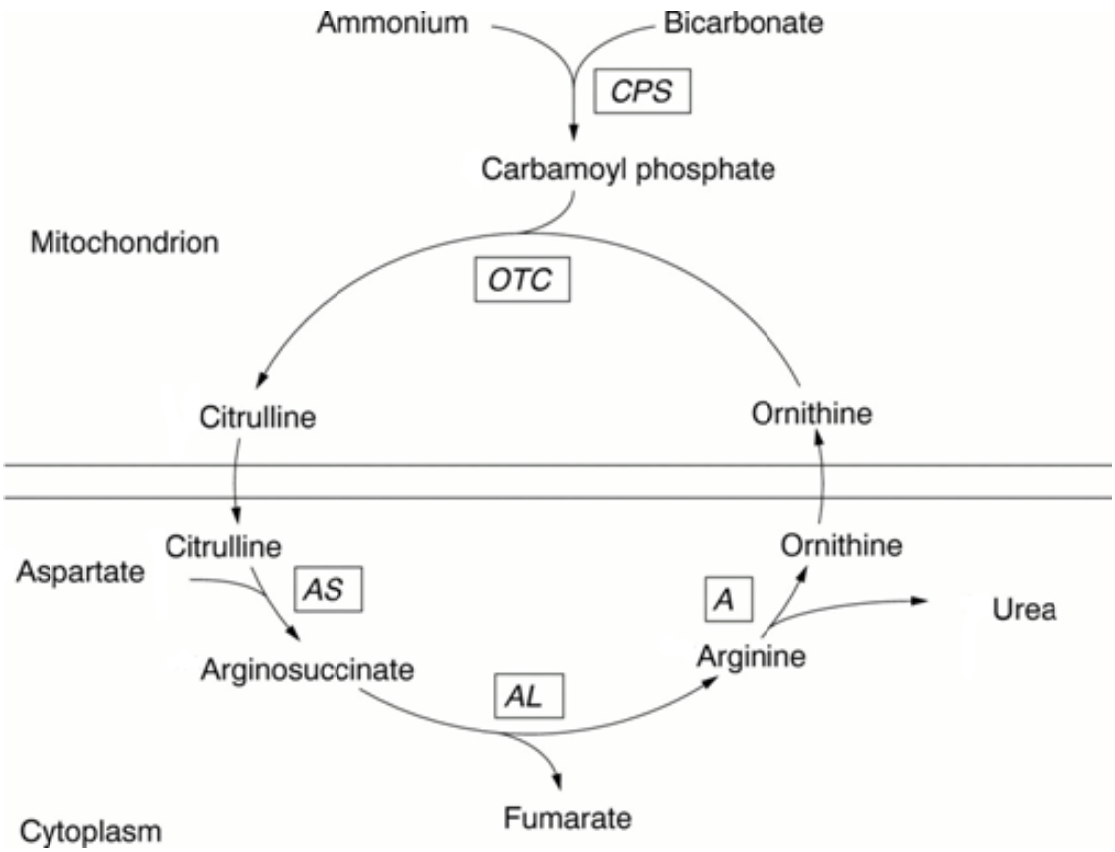


Figure 2: The ornithine cycle in the liver. CPS, carbamyl phosphate synthetase; OTC, ornithine transcarbamylase; AS, argininosuccinic acid synthetase; AL, argininosuccinic acid lyase; A, arginase (178).

Urea (see the figure 3 below) is considered as an end product of N metabolism and is excreted via the kidneys in urine. It is a solid, colorless, and odorless compound (although the ammonia that it gives off in the presence of water, including water vapor in the air, has a strong odor). Urea is highly soluble in water and is non-toxic. Dissolved in water, it is neither acidic nor alkaline. Urea is widely used as a fertilizer and as a convenient source of N.

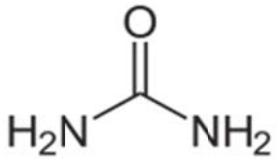


Figure 3: Structure of Urea

1.2.6 Urea and urea recycling in ruminants

In ruminants, urea is excreted in urine but is also recycled to the gut and is well known, after hydrolysis to CO_2 and ammonia, as being an important N-resource for microbial protein synthesis in the forestomach. Figure 4 summarizes the metabolism and reuse of urea in ruminants. Urea is synthesized in the liver and either excreted via the kidney or recycled into the gut via salivary secretion or by diffusion into the various compartment of the gastrointestinal tract, predominantly into the rumen (Figure 4).

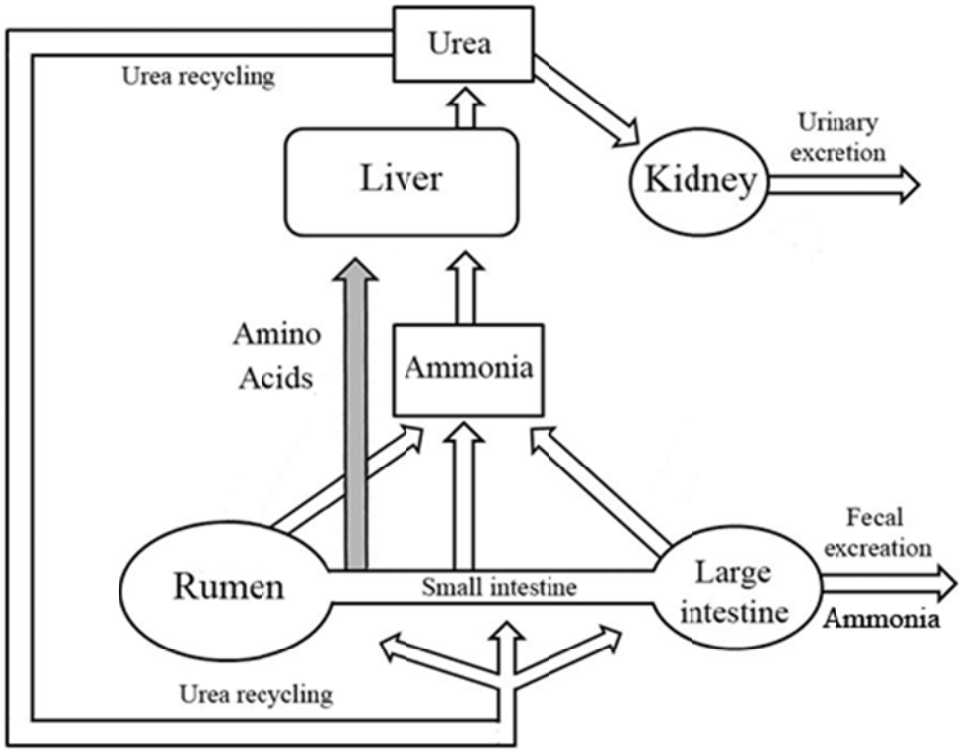


Figure 4: Scheme showing urea metabolism in ruminants. For details, see text. Adopted and modified from Harmeyer and Martens (66)

The important function of urea recycling in the digestive system of ruminants has stimulated a large number of publications dealing with a) total daily urea production (turnover or entry rate: UER), b) urea excretion via the kidney (UUE), c) urea recycling into the gastrointestinal tract (URR), and d) the fate of urea-N in the gastrointestinal tract. The various parameters of urea metabolism

are schematically shown in Figure 5.

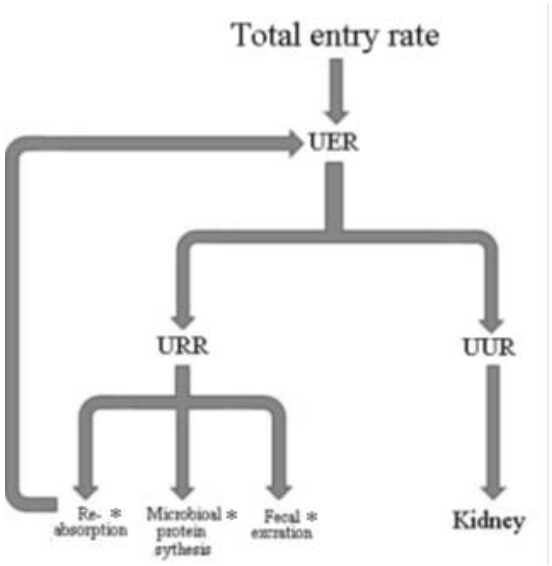


Figure 5: Parameter of urea kinetics in ruminants. UER: urea entry rate; URR: urea recycling rate; UUR: urea urinary excretion rate. Recycled urea is reabsorption as ammonia* after microbial hydrolysis of urea. Ammonia* used for microbial protein synthesis or excreted via feces. Adopted and modified from Harmeyer and Martens (66).

Harmeyer and Martens (66) have summarized, in a review, the corresponding data mainly from studies of small ruminants (sheep and goats). The urea entry rate (UER; g urea-N/d) turns out to be correlated with N-intake with the diet and accounts for some 70 % of N-intake (Figure 6). This high and constant UER (60-70 %) is relative independent of the quality of N-source and the amount of N in the diet.

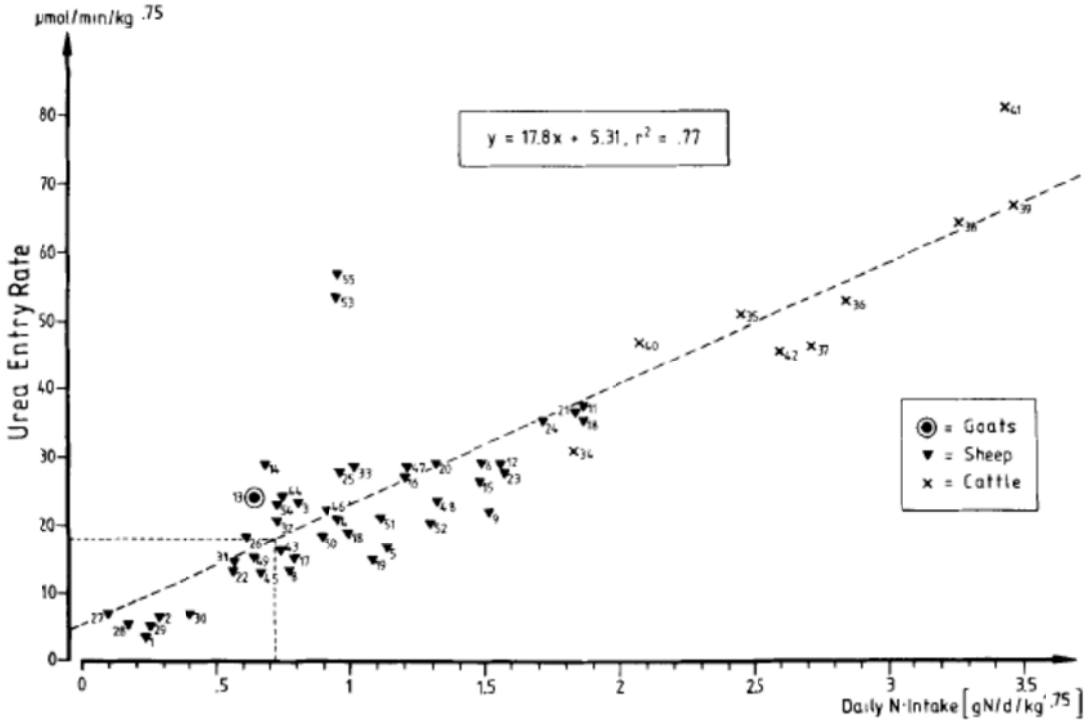


Figure 6. Compilation of data concerning N-intake and urea entry rate normalized to g N per day and per kg metabolic bodyweight ($kg^{.75}$). For example, a sheep with a body weight of 70 kg (24.2 $kg^{.75}$) and a N-intake of 60 g N/d has an N-intake of 2.48 gN/d/$kg^{.75}$. Urea entry is 1.542 Mol urea per day or 92.5 g urea or 43.2 r urea-N/d, which accounts for ca. 70% of N-intake. Adopted from Harmeyer and Martens (66).

Since 1980, the magnitude of UER has been confirmed in many studies, and the determined amount, namely UER ≈ 70% of N-intake, can be considered as a rule of thumb in nitrogen metabolism in ruminants. Furthermore, despite the constant UER, Harmeyer and Martens (66) realized that the two other parameters of urea metabolism, namely excretion via the kidneys or recycling, exhibit large and reciprocal variations. For example, urea excretion via the kidneys could account for most of UER, with only a small amount being recycled or – vice versa – almost all UER is recycled and negligible amounts are excreted in the urine. In 1980, the reasons of these alterations were only partly known and were considered to be related to fermentation products, CO_2, SCFA, and ammonia. Furthermore, nothing was known about the fate of the recycled urea-N.

The large variation of excretion via urine and by recycling was addressed in a review of Reynolds and Kristensen (135). A key factor determining urea recycling is obviously the crude protein (CP) content of the diet (Figure 7).

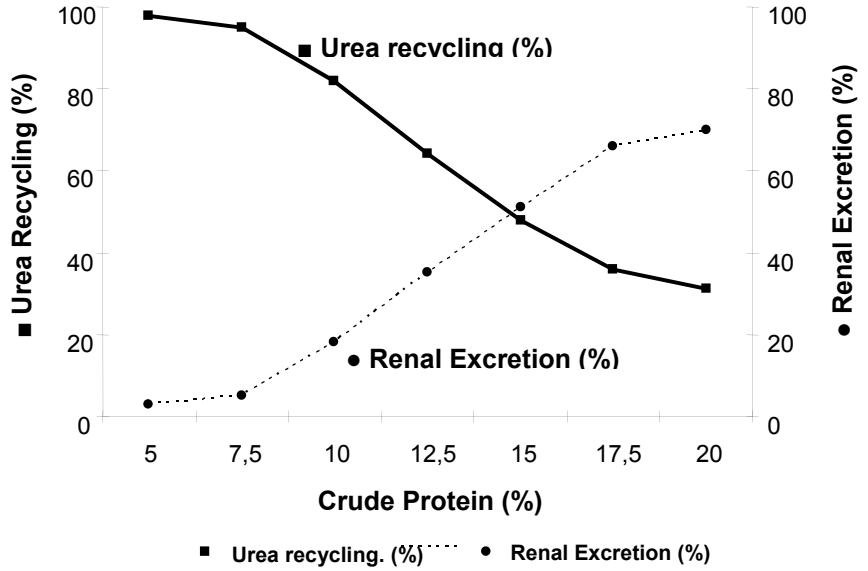


Figure 7: Schematic drawing of the compilation of data by Reynolds and Kristensen (135). Urea recycling clearly decreases with increasing CP content in the diet. Urea excretion via the kidneys is reciprocally increased.

The compilation of data by Reynolds and Kristensen (135) clearly shows that, at a low CP intake, almost all UER is recycled, and that this amount decreases with increasing CP. The amount of urea recycling changes reciprocally. The reason for these alterations was not clear, but good evidence has been presented that the concentration of the fermentation product ammonia increases with rising CP and probably modulates urea recycling (135).

Fate of recycled urea (Figure 8): As is well known, ammonia-N is the major N-precursor for the synthesis of microbial protein in the rumen, and urea-N is used for this purpose after hydrolysis by bacterial urease to ammonia and CO_2. Hence, recycled urea-N is, of course, used for microbial synthesis, particularly

at low CP intake. The magnitude of the re-use of urea-N exhibits some variation, but a recent summary of data has shown that some 50% of recycled urea-N is used for the synthesis of microbial protein, and some 40% is reabsorbed as ammonia and again detoxified to urea in the liver. The remaining 10% is excreted with the feces (89).

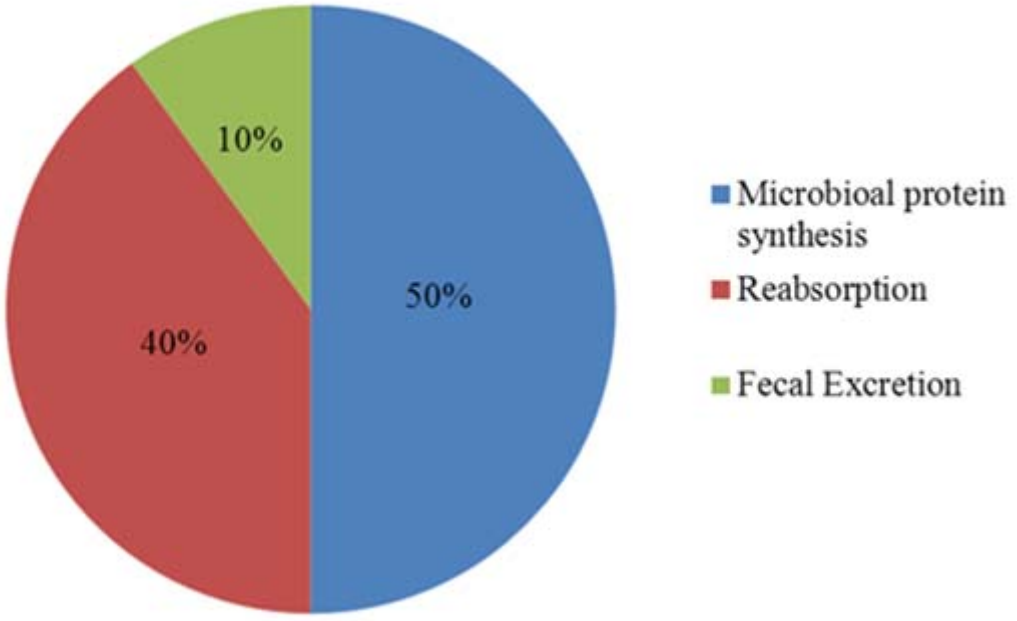


Figure 8: The fate of recycled urea-N. Some 50% is used for microbial synthesis, ca. 40% is reabsorbed as ammonia after intraruminally hydrolysis of urea by microbial urease, and 10% is excreted via feces. Data from Lapierre and Lobley (89).

In a summary of the data concerning urea turnover and recycling, three major observations are of interest:

1. The high amount of daily urea synthesis (g urea-N/d), which accounts for ca. 70 % of N-intake (g N/d). For example, a dairy cow with a daily milk production of 40 kg/d and a N-intake of some 600 g N/d, according to requirement, has an UER of some 400 g urea-N/d.

2. Urea handling exhibits a large variation and reciprocal changes: urea excretion via urine varies from 100 % during starvation to less than 10 % at

low protein intake, and urea recycling varies from zero to more than 90 %, respectively.

3. Most of the recycled urea-N is used as ammonia for microbial protein synthesis, but a significant amount is reabsorbed and must again be detoxified to urea. Some 10 % of recycled urea is excreted as ammonia via the feces.

The high and reciprocal variation of urea recycling and urea excretion has attracted the interest of scientists who are involved in nutrition and physiology and who wish to have a better understanding of the underlying mechanisms. Moreover, high urinary urea-N excretion has recently become a concern with regard to the contamination of the environment. Interestingly, this variation has been suggested to be caused by the alteration of urea transport across the rumen epithelium (135).

The data in Figure 7 suggest that the large variation in urinary excretion and of urea recycling is probably caused by the main fermentation products SCFAs, carbon dioxide (CO_2), and ammonia.

1.2.7 SCFA production and absorption in the rumen

Previous research has indicated that the SCFAs produced in the rumen are directly absorbed across the forestomach epithelium (128). In the rumen, 50% to 85% of the produced SCFAs are directly absorbed across the rumen epithelium; only 15% to 50% passes into the distal parts of the digestive system. Absorption of undissociated HSCFA via lipophilic diffusion has long been postulated to be the only relevant pathway of SCFA absorption (25, 61, 101). Because the permeability of the lipid bilayer membranes to the electrically charged SCFA anion ($SCFA^-$) is extremely low, passive diffusion

has to be attributed to the lipophilic protonated form: HSCFA (55, 172). Lipophilic diffusion couples the removal of $SCFA^-$ anions to the removal of protons at a ratio of 1:1, which constitutes a highly efficient way of proton extraction from the rumen (5). However, lipophilic diffusion is not the only way of SCFA absorption. The non-diffusional absorption of SCFA requires transport proteins and applies to the dissociated anions, $SCFA^-$. The further pathway for apical non-diffusional absorption of $SCFA^-$ has been clearly identified as the $SCFA^-/HCO_3^-$ exchanger (9, 57, 87). Acetate, especially, seems to use HCO_3^--dependent uptake for absorption (8, 10, 126). Masson and Phillipson (113) discovered that the absorption of SCFA leads to an increase of HCO_3^- in the rumen. However, this phenomenon was initially explained by carbonic acid dissociation that served to provide protons for a diffusive uptake of HSCFA, leaving HCO_3^- behind in the rumen (10). As has long been known, low ruminal pH stimulates SCFA absorption. However, definitive evidence for the direct interaction between $SCFA^-$ absorption and HCO_3^- secretion suggests that $SCFA^-$ absorption is attributable to a HCO_3^- gradient across the apical membrane and not to the pH gradient itself. In the absence of HCO_3^-, ruminal pH has no significant effect on acetate uptake (8). Meanwhile, apically exported HCO_3^- is probably replenished to a significant degree from extracellular sources, suggesting that HCO_3^- secretion by the ruminal epithelium involves apical $SCFA^-/HCO_3^-$ exchange and basolateral $Na^+HCO_3^-$ co-transport operating in series (8, 73).

1.2.8 SCFA, CO_2, and ruminal urea transport

Thorlacius et al. (167) used the method of the temporarily isolated and washed rumen of cows to study the effects of fermentation products on urea transport from blood to the rumen. The emptied rumen was filled with an artificial rumen fluid, which was bubbled with CO_2. The effect of CO_2 on urea transport from blood into the rumen increased with CO_2 (25%, 50%, and 100%) concentration

and was linearly related to the urea blood concentration. Furthermore, the presence of SCFA increased urea flux and a combination of CO_2 + SCFA stimulated urea flux even further. The underlying mechanisms of this effect on urea transport remained unclear at that time, but an increase of blood flow and hence transport of urea to the blood side of the epithelium was discussed as one explanation. In a later study, Dobson (41) rejected this hypothesis and an intra-epithelial mechanism was suggested.

Interactions between SCFA and urea recycling were observed in a study by Norton et al. (118). The authors infused Na-butyrate into the rumen of sheep and found an increase of urea recycling. The alteration of urea transport by butyrate is in agreement with further studies in which the intake of readily fermentable carbohydrates increased SCFA concentration in the ruminal fluid and urea recycling (118). The release of CO_2 and SCFA by microbial fermentation suggest that energy is available for the microbes in the rumen, and hence nitrogen is required for microbial growth and protein synthesis. Because most of the bacteria in the rumen depend on ammonia as a nitrogen source, the recycling of urea and its hydrolysis to ammonia are of physiological importance under these conditions. Ammonia can and is used for microbial growth.

However, this clear physiological implication does not explain the enhanced flux rate of urea across the rumen epithelium caused by CO_2 and SCFA. Hints for the intra-epithelial effects of these fermentation products have been deduced from studies of Na transport across the rumen epithelium. CO_2 and SCFA stimulate electroneutral Na transport (29, 38, 57). All these observations are consistent with the assumption that CO_2 and SCFA increase the intracellular availability of H^+ and decrease intracellular pH (pH_i), because CO_2 + H_2O is converted by carbo-anhydrase to H^+ and HCO_3^-, and because SCFA

uptake is mediated either by lipid diffusion in the undissociated form or exchanged as an anion via $SCFA^-/HCO_3^-$. In all cases, the increase of H^+ availability and the decrease of pH_i activates the Na^+/H^+ exchanger (NHE) and hence electroneutral Na transport (29, 38, 57, 109).

The interaction of luminal pH with SCFA and CO_2 on urea transport has been tested by Abdoun et al. (2). At a luminal pH of 6.4, the addition of SCFA (40 $mmol \cdot l^{-1}$) and CO_2/HCO_3^- (10% and 25 $mmol \cdot l^{-1}$) leads to a fourfold increase in urea flux. At pH 7.4, no significant effect of SCFA on the magnitude of the flux rates can be found. CO_2 has a slightly stimulating effect on urea transport. The stepwise reduction of luminal pH in the presence of $SCFA/CO_2$ from 7.4 to 5.4 leads to a bell-shaped modification of urea transport, with a peak at pH 6.2. Lowering the pH in the absence of $SCFA/CO_2$ has no effect. However, the mechanism of pH (including luminal and pH_i) on urea transporter is still unknown.

1.2.9 Ammonia

Ammonia is produced in all sections of the gut in animals and humans. In ruminants, ammonia is generated in the rumen fluid as a result of two main processes: 1) microbial degradation of nitrogenous compounds such as protein, peptides, amino acids, and nucleic acids; 2) microbial hydrolysis of urea recycled across the epithelia of the gastrointestinal tract from the blood and interstitial fluids and urea flowing into the rumen via saliva. In rumenal fluid, ammonia concentrations have been reported to range from 4 to 70 $mmol \cdot l^{-1}$, and more than 50% of the daily N-intake is absorbed in the form of ammonia (134).

Ammonia is also an essential nitrogen source for most of the rumen bacteria and hence for bacterial growth and protein synthesis. The early work of Bryant

and Robinson (24) indicated that about 90% of bacterial species in the rumen used ammonia as the main source of N for growth. Further studies have reported that free amino acids and peptides are probably directly incorporated into microbial protein without passing through the rumen ammonia pool (35, 104). Other studies have shown that, under feeding with protein sources, 40% – 70% of microbial N is derived from the rumen ammonia pool (72).

Ruminal microbial protein synthesis is saturated at an ammonia concentration of 3.6 $mmol \cdot l^{-1}$ (50 $mg \cdot l^{-1}$ NH_3-N)(141). Because ruminal ammonia concentrations exceed this value in many cases, the recycling of urea is not required under these conditions and is even unproductive, because the recycled urea is hydrolyzed, and the released ammonia is again reabsorbed and must be detoxified in the liver by the re-synthesis of urea. Hence, we can assume that urea recycling should depend on the ruminal ammonia concentration, and that ammonia is an important factor modulating urea recycling. Indeed, intraruminal infusion of NH_4Cl causes a significant decrease of urea recycling and increases urea synthesis and urea excretion (118). This early observation has been confirmed many times; in particular, the studies of Kennedy (79) have clearly shown that an increase of ruminal ammonia concentration inversely changes urea recycling. In recent *in vitro* studies with the isolated rumen epithelium of goats (116) and lambs (44), dietary nitrogen reduction has been demonstrated to enhance urea transport *in vitro* across rumen epithelium from the blood side to ruminal side.

These previous studies have clearly shown that luminal ammonia reduces urea recycling significantly. However, the mechanisms and details are still unclear. For example, in which form does ammonia inhibit urea recycling: in the ionized form (NH_4^+) or non-ionized form (NH_3)? Because luminal ammonia is mainly removed from the rumen (30%-65%) by absorption, the different

forms (ionized or non-ionized) might have different pathways across the rumen epithelium and lead to different effects on urea recycling. The predominant form of absorbed ammonia (NH_4^+ or NH_3) depends on the ruminal pH; NH_3 is mainly absorbed at a pH of > 7.00 by lipophilic diffusion and inhibits Na transport via Na/H exchange (NHE type 3: NHE3), because NH_3 is intracellularly protonated and hence H^+ are not available for NHE3 activity (1). Vice versa, at pH 6.4, NH_4^+ is predominantly taken up across an unspecific cation channel in the luminal membrane, and the release of H^+ in the cytosol increases NHE3 activity (1).

Again, for CO_2 and SCFA, the underlying mechanisms of altered urea transport are not known. Surprisingly, ammonia (NH_4^+) also stimulates electroneutral Na transport via NHE (1), because at a luminal pH of 6.4, ammonia is taken up from the lumen into the rumen epithelium via a K channel and/or an unspecific cation conductance and dissociates intracellularly, and the released H^+ is recycled via NHE (1).

The published data concerning the effects of fermentation products on urea transport or urea recycling clearly show effects that are of tremendous physiological importance: stimulation by CO_2 and SCFA and inhibition by ammonia NH_4^+ despite identical effects of pH_i.

1.2.10 Ruminal ammonia transport and potassium channel

Ammonia concentrations can increase within 2 h after a meal to 20 or 30 mmol/l, dependent on rumen degradable N-intake (135) and decrease rapidly thereafter, because of utilization by ruminal bacteria (35% to 65% of the decrease), efflux into the omasum (10% of the decrease), or absorption across the ruminal epithelium (79, 114, 123, 151). Absorption of ammonia across the apical membrane into the cytosol of rumenal epithelial cells equals the sum of

the parallel movement of the ionized (NH_4^+) and non-ionized (NH_3) forms. The relative concentrations and subsequent flux rates of NH_3 and NH_4^+ are modulated by the luminal pH according to the Henderson–Hasselbalch equation. The wide variations of luminal ammonia concentrations (maxim 70 mM) and luminal pH (5.4 to 7.4) cause corresponding changes of NH_3 and NH_4^+ concentrations and flux rates across the rumen epithelium.

Ammonia is a weak base with a pK of 9,20 (Leng and Nolan, 1984). The Henderson–Hasselbalch equation (Figure 9) shows that between pH 6 and 7, small amounts of ammonia exists in the non-ionized form NH_3 (0.1% and 1.3%, respectively), which will diffuse across the lipid layers of the cell membranes at low rates according the concentration. At pH values of > 7.0, the absorption of NH_3 increases with the luminal NH_3 concentration (21, 71, 133) and pH (58). The permeability of the rumen wall for ammonia is reduced at lower pH (21, 26, 71). Hence, NH_3 absorption remains stable, despite the increase in luminal ammonia concentration (71). These results are generally taken as evidence that one pathway of ammonia absorption across the epithelium of the rumen occurs by simple diffusion of the non-ionized lipid soluble form (NH_3).

$$\mathrm{pH} = \mathrm{pK}_a + \log\left(\frac{[\mathrm{A}^-]}{[\mathrm{HA}]}\right)$$

Figure 9: Henderson–Hasselbalch equation

However, convincing evidence has been presented that ammonia is also absorbed from the digestive tract, including the rumen, in the ionized form (NH_4^+) (71, 151). NH_4^+ is weakly lipid-soluble and needs the assistance of carriers or channels for its transport across the membranes of the epithelial cells. Bödeker and Kemkowski (18) have observed that the presence of luminal NH_4^+ causes positive short-circuit currents (I_{sc}) in sheep rumen epithelium *in vitro* indicating a transport of a cation from the mucosal to the

serosal side. They have also reported a reduction of the transepithelial ammonia fluxes in the presence of quinidine in the incubation solution. Quinidine is an unspecific K channel blocker, and its effect on NH_4^+ transport indicates passage through a channel. This conclusion of ammonia transport as an ion, namely NH_4^+, is supported by the alteration of the potential differences of the apical membrane, PD_a, by changes in the transepithelial potential difference, PD_t. The transepithelial potential difference of the rumen epithelium is clamped to +25 mV (blood site positive) , which depolarizes PD_a by some 15 mV (88) and hence is the driving force for NH_4^+ uptake. A PD_t of +25 mV causes a decrease in ammonia transport and suggests the transport of NH_4^+. Bödeker and Kemkowski (20) have concluded that NH_4^+ uptake across the apical membrane of the rumen epithelium occurs most likely via a K^+ channel; this initial suggestion was later demonstrated both *in vivo* and *in vitro*.

Sperber and Hyden firstly observed potassium secretion across the rumen epithelium following *in vivo* studies of ruminal chloride transport in 1952. K secretion was later observed both *in vivo* (53, 75) and *in vitro* (52, 95, 177) via a ouabain-sensitive mechanism (68). These studies led to the conclusion that K is taken up across the basolateral membrane with the Na/K-ATPase, and that most of the K is recycled via a K channel in the basolateral membrane. A small part of K passes the apical membrane across a cation channel and explains the small net secretion. However, the net movement of K depends on the K gradient across the epithelium. At low ruminal K concentrations, K is secreted, and at high luminal K concentrations, K absorption is correlated to the luminal K concentration probably by using the channels in the apical and basolateral membrane (75, 173, 174).

The experimental data of previous researches show that the PD_t across the rumen epithelium rises both *in vivo* (53, 107, 144, 145) and *in vitro* (52, 68, 95)

with increasing luminal K concentration and can be explained by the depolarization of PD_a (95). The depolarization of the apical membrane of the rumen epithelium in high potassium solutions coexists with the decrease in the fractional apical resistance.

1.2.11 Ruminal ammonia absorption and modulation by electrical gradients

Abdoun et al. (1) have used the Ussing chamber technique to measure the flux of ammonia in the mucosal–serosal direction across the isolated sheep rumen epithelium at various ammonia concentrations and pH values in order to identify the transported form and amount of ammonia (NH_3 or NH_4^+). The measurement of total ammonia flux at a mucosal concentration of 30 $mmol \cdot l^{-1}$ shows that the reduction of ammonia fluxes is less than the decrease in NH_3 concentration with a decreasing mucosal pH from 7.4, to 6.9, and 6.4 and suggests an additional flux of NH_4^+. NH_3 concentrations have been calculated with the Henderson–Hasselbalch equation as being 0.07 $mmol \cdot l^{-1}$ (pH 6.4), to 0.27 $mmol \cdot l^{-1}$ (pH 6.9), and 0.68 $mmol \cdot l^{-1}$ (pH 7.4), respectively, whereas the concentrations of NH_4^+ remain almost unchanged (> 29 $mmol \cdot l^{-1}$; total ammonia concentration 30 $mmol \cdot l^{-1}$). A linear correlation (1) between the measured total ammonia flux (designated as 'y') and the concentration of mucosal NH_3 (designated as 'x') has been obtained: $y = 4.1x + 0.7$ ($r = 0.99$): y = ammonia flux ($NH_3 + NH_4^+$); x = NH_3 concentration. The intercept 0.7 represents the flux rate of NH_4^+ at zero NH_3 concentration and permits the calculation of permeability for NH_4^+: $\approx 6.5 \times 10^{-6}$ cm/s. The flux rate of NH_3 is obtained by the calculation of the total ammonia flux minus (0.7 = NH_4^+ flux rate) and results in a transport rate of 2.8 $\mu eq \cdot cm^{-2} \cdot h^{-1}$ NH_3 (NH_3 concentration 0.68 $mmol \cdot l^{-1}$ at pH 7.4 at a total ammonia concentration of 30 $mmol \cdot l^{-1}$) and a permeability of 1.1×10^{-3} cm/s, which is ca. 175 times larger than the permeability of NH_4^+. Hence, despite the low proportion of NH_3 of 0.68 $mmol \cdot l^{-1}$

(= 29.32 mmol·l^{-1} NH_4^+), NH_3 is predominantly transported at pH 7.4 and, vice versa, NH_4^+ at pH 6.4.

As is to be expected from these calculations, the luminal pH determines the relative amounts of NH_3 and NH_4^+ entering the cytosol, which in turn determines the effect on intracellular pH, pH_i, by the protonation of NH_3 or the dissociation of NH_4^+ and hence Na transport via the apical sodium– hydrogen exchanger (NHE3) (1).

The above experimental results clearly show the transepithelial movement of NH_4^+. Hence, NH_4^+ transport across the rumen epithelium should be determined not only by the chemical gradient, but also by the electrical gradient. Schwartz and Tripolone (143) have made similar observations in bladder and report a significant increase from serosal to mucosal side and a decrease from mucosal to serosal side in the ammonia flux rate after a change of transepithelial potential difference (PD_t) to + 50mV (serosal side positive to mucosal side). The opposite changes occur when the PD_t is clamped to –50 mV. In the sheep rumen epithelium, ammonia fluxes from the mucosal to the serosal side are significantly reduced when PD_t is clamped to +25mV (polarity on the serosal side) compared with those measured at a PD_t of -25mV (18).

Recent studies support the assumption of NH_4^+ transport across the apical membrane via a potassium channel (1). The potential difference of the apical membrane (PD_a) works as the driving force. The results of Lang and Martens (95) have clearly demonstrated that the change of PD_t by 25mV causes an alteration of PD_a of some 15 mV. The applied PD_t of +25mV (blood site positive) in the studies of Bödeker and Kemkowski (18) changes (depolarization) the PD_a by some 15mV and leads to a reduction of NH_4^+ uptake attributable to the decreasing of the electrical driving force across the

apical membrane. However, a PD_t of -25mV enhances NH_4^+ flux rates corresponding to the increase of PD_a. Notably, the flow of NH_4^+ through the luminal potassium channel exhibits a competitive relationship with luminal K^+ (1). Hence, NH_4^+ absorption can be reduced at a low luminal pH (<6.4) combined with high luminal K^+ concentration.

1.2.12 Modulation of ammonia transport by CO_2 and SCFA

Ammonia absorption depends mainly on its concentration but can be enhanced by SCFAs and the CO_2/HCO_3^- in the rumen (20, 133). Bödeker et al. (20) suggest that the interaction between SCFAs and ammonia occurs just underneath the apical membrane of the epithelial cells. Because the intracellular pH of these cells is close to 7, SCFAs absorbed in their non-ionized form will dissociate and release protons. These released protons can be used to form NH_4^+ from the absorbed NH_3 (Figure 9). This process will result in a decrease in the intracellular NH_3 concentration and thereby will increase its absorption. Likewise, the intracellular release of HCO_3^- and H^+ from CO_2 and H_2O by the action of carbonic anhydrase might serve as a proton source for NH_4^+ formation, since the inhibition of carbonic anhydrase reduces the ammonia flux across rumen mucosa in vitro (19). Bödeker et al. (20) have also suggested that the passage of both $SCFA^-$ and HCO_3^- ions across the basolateral cell membrane in conjunction with NH_4^+ allows the electroneutral exit of all three compounds against an adverse electrical potential difference (Figure 10).

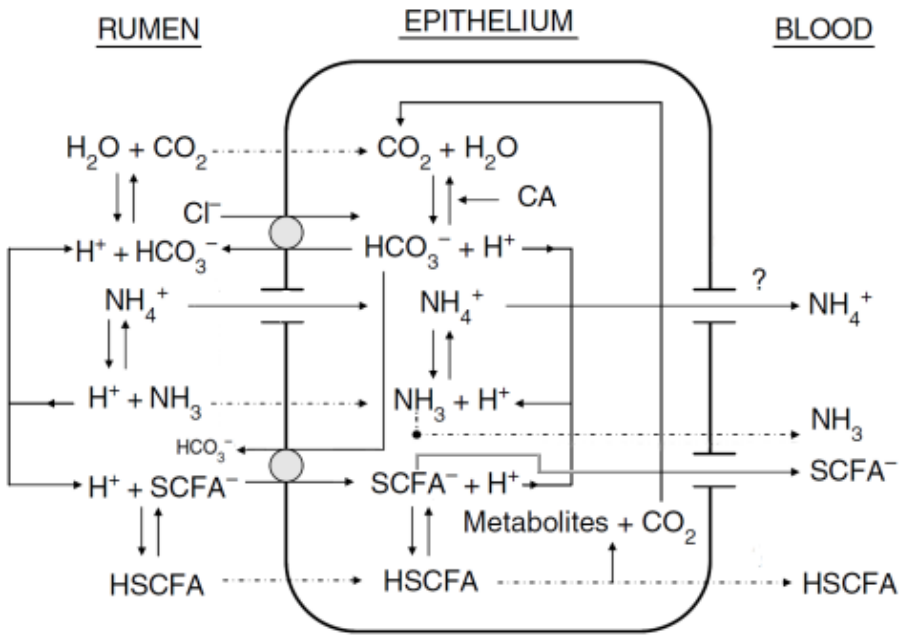


Fig.10 Scheme of pathways of NH_3 and NH_4^+ across the rumen epithelium and modulation by CO_2/HCO_3^- and SCFA according the data of Bödeker et al. (19, 20). The luminal uptake of NH_4^+ and NH_3 is mediated by a putative K channel or by diffusion across the apical membrane, respectively. The relative transport rates of both forms depend on the ruminal pH and the concentration of protons just above the luminal membrane. Availability of protons can be reduced by reaction with secreted HCO_3^- ($H^+ + HCO_3^- \leftrightarrow H_2O + CO_2$) or by the protonation of SCFA ($H^+ + SCFA^- \leftrightarrow HSCFA$). In both cases, the NH_3 concentration increases and hence also NH_3 uptake. Intracellularly, NH_3 will be protonated to NH_4^+ by protons from the dissociation of HSCFA or H_2CO_3 (catalyzed by the carboanhydrase = CA). This intracellular protonation of NH_3 maintains the NH_3 gradient and uptake across the luminal membrane. The exit of both forms of ammonia occurs either by lipid diffusion of NH_3 or as NH_4^+ across a basolateral cation channel.

Stimulation of NH_3 absorption by HCO_3^- has been reported in monogastric animals (33, 181). This suggests that HCO_3^- secretion in the colon titrates luminal NH_4^+ to NH_3 and leads to NH_3 diffusion from the lumen. The reported negative effect of carbonic anhydrase on ammonia transport by Bödeker et al. (20) can therefore be explained as: the reduced intracellular titration of NH_3 to NH_4^+ and the reduced luminal titration of NH_4^+ to NH_3.

The effects of SCFAs and CO_2 on ammonia absorption can also be explained

by their action on subepithelial blood flow (40) and irrigated capillary surface area (167). However, Remond et al. (133) have observed that, when butyric acid is supplied to the rumen contents or when CO_2 is blown into the rumen, the increase in ammonia flux across the rumen wall is always lower than the increase in ruminal blood flow produced by either of the two treatments. As mentioned above, Dobson (42) rejected the suggestion that the blood flow is th major determinant of changed transport rates.

Our current knowledge about ammonia transport permits the conclusion that both forms of ammonia, NH_3 and NH_4^+, are transported across the rumen epithelium, and that this transport is predominantly modulated by the luminal pH. Furthermore, interactions between ammonia transport and SCFA and/or CO_2 can be suggested.

1.2.13 Urea transporter

Early suggestions: Urea as a water-soluble molecule is evenly distributed between the extra- and intracellular space. No significant differences of urea concentration in blood and muscle fluid have been found in sheep, and the urea space accounts for some 50% of body weight and is almost identical with the body water volume (67). Movement of urea across the cell membranes out of or into the intracellular space was originally thought as passive diffusion by lipid-phase permeation. Urea was, mistakenly, considered to be freely permeable. However, in kidney, the transit time for tubular fluid through the collecting duct or for red blood cells through the vasa recta is too fast to allow urea concentrations to reach equilibrium solely by passive diffusion (84). Indeed, Chou et al. (30) have concluded, from their studies with the isolated inner medullary collecting duct of rat kidney, that urea is transported via a “specialized transporter, probably by an intrinsic membrane protein” as earlier demonstrated in toad urinary bladder (98) and in red blood cells (103). Since

this first evidence of urea-transporting proteins, a vast number of publications have appeared describing urea transporters in a variety of cells and organs. This knowledge has been summarized in reviews (12, 84, 115, 154, 161).

Urea transporters: According to the reviews mentioned above, urea transporters are members of a superfamily that mediates urea flux across cell membranes. This family has two major subgroups, designated *SLC14A1* (or UT-B) and *Slc14A2* (or UT-A). UT-B has 2 subtypes, namely UT-B1 and UT-B2, that differ by only a few nucleotides at their 3' end (36, 169). UT-A has gene splice variants that have been characterized: UT-A1 (148), UT-A2 (155), UT-A3 and UT-A4 (76), UT-A5 (51), and UT-A6 (156). UT-B and UT-A gene products are glycoproteins located in the kidney, and some isoforms are also found in various extra-renal tissues.

The molecular identity of the first member of this superfamily was determined by You et al. (182) in rabbit inner medulla. The protein encoded by this cDNA came to be known as UT-A2 and is encoded by the *Slc14a2* gene. Ritzhaupt et al. (136) first reported that a cDNA fragment amplified from sheep rumen cDNA was homologous to rat kidney UT-B (*Slc14A1*), which was firstly cloned by Olives et al. (124) from a human erythropoietic cell line, with 1173 bases in the open reading frame and encoding a protein with 43 kDa of molecular mass. However, the authors did not report the isolation of a full-length clone.

In ruminants, UT-B has been found in the kidney, liver, reticulum (Ludden et al., 2009), parotid gland (39, 102), small intestine, colon (102), and rumen epithelium (102, 105, 106, 116, 136, 137, 153, 162). Both subtypes of UT-B, namely UT-B1 and UT-B2, can be found in the rumen, with UT-B2 being the predominant variant (162). Once urea recycling was found to be modulated by CP intake (see above), many studies of UT-B expression in the rumen

epithelium of cattle, sheep and goats have been published since that time (102, 105, 106, 116, 137, 153) (see Table 1). In these studies, either the CP content of the diet has been changed, or the fermentation pattern of the rumen microbes has been altered, and the expression of UT-B has been monitored.

Table 1: Effect of feeding conditions on ruminal UT-B expression

Authors	Year	Animal	Variable Parameter	Ruminal UT-B expression
Marini et. al	2003	cow	Crude protein intake	Increase (protein)
Marini et. al	2004	lamb	Crude protein intake	No change (protein)
Ludden et. al	2009	lamb	Crude protein intake	Increase (protein)
Simmons et. al	2009	bovine	Fermentable carbohydrates	Increase (mRNA, protein)
Muscher et. al	2010	goat	Crude protein intake	No change (mRNA)
Røjen et. al	2011	cow	Crude protein intake	No change (mRNA, protein)

In summarizing these results, the following conclusions can be made:

1. UT-B 1 and 2 are expressed in the rumen, with UT-B2 being the predominant variant (162).

2. UT-B is expressed in the membranes of all rumen epithelial cell layers (153, 162); this permits urea transport from cell to cell in the multilayered rumen epithelium. Hence, UT-B is suggested to be responsible for transepithelial urea transport.

3. As is well established, urea transport across rumen epithelium is modulated by the dietary N-intake (135). However, the modulation of UT-B expression in rumen epithelium by dietary N-intake is still disputed. Some researchers report the up-regulation of ruminal UT-B expression by increasing dietary N-intake (102, 106, 153). However, some studies indicate that increasing dietary N-intake has no significant effect of UT-B(107) expression in

rumen epithelium (105, 116, 137). The reasons for this discrepancy are still unknown. Furthermore, these findings do not explain the acute modulation of urea transport *in vivo* and *in vitro* by SCFA, CO_2, ammonia, or luminal pH (2, 42, 66, 132, 167).

Structure of UT: The knowledge about the sequence of the UT proteins has encouraged scientists to characterize the structure of a variety of urea transporters (51, 124, 149, 156, 182). All of UT-A and UT-B isoforms share significant structural homology. The hypothetical primary structure predicts 10 transmembrane domains organized into two internal hydrophobic repeats with an extracellular connecting loop, intracellular amino and carboxy termini, and a single N-glycosylation site with many protein kinase A (PKA) and protein kinase C (PKC) phosphorylation sites (124, 182) (Figure 11)

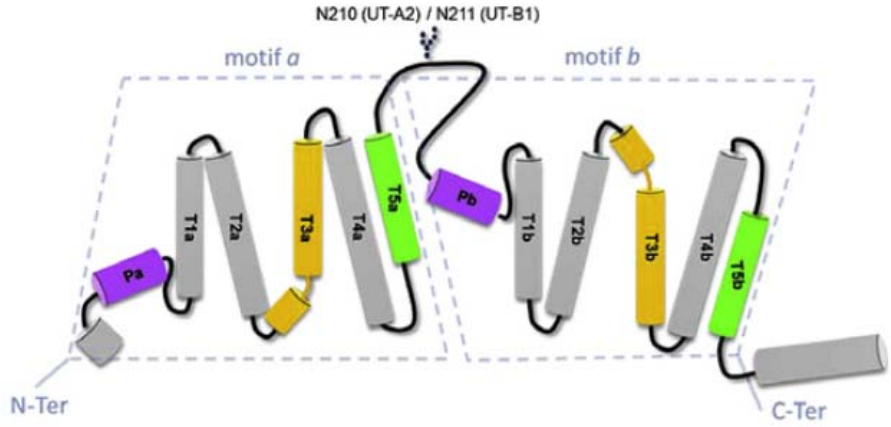


Figure 11: Scheme of membrane topology of human urea transporter UT-A1. It includes duplication with five membrane-spanning segments connected by a hydrophobic extracellular loop. The protein carries several putative N-glycosylation (N 271, N 733) and tyrosine kinase (TK, white star), protein kinase A (PKA, yellow star) and protein kinase C (PKC, blue star) phosphorylation sites, and the amino- and carboxy termini are located intracellularly (147).

In 2009, Levin determined the high-resolution structure of a bacterial (*Desulfovibrio vulgaris*) homolog of the kidney urea transporter (dvUT) (97).

This structure was a milestone in increasing our understanding of the molecular transport mechanism of urea transporters and has been discussed in an editorial by Knepper and Mindel (81, 86) They propose, in accordance with the data of Levin et al. (97), a “molecular coin slot for urea” (Figure 12)

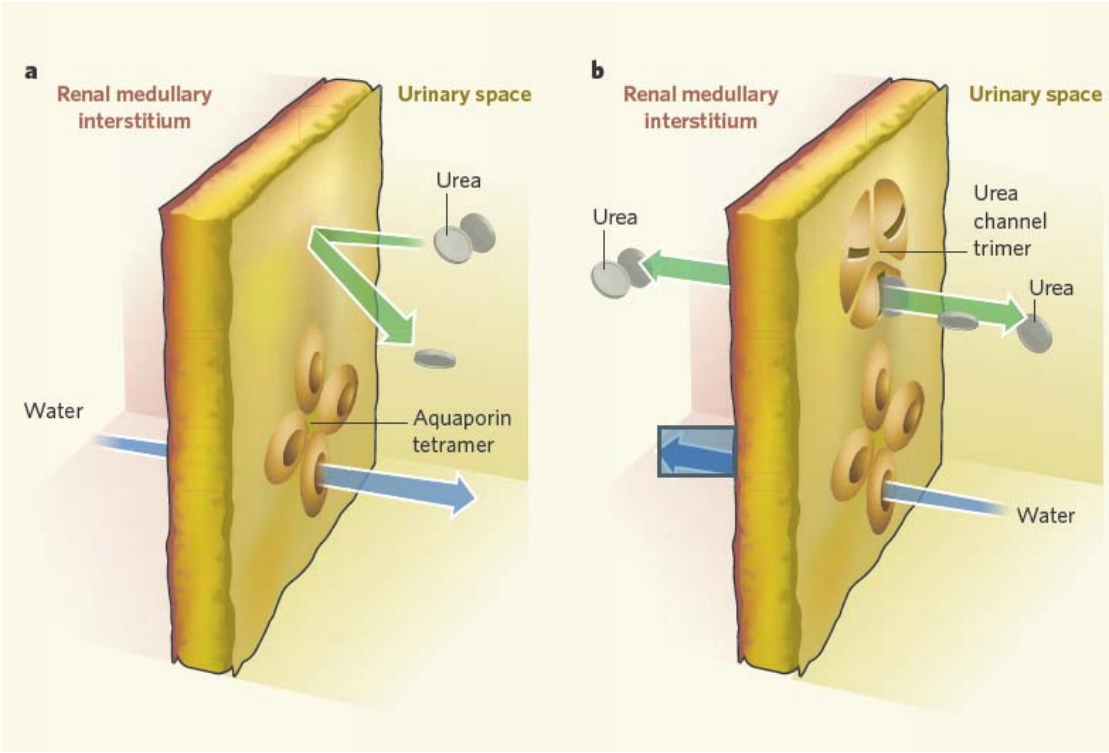


Figure 12: The urea transporter model from the bacterium Desulfovibrio vulgaris (dvUT) proposed by Knepper and Mindell (86) according the data of Levin et al. (97).

Urea channels are located as a trimer in the membrane, and the planar urea molecule passes through a urea channel, which resembles a “coin slot” of coin-operated vending-machines. This urea transporter homolog from the bacterium *Desulfovibrio vulgaris* (dvUT) has been shown to have similar transport properties as mammalian urea transporters and is now obviously accepted as a general model, because, in a recent review on UT structure, Shayakul et al. (147) have applied this scheme for the human UT-A1. A corresponding model of UT-B and, particularly, of bovine and ovine UT-B is still lacking, but the conservation of the selectivity filter of UT within a species and

between species supports the assumption of a similar structure.

1.2.14 Regulation of UT activities

The evidence of urea transporters in a variety of cells and organs, their precise location within an organ as, for example, in the kidney (147), and the known phenotypic alteration of urea transport have opened the door for the exact determination of UT activities. As is well established, vasopressin enhances urea transport in various cells (collecting duct, vasa recta) of the kidney, and this effect is mediated by the phosphorylation of UT. The details of these steps of regulation are summarized in recent reviews (12, 84) but are restricted to UT-A subtypes.

Surprisingly, little is known about regulation of UT-B. The careful study by Tickle et al. (168) of bovine UT-B2 expressed in a stable MDCK cell line has examined the effects of various modulators on urea transport. Pre-exposure or acute addition of vasopressin does not influence urea transport. Furthermore, an increase of cAMP, intracellular Ca^{2+}, or of protein kinase activity does not change urea transport clearly indicating that the regulation of this transporter (bovine UT-B2) does not fit into the scheme of UT-A regulation. Hence, Tickle et al. (168) have concluded that “Further work is now required to determine exactly how gastrointestinal (bovine) UT-B function is controlled”.

The current knowledge about urea recycling and urea transport across the rumen epithelium allows the following conclusions to be made:

a. Urea is transported by passive diffusion across the rumen epithelium.
b. The transporters UT-B1 and UT-B2, which have been detected in the rumen epithelium, probably mediate urea transport across this epithelium.

c. Urea recycling and hence ruminal urea transport are modulated by the diet.
d. Ruminal urea transport is modulated *in vivo* and *in vitro* by fermentation products such as SCFA, CO_2, and ammonia.
e. Knowledge of the regulation of UT-B is still lacking, despite the large changes in urea transport induced by fermentation products, luminal pH, and particularly ammonia, which appears to be the major cause of the reciprocal changes of urea recycling and renal excretion (see Figure 7).

The aim of the current study has therefore been to investigate the direct and short-term effects of ammonia on ruminal urea transport *in vitro*. The following questions have been addressed.

a) Can the inhibitory effect of ammonia on ruminal urea transport be confirmed *in vitro*?

b) As the stimulating effect of CO_2 and SCFA on urea transport depends on the luminal pH (2), do similar correlations exist between luminal pH and the inhibitory effects of ammonia on urea transport?

c) Is it possible to demonstrate interactions between SCFAs (stimulation) and ammonia (inhibition) with regard to urea transport?

d) In what way does ammonia affect intracellular pH (pH_i), and might this play a role in modulating urea transport across the ruminal epithelium?

Chapter 2

Material and Methods

2.1 Experimental animals and feeding

Experiments were conducted in accordance with the German law for the care and use of experimental animals and performed essentially as previously described (50). The sheep were 6-10 months old at the time of the experiments with a range of body weight between 33 kg and 50 kg. All animals were fed a pure hay diet ad libitum over a period of six weeks containing (per kg dry matter (DM)): 144 g crude protein, 28 g fat, 277 g crude fibre, 89 g ash, 29 g potassium, 2.2 g sodium, and 8.5 MJ metabolizable energy (ME). At the beginning of the experimental period, hay intake was 1000 g per animal and day (88 % DM) and was offered in two portions at 7.00 a.m. and 3.00 p.m. equaling an intake of 7.5 MJ ME, which is slightly above requirements (Gesellschaft für Ernährungsphysiologie (GfE)). One week before each experiment, the sheep were kept individually in pens in the same room on straw in order to control their feed intake. The sheep could see each other (no isolation). The concentrate-fed sheep received 400 g hay and 400 g concentrate twice a day (7.00 a.m. and 3.00 p.m.), which amounted to 11.2 MJ ME per animal and day and permitted a growth rate of ca. 200 g/day**.** The supplemented concentrate contained per kg DM: 176 g crude protein, 143 g crude fibre, 33 g fat , 104 g ash, and 6.5 MJ ME. The mixed diet (hay + concentrate) was stepwise changed within 4 days and was fed to the sheep 2 – 3 weeks before an experiment (50).

2.2 Rumen epithelium isolation and incubation

The detail of rumen epithelium incubation has been described by Abdoun et al. (2). The experimental sheep were slaughtered in a local slaughterhouse, and the reticulo-rumen was removed from the abdominal cavity within 2-3 min. A piece of rumen wall (about 300 cm^2) was taken from the ventral sac and cleaned in transport buffer (see the buffer list below, Tables 2, 3, and 4) stripped from the muscle layers. The tissues were transported to the laboratory

in a buffer solution that contained (in $mmol \cdot l^{-1}$): 115 NaCl, 25 $NaHCO_3$, 0.4 NaH_2PO_4, 2.4 Na_2HPO_4, 5 KCl, 5 Glucose, 1.2 $CaCl_2$, 1.2 $MgCl_2$; pH 7.4 at 38.0°C, adjusted to 280 $mosmol \cdot l^{-1}$ with mannitol. The solution was gassed with 95% O_2 - 5% CO_2. Pieces of epithelia (3×3 cm) were mounted between the two halves of an Ussing chamber with an exposed area of 3.14 cm^2. The mounted tissue was bathed on each side with 16 ml buffer solution by using a gas-lift system and gassed with a mixture of O_2/CO_2 (95:5) at 38°C.

2.3 Ussing chamber technique

All the experiments were carried out on isolated ruminal epithelial tissue with the conventional Ussing chamber technique developed by the Danish scientist Hans Ussing . This method has been modified many times for investigations of rumen and other forestomach epithelial tissues (54, 160). The chamber consists of two equal halves between which the epithelia are mounted dividing the chamber into two equal spaces (luminal = apical = mucosal; blood side = basolateral = serosal). In these experiments, the exposed area of the epithelium was 3.14 cm^2. Silicon rings placed on both sides between the epithelium and the chamber were used to minimize edge damage. The usual procedure for all the experiments was to equilibrate the tissues under the short-circuited condition for not less than 30 min with the control buffer solution, so that all the electrophysiological values became relatively stable. After this incubation period, only those epithelial tissues that had a conductance (G_t) of not more than 8.0 $mS \cdot cm^{-2}$ and a short-circuit current (I_{sc}) of not less than 0.5 $\mu eq \cdot cm^{-2} \cdot h^{-1}$ were subsequently used in the experiments described. Under these conditions, the epithelial tissues remained stable for a relatively longer period of time . Epithelial tissues that deviated markedly from the above-mentioned electrophysiological values were considered to have been improperly prepared, transported, or handled. Once relatively stable electrophysiological parameters had been achieved, epithelial tissues with

approximately the same electrophysiology (G_t and I_{sc}: less than 20%) were paired for the determination of unidirectional ion fluxes (J_{ms}: mucosal-serosal, or J_{sm}: serosal-mucosal). For each set of experiments, 12 epithelial pairs were available (when 4 clamps were used in parallel). Since the heterogeneous origin of sheep causes unavoidable variations in the electrophysiological values (142), and because of variations among tissues within the same animal, control and experimental groups were performed with tissues from the same animal.

The standard experimental buffer contained (in $mmol \cdot l^{-1}$): 95 NaCl, 25 Na-acetate, 10 Na-propionate, 5 Na-butyrate, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 0.4 NaH_2PO_4, 2.4 Na_2HPO_4, 2.5 glutamine, 5 KCl, 5 glucose, 8 MOPS, 5 NH_4Cl, and 1 urea, adjusted to pH 7.4 and bubbled with O_2. In solutions containing SCFA and bicarbonate (SCFA & bicarbonate), an equimolar amount of NaCl was replaced by 25 $mmol \cdot l^{-1}$ $NaHCO_3$ equilibrated with 95% O_2 - 5% CO_2. In high potassium buffer (buffer 4), 65 $mmol \cdot l^{-1}$ KCl was used to replace an equimolar amount of NaCl. High SCFA & bicarbonate buffer (5) contained (in $mmol \cdot l^{-1}$) 45 NaCl, 50 Na-acetate, 20 Na-propionate, 10 Na-butyrate with all other components as in buffer 2. In the groups with lower NH_4^+, appropriate amounts of NH_4Cl were replaced by NMDG-Cl (N-methyl-D-glucamine chloride) (for details of buffer composition, see Table 2, 3 & 4). Mannitol was used to adjust the osmolarity of all solutions to 300 $mosmol \cdot l^{-1}$ (Osmomat 030-D, GONOTEC Berlin, Germany). The urease inhibitor phenyl phosphorodiamidate (0.1 in $mmol \cdot l^{-1}$) was obtained from ABCR (Karlsruhe, Germany). All reagents were of analytical grade.

2.3.1 Electrical measurements

Electrical measurements were continuously obtained 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. 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 for 1s duration. If not specified otherwise, all measurements were performed under classic short-circuit conditions

2.3.2 Open circuit conditions

In this method, current pulses of 100 µA amplitude (0.5 sec duration) were applied to cause a change in PD_t, and from this, the tissue conductance (G_t) could be calculated according to Ohm´s law. All the experiments began in this mode for at least for 30 min in order for the tissues to equilibrate.

$$Gt = \frac{\Delta I}{\Delta PDt}$$

where

G_t = Tissue conductance ($mS \cdot cm^{-2}$)

ΔI = Current pulses (µA)

ΔPD_t = Change in transepithelial potential difference (mV)

2.3.4 Short-circuit conditions

This technique is a particular form of the *voltage clamp* technique in which the transepithelial potential difference (PD_t) is clamped to 0.0 mV through the application of an external current. Under these conditions, the short-circuit current (I_{sc}) is equivalent to the sum of all electrogenic ion movement across the epithelial tissue. There is no electrical gradient across the epithelial tissue,

and if the buffer solutions on both sides of the epithelial tissue are identical, there is also no chemical gradient across the epithelial tissue. When a net transport of an ion or nutrient is measured under these conditions (= no passive driving forces), the transport mechanism of the ion is considered as active or secondary active.

2.3.5 Calculation of ion flux rates

The unidirectional flux rate (J) per h and per cm^2 [J_{ms} (mucosal-serosal) and J_{sm} (serosal-mucosal) flux rates] were calculated from the samples taken from the cold (non-radioactive) sides according to the following formula:

$$J = \frac{P_2 \bullet V_b / V_s - \left[P_1 \bullet \left(V_b - V_s\right)/V_s\right]}{AK_{spec}.A.t}$$

where

J = Transport rate or flux [$\mu mol \cdot cm^{-2} \cdot h^{-1}$]

P_1 = Counts per min at the beginning of flux period

P_2 = Counts per min at the end of the flux period

V_b = Buffer volume (16ml)

V_s = Sample volume (1.0ml)

Ak_{spec} = Specific radioactivity in cpm/µM

A = Chamber free surface area (3.14cm^2)

t = Duration of the flux period (0.5 h)

The specific radioactivity was calculated from the mean of the hot sample radioactivity [(H1+H2/2)] (see below for an explanation of H1 and H2):

$$\text{Specific Radioactivity } (AK_{spec}) = \frac{cpm_H}{V_H \cdot c}$$

where

Cpm_H = Radioactivity of the hot sample (Cpm)

V_H = Volume of the hot sample in ml

C = Concentration of the measured non-radioactive isotope in the buffer solution ($mmol \cdot l^{-1}$ or $\mu mol \cdot l^{-1}$)

The net flux rate was calculated from the unidirectional flux rate as follows:

$$J_{net} = J_{ms} - J_{sm} \; [\mu mol \cdot cm^{-2} \cdot h^{-1}]$$

A positive net flux indicated absorption, whereas a negative net flux indicated secretion.

2.3.6 Measurement of fluxes

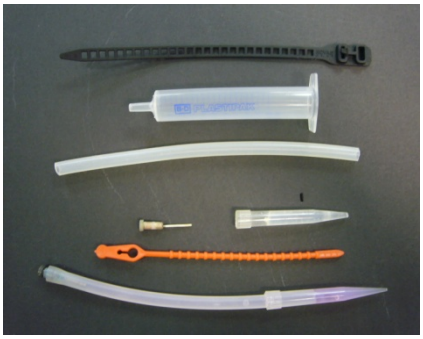
Since the Ussing chamber technique only allows the measurement of unidirectional fluxes, i.e., mucosal to serosal (J_{ms}) or serosal to mucosal (J_{sm}), careful pairing of the epithelia is important. Hence, tissues were paired with differences of G_t < 20%. For flux measurements, 70 kBq of the radioactive isotope ^{22}Na (Amersham Buchler, Braunschweig), 60 kBq of ^{14}C-acetate, or 46.25 kBq of ^{14}C-labeled urea were added to the marked side (mucosal for J_{ms} and serosal for J_{sm}), called the “hot side”. The other side was referred to as the “cold side”. At 15 min after the addition of the radioisotope, 100-µl aliquots were taken from the hot side (referred to as “H1”), and this was repeated at the end of the last flux periods (called the “H2”). These were used to calculate the specific activity. Periodic samples of 1 ml from the cold side were taken at an interval of 30 min, at least 3 times (called fluxes). The same volume was replaced in the reservoir with the respective experimental buffer to maintain a

constant volume. ^{22}Na was assayed by using a well-type crystal counter (LKB; Wallace-Perkin Elmer, Überlingen, Germany), and ^{14}C-labeled urea and acetate was assayed in scintillation liquid (Rotiszint, Roth–Karlsruhe, Germany) by using a β-counter (LKB Wallace-Perkin-Elmer; Überlingen, Germany).

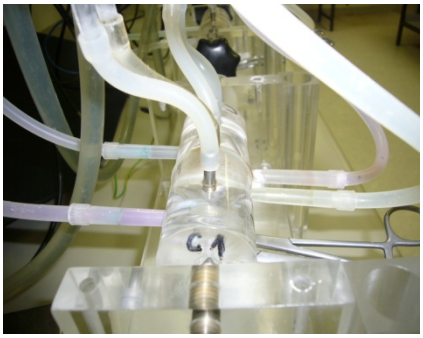
2.3.7 Modifications of Ussing chamber technique

Details of the Ussing chamber experiment have been described above. A modified version of attachment tubes were used during the time course of the study. Gelrite® (Roth, Karlsruhe) (0.8%) was dissolved in boiling water with 0.1% $MgSO_4.7H_2O$, for polymerization (agar), and 1.5 mmol·l^{-1} KCl was added to it. Later, this material was filled half way up 100-1000µl pipette tips, allowed to cool at room temperature and stored in 1.5 mmol·l^{-1} KCl solution in the refrigerator (4°C). The conventional front and rear side bridges were replaced with silicone tubing (inner diameter 4 mm, outer diameter 7 mm) of approximately 20 cm in length. For the rear side bridges, the tubing was filled with 1.5 mmol·l^{-1} KCl, attached to the pipette tips, and closed with a silver wire adaptor preventing larger air bubbles. For the front bridges, 10-ml syringe cylinders were connected to the silicon tubing, which was closed with pipette tips filled with 1.5 mmol·l^{-1} KCl, and then the electrodes were placed into it (see Fig 13). For the better insertion of the pipette tips, the chambers were drilled with a V-shaped drill to create conical holes. This facilitated insertion of the pipette tips and their fixation to the chambers. The advantage of this pipette attachment system was that a uniform distance was obtained within and between the chambers or tissues (when the epithelium was in place) to achieve approximately alike PDs within the chamber. This method is more secure in terms of the prevention of the leakage of buffer, and it is quick and easy to handle. The fluid resistance between all the clamps (24 in our laboratory) is almost within the same range compared with the same distance between the tips of the bridge. With this setup, namely having stiff pipette tips

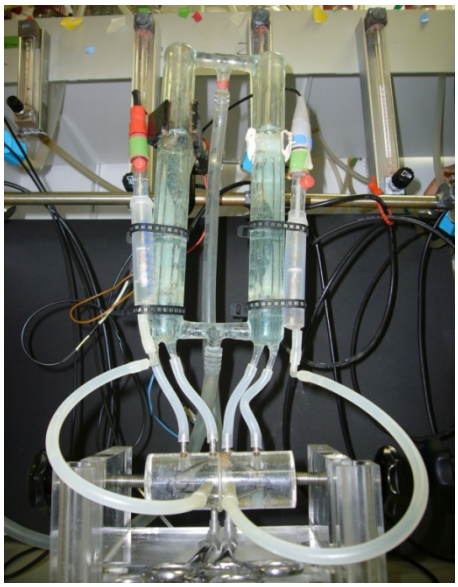
attached to the chambers and silicone tubes filled with KCl, better conductivity and stability can be achieved. This modified equipment has been tested in many experiments, and the information obtained is reproducible.



A



B



C

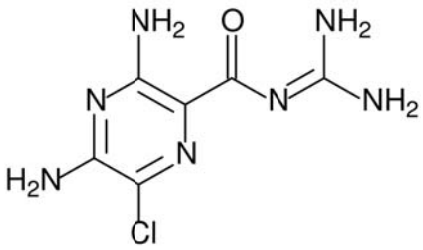
Fig. 13: Modified Ussing Chamber
A: Various attachment tubings used; B: Side view showing the front and rear bridges; C: Complete setup of Ussing Chamber with modified tubing attached.

2.4 Inhibitors

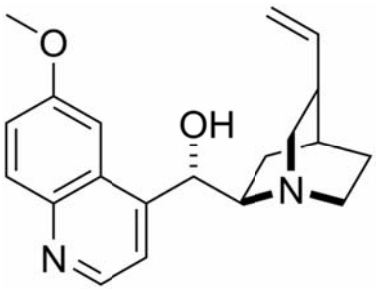
All the inhibitors were handled carefully and dissolved in DMSO (dimethylsulfoxide) shortly before use in the experiments. Adequate time (30

min) was given to the epithelium after the addition of inhibitors for the desired affect to be achieved.

Amiloride ($C_6H_8ClN_7O$): Amiloride is a potassium-sparing diuretic exerting its effect by blocking the epithelial Na channel (ENaC) at low concentrations (micromolar) and the NHE at high concentrations (> 0.1 mmol·l^{-1}). It was generally used at a concentration of 1.0 mmol·l^{-1} on the mucosal side of the epithelium.

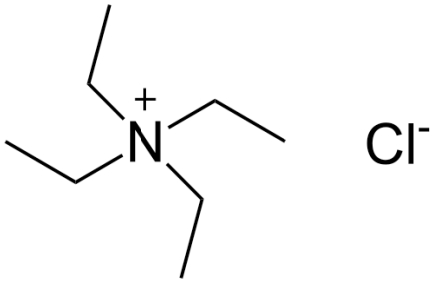


Quinidine ($C_{20}H_{24}N_2O_2$): Quinidine is an unspecific cation channel blocker. It primarily works by blocking the fast inward sodium current (I_{Na}). Quinidine's effect on I_{Na} is known as a "use-dependent block". Quinidine also blocks the slowly inactivating, tetrodotoxin-sensitive Na current, the slow inward calcium current (I_{Ca}), the rapid (I_{Kr}) and slow (I_{Ks}) components of the delayed potassium rectifier current, the inward potassium rectifier current (I_{KI}), the ATP-sensitive potassium channel (I_{KATP}), and I_{to}.

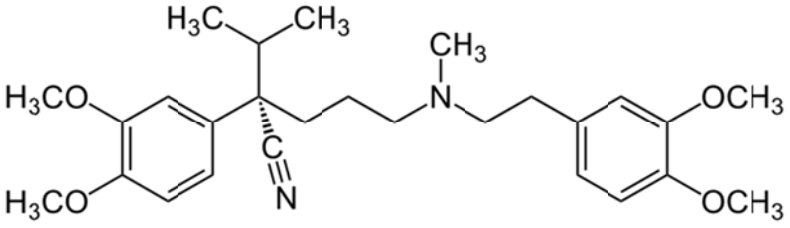


Tetraethyl ammonium chloride (TEA-Cl; $C_8H_{20}NCl$): Tetraethyl ammonium chloride is a quaternary alkyl ammonium compound. It is a ganglion-blocking agent and is widely used in neuroscience research. TEA chloride has been

used in studies as a K^+ inhibitor.



Verapamil ($C_{27}H_{38}N_2O_4$): Verapamil is an L-type calcium channel blocker of the phenylalkylamine class. It has been used in the treatment of hypertension, angina pectoris, cardiac arrhythmia, and most recently, cluster headaches. It is also an effective preventive medication for migraine. Verapamil has also been used as a vasodilator during the cryopreservation of blood vessels. It is a class IV antiarrhythmic and is more effective than digoxin in controlling ventricular rate.



2.5 Full sequence of UT-B

Total RNA from sheep rumen epithelium was isolated by using a commercially prepared system (Nucleospin RNA II kit, Macherey & Nagel®), and any fragments of genomic DNA that could contaminate the RNA were removed by incubating the preparation with DNase (DNase I reaction mixture) for 15 min. RNA integrity was determined by using a commercially prepared kit (RNA 6000 Nano kit, Agilent), which allocated an integrity number to each RNA sample (RNA integrity number, RIN). RNA samples with a RIN of not less than 8.5 were treated with deoxyribonuclease and reverse-transcribed with reverse transcriptase to prepare complementary DNA (cDNA) in a commercially available system (iScript cDNA synthesis kit, Bio-Rad®).

cDNA of sheep rumen epithelium was used as a template for UT-B sequencing. The polymerase chain reaction (PCR) buffer and HotStar Hifi Polymerase were provided by Qiagen, Hilden, Germany. The PCR primers were as below:

UT-B1 left: 5'-tgagcacttcccacagctta-3' (sense)
UT-B1 right: 3'-gagtcccaccagaatcagga-5' (antisense)
UT-B2 left: 5'-tgtttgtcagcaaccccata-3' (sense)
UT-B2 right: 3'-tgctcaacacggagttcaag-5' (antisense)
UT-B3 left: 5'-tgacgtgtccagtcttctcg-3' (sense)
UT-B3 right: 3'-cctgctattatccccagcaa-5' (antisense)
UT-B4 left: 5'-ccttgctggggataatagca-3' (sense)
UT-B4 right: 3'-ggcagctcgtcagaaatga-5' (antisense)
UT-B3 left new: 5'-gttattccctgtatctgctatgtcc-3' (sense)

The PCR products were tested by being run on a 1.5 % agarose gel, which was sent to Eurofins MWG Operon® for sequence analysis.

2.6 Statistics

In our study, each experimental protocol was tested on at least 3 experimental animals. Several samples of epithelial tissues were obtained from each animal to be used in different Ussing Chambers. "N" refers to the number of experimental animals, whereas "n" refers to the number of epithelial tissues per treatment group.

The data were calculated by Sigma Plot 10.0 for Windows (SPSS Inc, Chicago, Illinois, USA). Results are given as means ± SD (standard deviation). The comparison between the groups was carried out in the form of a Student's t-test or paired t-test. P values < 0.05 were considered as being significant.

Table 2: Composition of the buffer solutions of the mucosal side

Chemicals	Concentration (mmol·l^{-1})								
	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
$NaHCO_3$	0	25	25	25	0	0	0	25	25
NaH_2PO_4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Na_2HPO_4	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.4
KCl	┐	5	5	65	5	5	5	5	5
Glucose	5	5	5	5	5	5	5	5	5
MOPs[a]	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_6ClN[b]	0	0	0	0	0	0	0	0	0
NH_4Cl[c]	0 5	0 5	0 2	0 2	0 5	0 5	0 5	0 30	X[d]
NMDG-Cl[c]	5 0	5 0	2 0	2 0	5 0	5 0	5 0	30 0	4

a MOPs: 3-(N-morpholino) propanesulfonic acid ($C_7H_{15}NO_4S$)

b CH_6ClN: Methylammonium chloride

c Ammonia was replaced by NMDG-Cl (*N*-Methyl-D-glucamine chloride $C_7H_{17}NO_5Cl$)

d Luminal ammonia concentrations 0.1, 0.2, 0.4, 0.6, and 1 mmol·l^{-1} corresponding the experimental design.

Table 3: Composition of the buffer solutions of the mucosal side

Chemicals	Concentration (mmol·l^{-1})																	
	Buffer 10				Buffer 11				Buffer 12					Buffer 13			Buffer14	
NaCl	60				60				60					70			70	
$NaHCO_3$	25				25				25					25			25	
NaH_2PO_4	0.4				0.4				0.4					0.4			0.4	
Na_2HPO_4	2.4				2.4				2.4					2.4			2.4	
KCl	5				5				5					5			5	
Glucose	5				5				5					5			5	
MOPs[a]	8				8				8					8			8	
Urea	5	10	15	20	0	10	15	20	1					1			1	
Mannitol	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_6ClN[b]	0				0				0					0			5	
NH_4Cl[b]	0	0	0	0	5	5	5	5	0	1	2.5	5	15	0	2.5	5	0	
NMDG-Cl[b]	5	5	5	5	0	0	0	0	15	14	12.5	10	0	5	2.5	0	0	

a MOPs: 3-(N-morpholino) propanesulfonic acid ($C_7H_{15}NO_4S$)

b CH_6ClN: Methylammonium chloride

c Ammonia was replaced by NMDG-Cl (*N*-Methyl-D-glucamine chloride, $C_7H_{17}NO_5Cl$)

d Luminal ammonia concentrations 0.1, 0.2, 0.4, 0.6, and 1 mmol·l^{-1} corresponding the experimental design.

Table 4: Composition of the buffer solution of the serosal side

Chemicals	Concentration ($mmol \cdot l^{-1}$)
NaCl	115
$NaHCO_3$	25
NaH_2PO_4	0.4
Na_2HPO_4	2.4
KCl	5
Glucose	5
Urea	1
MOPs[a]	8
Na-Acetate	0
Na- Propionate	0
Na-Butyrate	0
Glutamine	2.5
$CaCl_2$	1.2
$MgCl_2$	1.2
NH_4Cl[b]	0
NMDG-Cl[b]	0

a MOPs: 3-(N-morpholino) propanesulfonic acid ($C_7H_{15}NO_4S$)
b Ammonia was replaced by NMDG-Cl (*N*-Methyl-D-glucamine chloride, $C_7H_{17}NO_5Cl$)

Chapter 3

Results

3.1 Urea flux measurements in Ussing chamber

3.1.1 Effect of ammonia on urea flux at pH 7.4 and 6.4

Table 5 summarizes the effect of luminal ammonia on unidirectional urea flux rates across the rumen epithelium at luminal pH 7.4 and 6.4. Urea transport across the rumen epithelium is a passive diffusion down the urea concentration gradient (2). Hence, the unidirectional flux rates, J_{ms} and J_{sm}, were almost identical, and no significant net transport, J_{net}, was observed with urea concentration of 1 mmol·l^{-1} on both sides of the epithelium. At luminal pH 7.4, luminal ammonia at 30 mmol·l^{-1} did not change urea flux rates significantly. In the ammonia and ammonia-free groups, urea flux rates varied in both direction (J_{ms} and J_{sm}) between 21 to 23 nmol·cm^{-2}·h^{-1} (Table 5). However, the decrease of luminal pH from 7.4 to 6.4 significantly enhanced urea transport rates in both directions ca. five times (J_{ms} 122.53 ± 32.81 and J_{sm} 117.6 ± 31.32 nmol·cm^{-2}·h^{-1}). In the presence of 30 mmol·l^{-1} luminal ammonia and at pH 6.4, urea transport rate was also significantly increased (J_{ms} 39.78 ± 13.06 and J_{sm} 47.60 ± 11.81 nmol·cm^{-2}·h^{-1}). However, this stimulation was much lower than the corresponding increase of the control group (47.60 ± 11.81 vs. 117.66 ± 31.32 nmol·cm^{-2}·h^{-1}; only J_{ms} was considered, because this flux direction is of physiological importance) indicating that a luminal ammonia level of 30 mmol·l^{-1} significantly inhibited J_{sm} urea transport by ca. 60 %. The presence of luminal ammonia also increased the current (I_{sc}) significantly indicating electrogenic NH_4^+ transport down its concentration gradient.

Table 5: Effect of luminal ammonia (30 mmol·l^{-1}) on unidirectional urea flux rates across the rumen epithelium (urea concentration 1 mmol·l^{-1}; mean ± SD; luminal pH 7.4 and 6.4; buffer 8; N = number of sheep, n = number of tissues).

NH_4Cl mmol·l^{-1}	pH	J_{ms}	J_{sm}	J_{net}	I_{sc}	G_t	N/n
		nmol·cm^{-2}·h^{-1}			µeq·cm^{-2}·h^{-1}	mS·cm^{-2}	
0	7.4	22.56^{a} ± 9.00	23.16^{a} ± 8.79	- 0.60 ± 6.60	0.72^{a} ± 0.18	2.56^{a} ± 0.51	3/9
30	7.4	22.69^{a} ± 6.30	21.45^{a} ± 4.59	1.24 ± 8.94	1.25^{b} ± 0.54	2.41^{a} ± 0.36	3/9
0	6.4	122.53^{b} ± 32.81	117.66^{b} ± 31.32	4.87 ± 29.86	0.87^{a} ± 0.17	2.18^{a} ± 0.52	4/12
30	6.4	39.78^{c} ± 13.06	47.60^{c} ± 11.81	- 7.82 ± 18.95	1.20^{b} ± 0.17	2.17^{a} ± 0.52	4/12

Values in the same column bearing different superscripts are significantly different at control group ($p < 0.05$).

The applied ammonia concentration of 30 mmol·l^{-1} was extremely high. A determination of the possible effects of lower and more physiological concentrations of ammonia was also of interest.

Figure 14 shows the effect of luminal ammonia concentration (1, 2.5, 5, and 15 mmol·l^{-1}) on urea transport rates of two series of experiments. In the first series, we tested 0, 2.5 and 5 mmol·l^{-1} luminal ammonia. The relatively low concentration of 2.5 mmol·l^{-1} ammonia significantly reduced J_{sm} urea from 142.23 ± 7.48 to 58.22 ± 3.08 nmol·cm^{-2}·h^{-1}. A further inhibition on urea transport rate to 42.07 ± 2.75 nmol·cm^{-2}·h^{-1} was produced by 5.0 mmol·l^{-1} ammonia (Figure 14). The strong inhibition of urea transport by 2.5 mmol·l^{-1} ammonia suggested that even lower concentrations might have effects.

Therefore, in the second series of experiments, 0, 1, 2.5, 5, and 15 mmol·l^{-1} ammonia were compared. The results showed that even 1 mmol·l^{-1} ammonia significantly reduced J_{sm} urea by ca. 40 % from 142.23 ± 07.48 to 85.00 ± 12.61 nmol·cm^{-2}·h^{-1}. No significant differences were observed between luminal ammonia 5 and 15 mmol·l^{-1} (Figure 14).

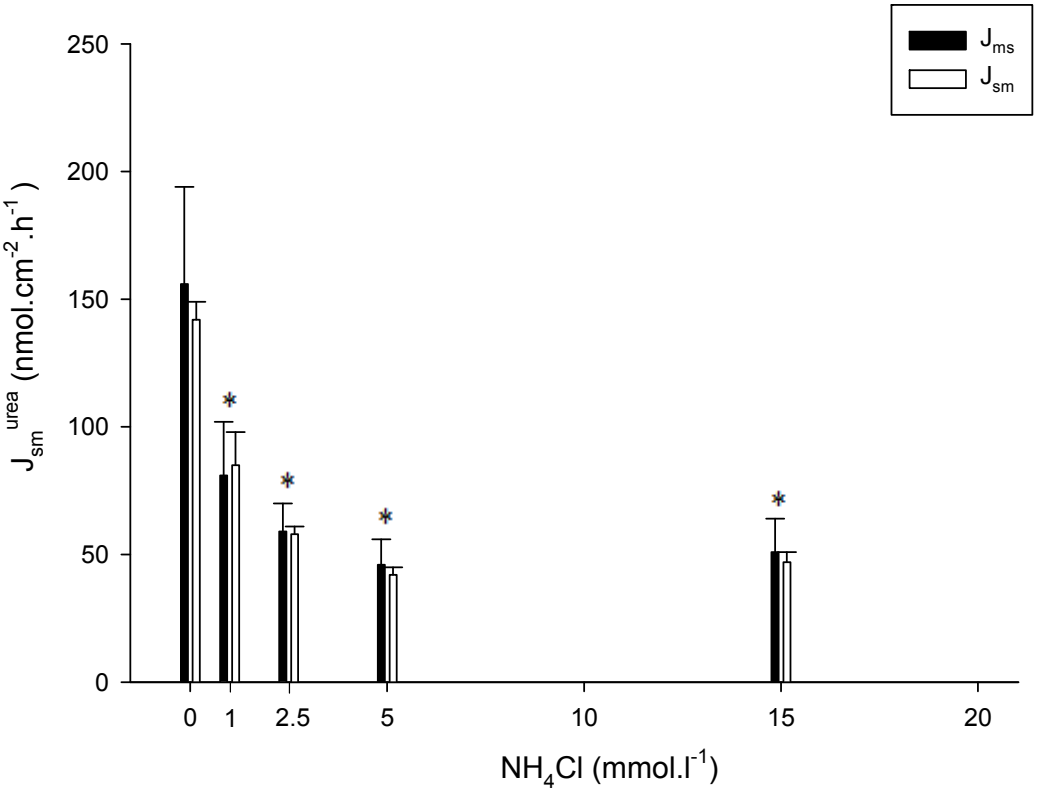


Figure 14: Effect of increasing luminal ammonium on urea flux rates across rumen epithelium (urea concentration 1 mmol·l^{-1}; mean ± SD; luminal pH 6.4; buffer 12 & 13). * This figure combines results from 2 series of experiments: 1) 0, 2.5, and 5.0 mmol·l^{-1} luminal ammonium (3 sheep; 6 – 9 tissues). 2) 0, 1, 2.5, 5.0, and 15 mmol·l^{-1} (3 sheep; 6 – 9 tissues). * J_{sm} and J_{ms} urea significant different ($p < 0.05$) from control (0 mmol·l^{-1}).

The pronounced effect of 1 mmol·l^{-1} on urea transport was unexpected and led to the conclusion that even lower concentrations were able to modulate urea transport. Therefore, we used 0 (control), 0.1, 0.2, 0.4, 0.6, and 1 mmol·l^{-1} ammonia. Compared with the ammonia-free group, the results (see Figure 15) indicated that 0.1 mmol·l^{-1} luminal ammonia surprisingly stimulated fluxes of J_{sm} urea significantly ($p = 0.045$), and that 0.2 mmol·l^{-1} luminal ammonium had slight, but non-significant ($p = 0.054$), stimulating effect. Higher (0.4, 0.6 and 1.0 mmol·l^{-1}) luminal ammonia concentrations inhibited J_{sm} urea. The inhibition was enhanced with the increase of luminal ammonia concentrations.

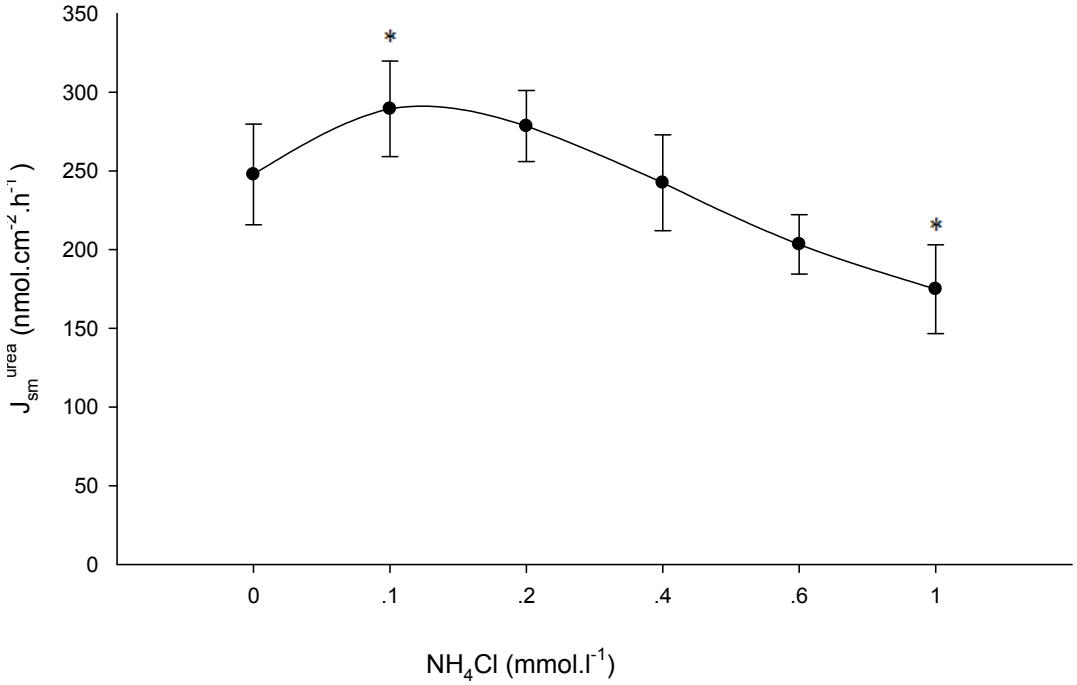


Figure 15: Effect of low luminal ammonia concentrations on urea flux rates (urea concentration 1 mmol·l^{-1}; mean ± SD; buffer 9; luminal pH 6.4; 3 sheep; 7 – 12 tissues). * J_{sm} urea significant different ($p < 0.05$) from control (0 mmol·l^{-1}).

In the previous experiments 1 mmol·l^{-1} urea was used on both sides of the epithelium. The inhibition of urea transport by ammonia could be explained by a simple interaction between ammonia and the urea transporter. Hence, the negative effect of ammonia might depend on the urea concentration. Therefore, increasing urea concentrations (5, 10, 15 and 20 mmol·l^{-1}) were used, and the transport of urea in the presence of 5 mmol·l^{-1} luminal ammonia was determined. The results indicated that J_{sm} urea linearly increased with serosal urea concentration, a finding that again supported passive urea transport (Figure 16). In the presence of 5 mmol·l^{-1} luminal ammonia, J_{sm} urea was significantly reduced at all urea concentrations by ca. 60 %, in close agreement with the relative changes of the previous experiments at 5 mmol·l^{-1} ammonia (Figure 14). The relative constant inhibition of urea transport at all urea concentrations does not support the assumption of an interaction between ammonia and urea at the urea transporter.

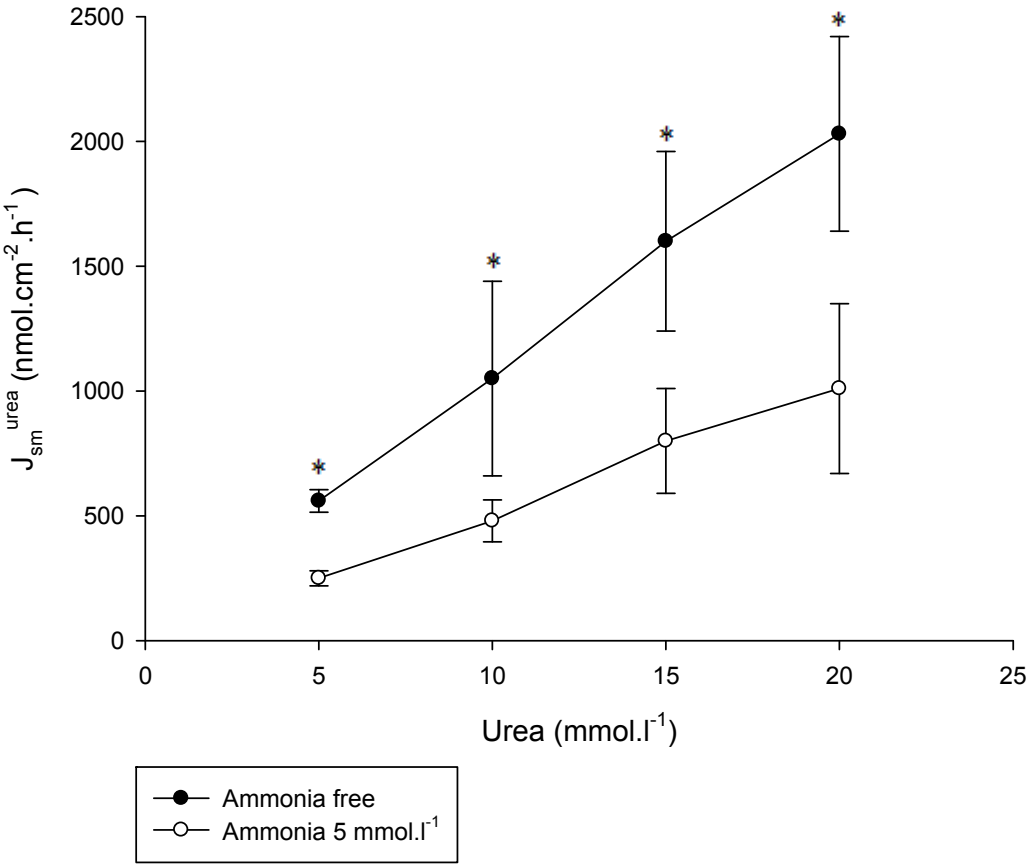


Figure 16: Correlation between serosal urea concentration and urea transport without (y = 99.2x + 70; r^2 = 0.998) and with 5 mmol·l^{-1} luminal ammonia (y = 52x – 15; r^2 = 0.9938); (mean ± SD; buffer 10 & 11; 3 sheep; 7 – 9 tissues). * J_{sm} urea significant different ($p < 0.05$).

3.1.2 Effect of methylammonium chloride

The obtained results in table 1, 2 and figure 1, 3 clearly show that luminal ammonia inhibits urea transport. Other studies have demonstrated that analogs of ammonia are transported, in addition to ammonia (22). Therefore, ammonium chloride was replaced by methylammonium chloride (CH_6ClN), which also released protons in the cell. Methylammonium chloride has a similar structure and is transported as ammonium chloride but has a lower transport rate. The results in Table 6 clearly show that methylammonium chloride significantly inhibits J_{ms} urea, but to a smaller extend than ammonia ($p = 0.02$) (Table 6).

Table 6: Effect of luminal methylammonium chloride (CH_6ClN) or ammonia on urea flux rates (urea concentration 1 mmol·l^{-1}; mean ± SD; luminal pH 6.4; buffer 2 & 14; N = number of sheep, n = number of tissues).

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
5 mmol·l^{-1} NH_4Cl	98.97^{a} ± 18.55^{b}	1.57 ± 0.29	3.41 ± 0.89	3/20
5 mmol·l^{-1} CH_6ClN	155.38^{a,b} ± 32.66^{c}	1.50 ± 0.19	2.74 ± 0.41	3/20

Values in the same column bearing different superscripts are significantly different at control group ($p < 0.05$).

3.1.3 Effect of transepithelial potential difference, PD_t, on urea transport rates

Ammonia occurs in the rumen fluid in two forms: NH_3 or NH_4^+. With a pK of 9.20, NH_4^+ is the predominant form at physiological pH values in the rumen fluid and accounts for 99.9 % at pH 6.4. Luminal ammonium (NH_4^+) has been suggested to enter the rumen epithelium cell via a cation channel (18), and luminal uptake of NH_4^+ is probably driven by the potential differences of the apical membrane, PD_a. The change in transepithelial potential differences (PD_t) and hence of PD_a had an effect on NH_4^+ transport (18), and the PD-dependent change of NH_4^+ transport might have also indirectly altered urea transport. We therefore studied the effect of PD_t on urea transport. Hyperpolarization of PD_t caused a decrease of PD_a and hence the driving force for NH_4^+ uptake, and vice versa.

Table 7 reveals that, in the presence of 2 mmol·l^{-1} luminal ammonia urea, the transport rate was significantly enhanced by a PD_t of + 25 mV and depolarization of PD_a by some 15 mV (Lang and Martens, 1999) from 92.65 ± 19.48 to 108.54 ± 27.87 nmol·cm^{-2}·h^{-1} and was reduced by – 25 mV PD_t to 75.30 ± 13.55 nmol·cm^{-2}·h^{-1}. Hence, changing the driving forces of NH_4^+ uptake caused corresponding alterations of urea transport.

Table 7: Effects of transepithelial potential difference, PD_t, on J_{sm} urea flux rates in the presence of luminal ammonia (urea concentration 1 mmol·l^{-1}; mean ± SD; luminal pH 6.4; buffer 3; N = number of sheep, n = number of tissues).

NH_4Cl mmol·l^{-1}	PD_t mV	J_{sm} nmol·cm^{-2}·h^{-1}	I 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^b	-4.44 ± 1.18^b	3.43 ± 1.04	3/24

Values in the same column bearing different superscripts are significantly different at control group ($p < 0.05$).

However, the data in Table 8 show that, in the presence of 5 mmol·l^{-1} ammonia at the lumen side, corresponding variations of PD_t did not change J_{sm} urea significantly ($p = 0.14$; Table 8).

Table 8: Effects of transepithelial potential difference (PD_t) on urea flux rates in the presence of 5 mmol·l^{-1} luminal ammonia (urea concentration 1 mmol·l^{-1}; mean ± SD; luminal pH 6.4; buffer 2; N = number of sheep, n = number of tissues).

NH_4Cl mmol·l^{-1}	PD_t mV	J_{sm} nmol·cm^{-2}·h^{-1}	I 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^b	4.24 ± 0.99	3/22

Values in the same column bearing different superscripts are significantly different at control group ($p < 0.05$).

3.1.4 Effect of luminal K concentration on urea transport

K^+ is a major cation in the ruminal fluid, and an increase of luminal K^+ is well established to depolarize PD_a and to hyperpolarize PD_t (95, 107). We tested the effects of luminal K^+ on urea flux rates by raising luminal K^+ concentration from 5 to 65 mmol·l^{-1}. The results in Table 9 clearly show that 65 mmol·l^{-1} luminal K^+ leads to a significant increase of J_{sm} urea ($p = 0.025$) from 86.61 ±

21.84 to 111.14 ± 19.81 nmol·cm^{-2}·h^{-1} under open circuit condition at 2 mmol·l^{-1} ammonia.

Table 9: Effect of luminal K^+ on urea flux rates under open circuit conditions (urea concentration 1 mmol·l^{-1}; mean ± SD; luminal pH 6.4; buffer 3 & 4; N = number of sheep, n = number of tissues).

NH_4Cl	K^+	J_{sm}	PD_t	G_t	
mmol·l^{-1}		nmol·cm^{-2}·h^{-1}	mV	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 in the same column bearing different superscripts are significantly different at control group ($p < 0.05$).

However, in Table 10 the data show that no effect of 65 mmol·l^{-1} luminal K^+ was observed ($p = 0.128$) under short circuit conditions.

Table 10: Effect of luminal K^+ concentrations on urea flux rates under short circuit conditions (urea concentration 1 mmol·l^{-1}; mean ± SD; luminal pH 6.4; buffer 3 & 4; N = number of sheep, n = number of tissues).

NH_4Cl	K^+	J_{sm}	I_{sc}	G_t	
mmol·l^{-1}		nmol·cm^{-2}·h^{-1}	µeq·cm^{-2}·h^{-1}	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 in the same column bearing different superscripts are significantly different at control group ($p < 0.05$).

3.1.5 Modulation of urea transport by short chain fatty acids (SCFA)

SCFAs are known to stimulate urea transport *in vivo* (2, 41, 118, 167) and *in vitro* (2). The results of the current study and previous *in vivo* experiments (80) clearly show an inhibitory effect of ammonia on urea transport. Hence, both fermentation products modulate urea transport in an opposite way. This

interaction was tested by increasing the luminal SCFA concentration (0, 40, and 80 mmol·l^{-1}) without and with a luminal ammonia concentration of 5 mmol·l^{-1} 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 (Figure 17).

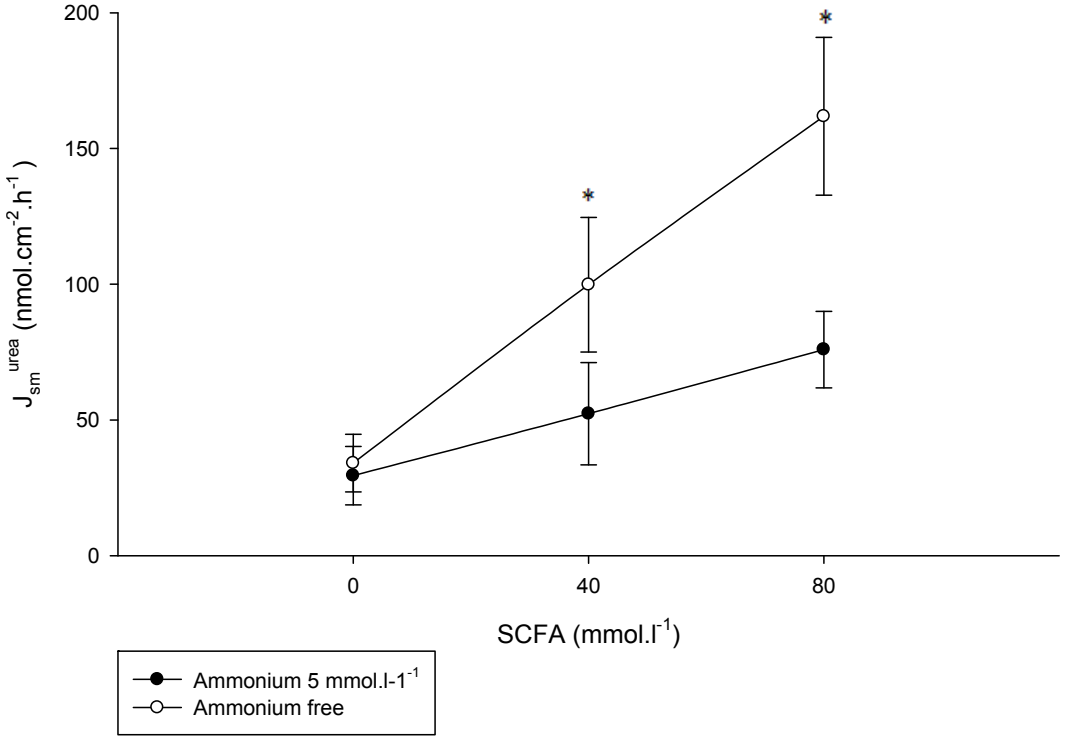


Figure 17: Effects of luminal SCFA concentrations on urea flux rates without (y = 1.60x + 34.69, r^2 = 0.99) and with 5 mmol·l^{-1} luminal ammonia (y = 0.58x + 29.34, r^2 = 0.99); (urea concentration 1 mmol·l^{-1}; mean ± SD; buffer 5, 6 & 7; luminal pH 6.4; 4 sheep; 13 – 16 tissues). * J_{sm} urea significant different ($p < 0.05$).

However, the increment of J_{sm} urea was significantly lower (p = 0.015 and 0.011) in the ammonia group, and increasing the SCFA concentrations did not change the relative inhibition of urea transport by ammonia, which was ca. 63 % at 40 or 80 mmol·l^{-1} SCFA (Table 11). In the absence of SCFA no effect of ammonia on urea transport was observed (Figure 17).

Table 11: Effect of SCFA concentration on urea transport in the presence and absence of 5 mmol·l^{-1} luminal ammonia (serosal urea concentration 1 mmol·l^{-1}; mean ± SD; luminal pH 6.4; buffer 1; N and n see Figure. 3)

	SCFA 40 mmol·l^{-1} nmol·cm^{-2}·h^{-1}	SCFA 80 mmol·l^{-1} nmol·cm^{-2}·h^{-1}
NH_4Cl free	63.87	125.93
NH_4Cl 5 mmol·l^{-1}	22.82	46.45
Inhibition (%)	64.3 %	63.1 %

3.1.6 Urea transport and tissue conductance

The results of the previous experiments exhibited large variations in urea transport depending on the experimental conditions. The data of Abdoun (2010) revealed no significant correlation (r^2 = 0.09) between transepithelial conductance (G_t) and serosal to mucosal (J_{sm}) urea flux, and no correlation was found between urea and mannitol fluxes as a marker for paracellular transport. Therefore, these results suggest that urea is transported across the rumen epithelium probably and solely via a transcellular pathway. Tissue conductance, G_t, was again tested as a possible parameter of changed urea transport. J_{sm} urea did not show a correlation with G_t (r^2 = 0.015, Figure 18) in tissue without luminal ammonia. This was also the case in the presence of 5 mmol·l^{-1} luminal ammonia (r^2 = 0.032, Figure 19). Hence, our data support Abdoun's result (2) that urea is transported across the rumen epithelium via a transcellular pathway.

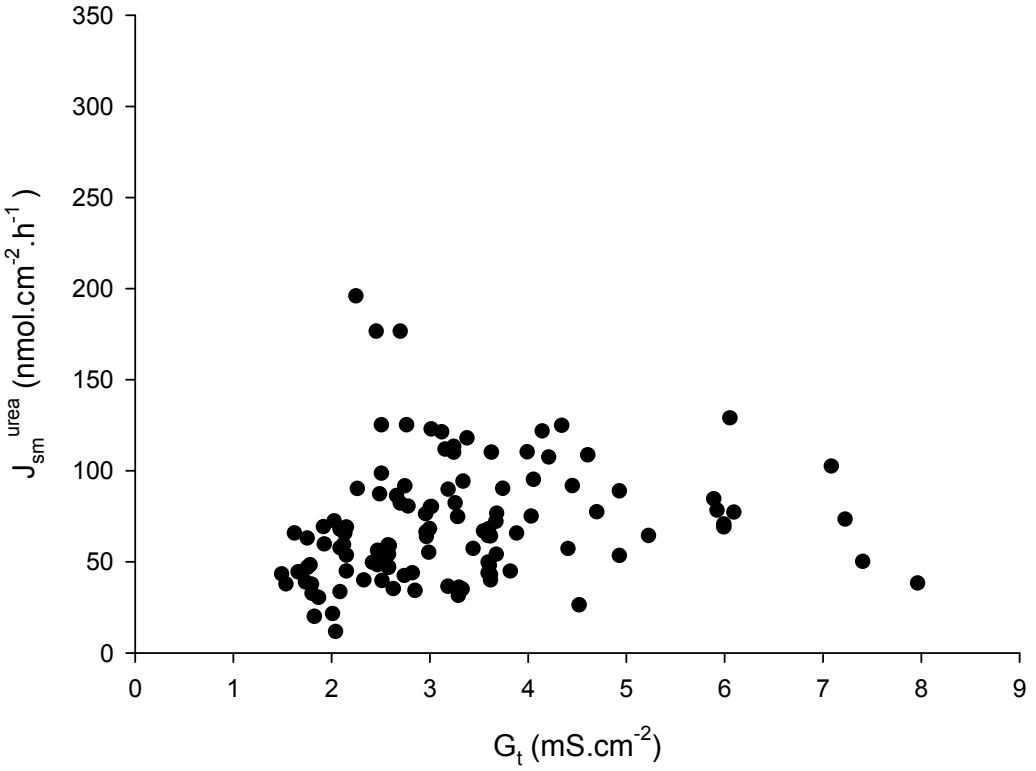


Figure 18: Tissue conductance (G_t) and J_{sm} urea flux rates across the rumen epithelium in the presence of short chain fatty acids without luminal ammonia (r^2 = 0.015; 15 sheep, 85 tissues).

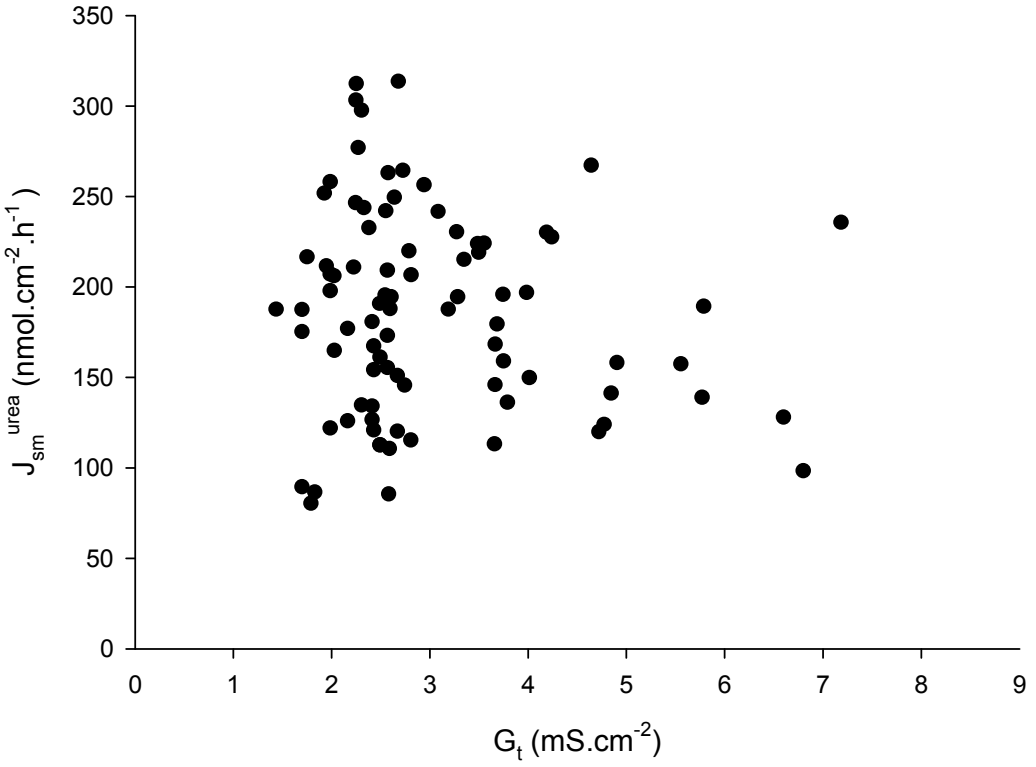


Figure 19: Tissue conductance (G_t) and J_{sm} urea flux rates across the rumen epithelium in the presence of short chain fatty acids and 5 mmol·l^{-1} luminal ammonia (r^2 = 0.032; 18 sheep, 117 tissues).

3.1.7 Modulation of urea transport by luminal pH

The results of experiment 1 (see Table 5) and earlier work from our laboratory clearly demonstrated that the effect of fermentation products such as SCFA and ammonia depends on the luminal pH (1). We therefore tested the effect of decreasing luminal pH from 7.4 to 5.4 on J_{sm} urea with (5 mmol·l^{-1}) and without luminal ammonia. In addition to SCFA and ammonia, the luminal pH modulates urea transport resulting in a bell-shape alteration of J_{sm} urea (Figure 20). In both the presence and absence of ammonia, J_{sm} urea progressively increased with a 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 significantly differed 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).

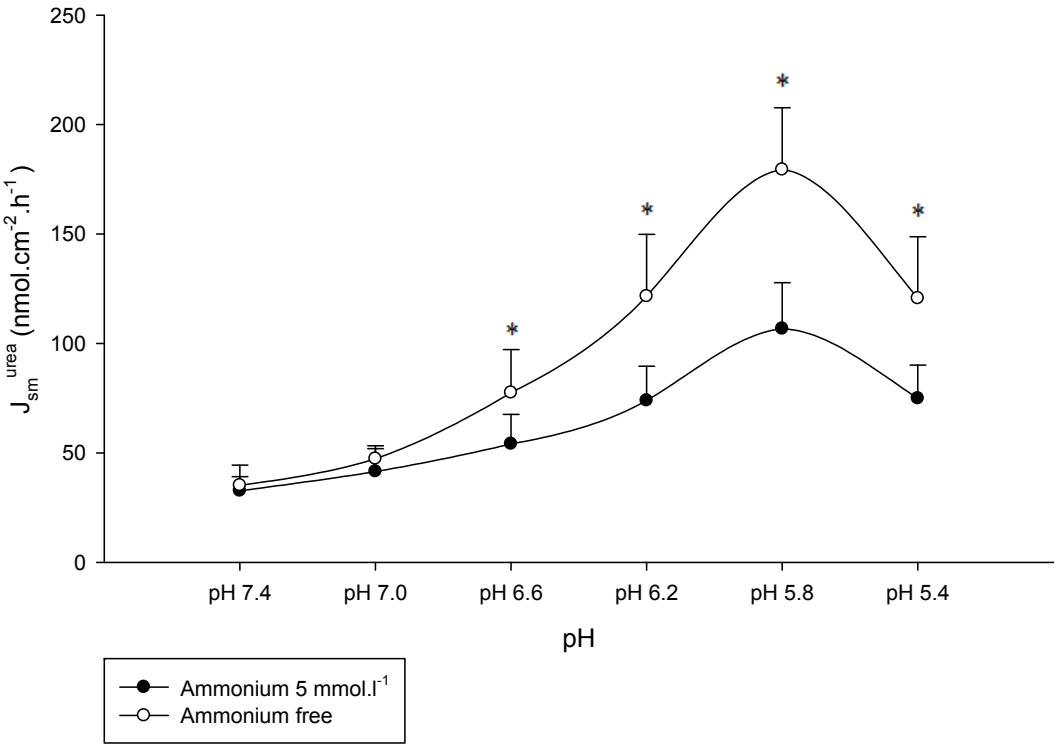


Figure 20: Effect of luminal ammonia and pH on urea flux rates (urea concentration 1 mmol·l^{-1}; mean ± SD; buffer 1; luminal pH 6.4; 4 sheep; 6 – 8 tissues). * J_{sm} urea significant different ($p < 0.05$) between treatment.

We repeated experiments of decreasing luminal pH (from 7.4 to 5.4) with tissue of concentrate-fed sheep and obtained two similar bell-shape-curves (Figure 21), although the peak of of J_{sm} urea was shifted from pH 5.8 to 6.2.

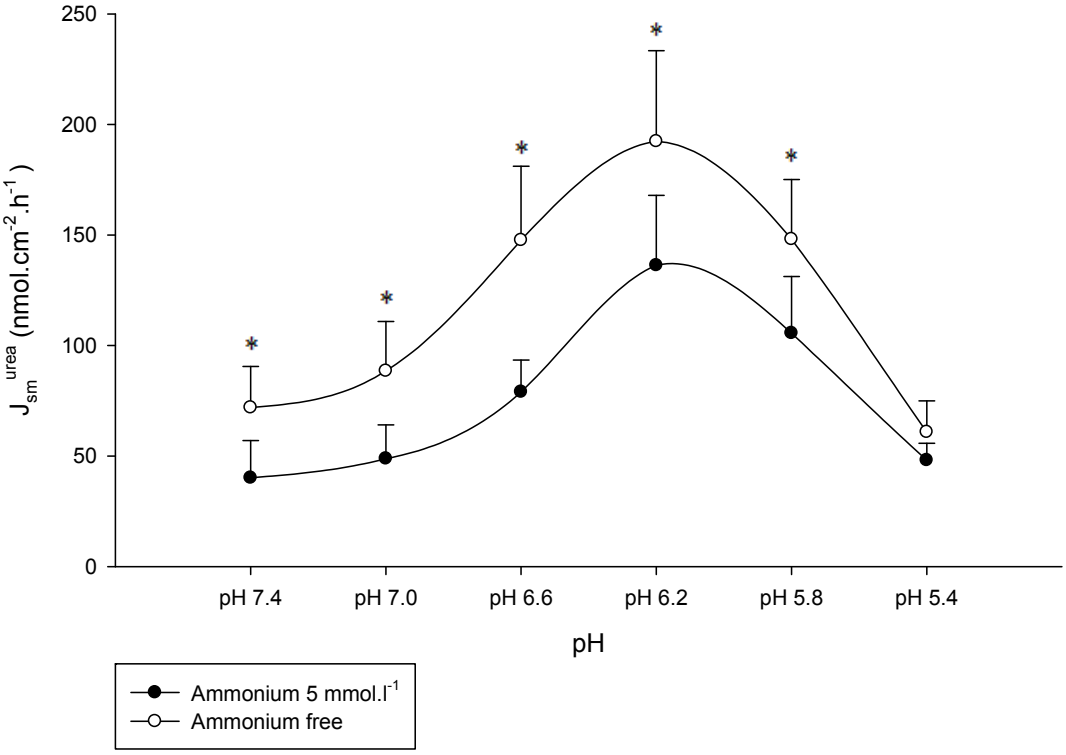


Figure 21: Effect of luminal ammonia and pH on urea flux rates in tissues of concentrate fed sheep (urea concentration 1 mmol·l^{-1}; mean ± SD; buffer 1; luminal pH 6.4; 2 sheep; 6 – 8 tissues). * J_{sm} urea significant different ($p < 0.05$) between treatment.

A low luminal pH is known to cause changes in the integrity of the rumen epithelium (56). This was also the case in the present study. Figure 22 shows that a decrease of luminal pH from 7.4 to 6.6 slightly reduced G_t. A pH of 5.8 and 5.4 significantly increased G_t from 3.97 ± 0.63 (pH 7.4) to 6.59 ± 1.76 (pH 5.8) and 6.97 ± 2.00 (pH 5.4), respectively. The increase of total tissue conductance, G_t, can (at least partly) be explained by alterations of the paracellular conductance, G_p, and hence should increase paracellular permeability for urea[2]. However, as shown in Figure 23, no correlation could be found between G_t and J_{sm} urea.

[2] Tissue conductance, G_t, is the sum of cellular (G_c) and paracellular conductance, G_p: $G_t = G_c + Gp$

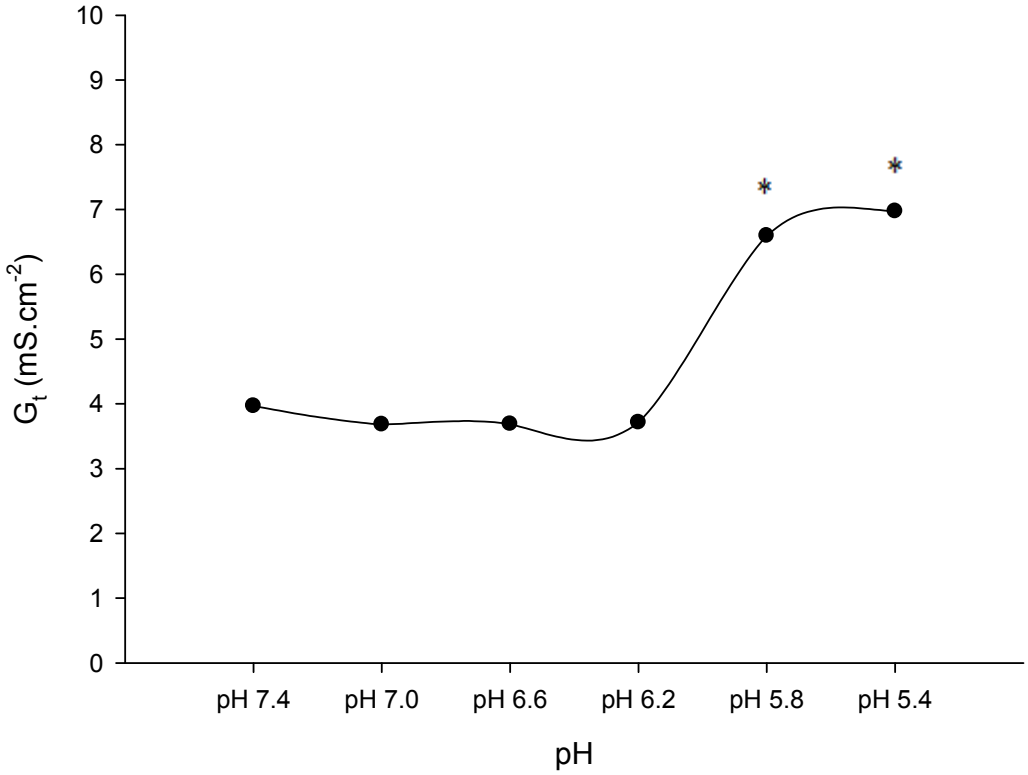


Figure 22: Effect of luminal pH on tissue conductance (G_t). (serosal urea concentration 1 mmol·l^{-1}; mean ± SD; buffer 1; luminal pH 6.4; 4 sheep; 6-8 tissues). * G_t significant different ($p < 0.05$) from pH 7.4

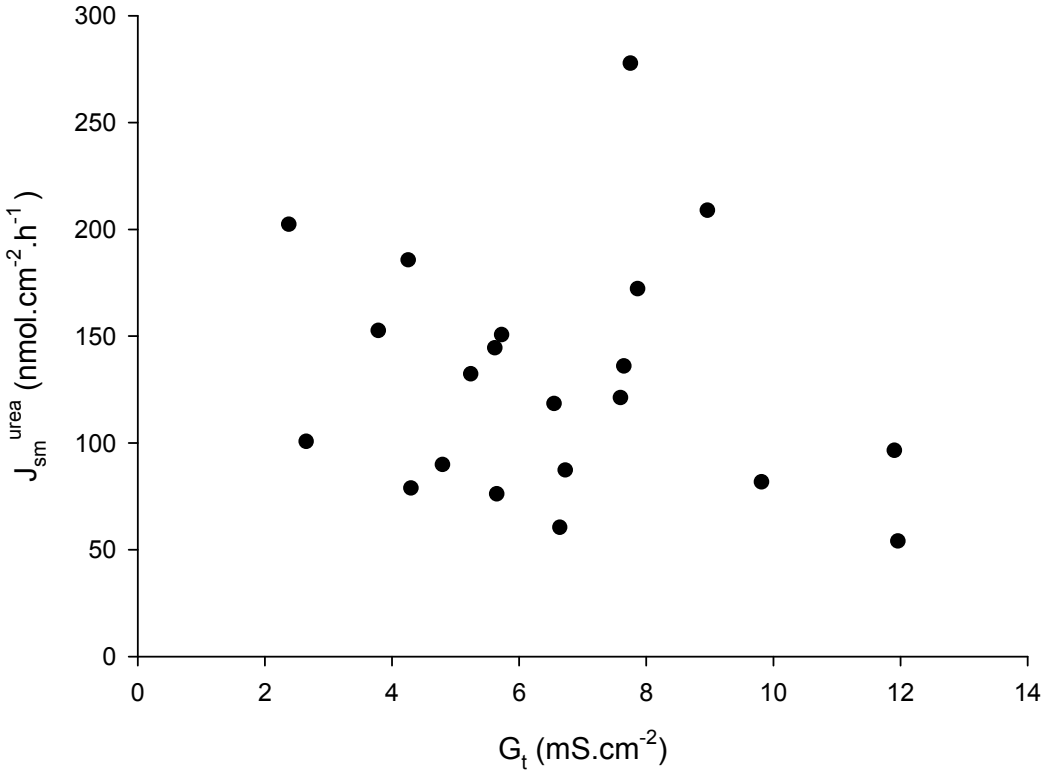


Figure 23: Tissue conductance (G_t) and J_{sm} urea flux rates across the rumen epithelium at pH 5.8 and 5.4 (urea concentration 1 mmol·l^{-1}; mean ± SD; buffer 1r^2 = 0.031; 4 sheep; 21 tissues).

3.1.8 Effect of channel blockers

Previous studies (1, 2, 20) and the results of the present study support the assumption of NH_4^+ uptake across the luminal membrane, probably through an up-to-now unknown cation channel (1). Hence, blocking this putative channel should modulate ammonia uptake and urea transport. Four inhibitors, namely amiloride ($C_6H_8ClN_7O$; 0.2, 0.6, 0.8, 1 mmol.l^{-1}), TEA-Cl ($C_8H_{20}NCl$; 10 mmol.l^{-1}), quinidine ($C_{20}H_{24}N_2O_2$; 1 mmol.l^{-1}), and verapamil ($C_{27}H_{38}N_2O_4$; 0.25 and 1mmol.l^{-1}), were added to the mucosal side of the rumen preparation in the presence of 5 mM ammonia. None of these blockers (Table 12, 13 & 14; amiloride data not shown) significantly changed urea transport rates.

Table 12: Effect of K^+ channel blocker TEA-Cl ($C_8H_{20}NCl$) on urea transport rate. (serosal urea concentration 1 mmol·l^{-1}; mean ± SD, luminal pH 6.4; buffer 2; N = number of sheep, n = number of tissues)

NH_4Cl	TEA-Cl	J_{sm} urea	I_{sc}	G_t	
mmol·l^{-1}		nmol·cm^{-2}·h^{-1}	µeq·cm^{-2}·h^{-1}	mS·cm^{-2}	N/n
0	0	151.69 ± 33.78	1.22 ± 0.30	4.47 ± 1.51	3/22
5	0	53.14 ± 10.53	1.18 ± 0.23	3.24 ± 0.99	3/23
5	10	51.40 ± 13.33	1.25 ± 0.31	2.95 ± 0.50	24

Table 13: Effect of K^+ channel blocker quinidine ($C_{20}H_{24}N_2O_2$) on urea transport rate. (serosal urea concentration 1 mmol·l^{-1}; mean ± SD, luminal pH 6.4; buffer 2; N = number of sheep, n = number of tissues)

NH_4Cl	Quinidine	J_{sm} urea	I_{sc}	G_t	
mmol·l^{-1}		nmol·cm^{-2}·h^{-1}	µeq·cm^{-2}·h^{-1}	mS·cm^{-2}	N/n
0	0	117.61 ± 36.7	0.97 ± 0.29	3.32 ± 0.90	2/8
0	1	93.01 ± 25.70	0.51 ± 0.17	3.23 ± 0.66	2/10
5	0	57.60 ± 11.14	1.03 ± 0.31	3.23 ± 1.16	2/9
5	1	45.50 ± 12.61	0.51 ± 0.16	2.34 ± 0.48	2/11

Table 14: Effect of 0.25 mmol·l^{-1} luminal verapamil ($C_{27}H_{38}N_2O_4$) on urea flux rates (serosal urea concentration 1 mmol·l^{-1}; mean ± SD, luminal pH 6.4; buffer 2; N = number of sheep, n = number of tissues).

NH_4Cl	Verapamil	J_{sm} urea	I_{sc}	G_t	
mmol·l^{-1}		nmol·cm^{-2}·h^{-1}	µeq·cm^{-2}·h^{-1}	mS·cm^{-2}	N/n
0	0	214.41 ± 33.33	1.13 ± 0.70	3.75 ± 1.10	2/10
0	0.25	214.88 ± 39.72	0.90 ± 0.22	3.36 ± 0.65	2/10
5	0	143.76 ± 34.04	1.23 ± 0.18	4.02 ± 0.91	2/10
5	0.25	148.17 ± 31.43	0.74 ± 0.22	2.47 ± 0.28	2/10

However, an increase in the luminal verapamil concentration to 1 mmol·l^{-1} (see

Table 15) significantly diminished the effect of ammonia ($p = 0.025$), but it numerically reduced J_{sm} urea in the absence of ammonia, suggesting some unspecific effects of this channel blocker (see discussion).

Table 15: Effect of 1 mmol·l^{-1} luminal verapamil on urea flux rates (urea concentration 1 mmol·l^{-1}; mean ± SD, luminal pH 6.4; buffer 2; N = number of sheep, n = number of tissues).

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

[a, b, c] Significant from 5 mmol·l^{-1} ammonia: $p < 0.05$

3.2 Sequencing of urea transport B in sheep

Ovine cDNA was synthesized by reverse transcription of RNA isolated from sheep rumen epithelium. The *Bos taurus* urea-transporter-coding sequence (GenBank: AY624602) was used for the design of four polymerase chain reaction (PCR) primer pairs. PCR was performed by using a proofreading polymerase (HotStar high fidelity polymerase, Qiagen, Hilden, Germany) producing four overlapping fragments that were sequenced in both directions (5' to 3', 3' to 5'). These obtained sequences were combined to one strand (1155 bases in length) and given GenBank accession number GQ118969. The alignment with *Bos taurus* urea transporter showed 95.9% identity (Figure 24 and Figure 25).

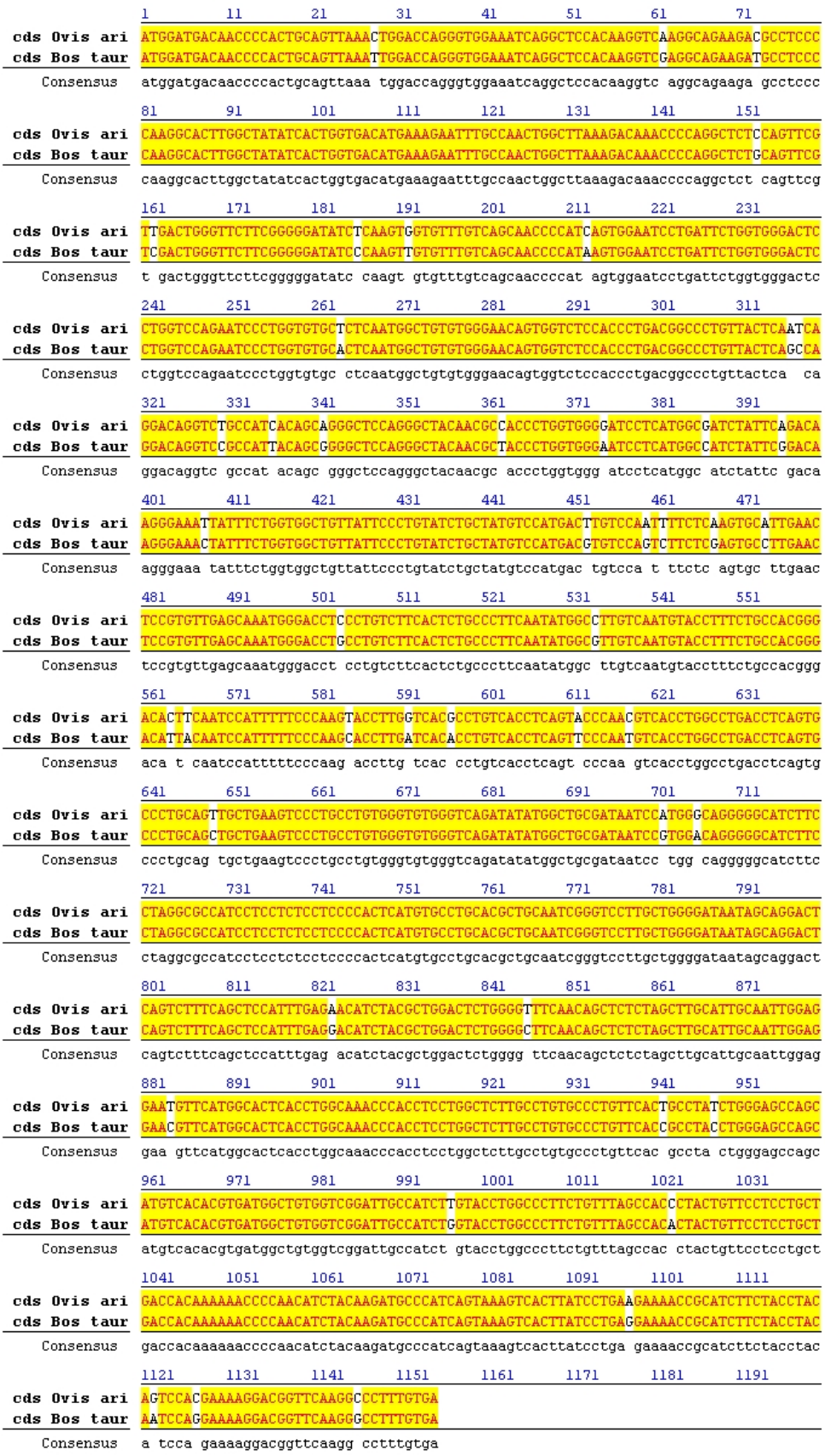


Figure 24: Comparison of *Ovis aris* UT-B cDNA sequence with *Bos Taurus.* The alignment with *Bos taurus* urea transporter showed 95.9% identity

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Bos,         1 MDDNPTAVKLDQGGNQAPQGRGRRCLPKALGYITGDMKEFANWLKDKPQALQFVDWVLRG
Ovis,        1 MDDNPTAVKLDQGGNQAPQGQGRRRLPKALGYITGDMKEFANWLKDKPQALQFVDWVLRG
               ****************** *** **********************************
Bos,        61 ISQVVFVSNPISGILILVGLLVQNPWCALNGCVGTVVSTLTALLLSQDRSAITAGLQGYN
Ovis,       61 ISQVVFVSNPISGILILVGLLVQNPWCALNGCVGTVVSTLTALLLNQDRSAITAGLQGYN
              ****************************************** **************
Bos,       121 ATLVGILMAIYSDKGNYFWWLLFPVSAMSMTCPVFSSALNSVLSKWDLPVFTLPFNMALS
Ovis,      121 ATLVGILMAIYSDKGNYFWWLLFPVSAMSMTCPIFSSALNSVLSKWDLPVFTLPFNMALS
               ******************************** ************************
Bos,       181 MYLSATGHYNPFFPSTLITPVTSVPNVTWPDLSALQLLKSLPVGVGQIYGCDNPWTGGIF
Ovis,      181 MYLSATGHFNPFFPSTLVTPVTSVPNVTWPDLSALQLLKSLPVGVGQIYGCDNPWAGGIF
               ******** ******** ************************************ ****
Bos,       241 LGAILLSSPLMCLHAAIGSLLGIIAGLSLSAPFEDIYAGLWGFNSSLACIAIGGTFMALT
Ovis,      241 LGAILLSSPLMCLHAAIGSLLGIIAGLSLSAPFENIYAGLWGFNSSLACIAIGGMFMALT
               ********************************* ****************** *****
Bos,       301 WQTHLLALACALFTAYLGASMSHVMAVVGLPSGTWPFCLATLLFLLLTTKNPNIYKMPIS
Ovis,      301 WQTHLLALACALFTAYLGASMSHVMAVVGLPSCTWPFCLATLLFLLLTTKNPNIYKMPIS
               ******************************* ************************
Bos,       361 KVTYPEENRIFYLQSRKRTVQGPL
Ovis,      361 KVTYPEENRIFYLQSTKRTVQGPL
               ************** ********
```

Figure 25: Comparison of *Ovis ari* UT-B protein sequence with *Bos Taurus.*

3.3 Interaction between ammonia and SCFA

SCFAs have been demonstrated to enhance ammonia absorption (18). Because both fermentation products (SCFA and ammonia) stimulate Na transport via NHE (3, 110), and because NHE activity increases SCFA absorption (4), luminal ammonia has been suggested to stimulate SCFA transport via altered NHE activity. However, luminal ammonia (0, 5 and 10

mmol·l^{-1}) did not change SCFA transport (Table 16).

Table 16: Effect of luminal ammonia concentration on short chain fatty acids (SCFA) transport (Luminal acetate concentration 25 mmol·l^{-1}; SCFA concentration 40 mmol·l^{-1}; mean ± SD, luminal pH 6.4; buffer 2; N = number of sheep, n = number of tissues).

NH_4Cl mmol·l^{-1}	J_{ms} Acetate (µmol·cm^{-2}· h^{-1})	I_{sc} (µeq·cm^{-2}·h^{-1})	G_t (mS·cm^{-2})	N/n
0	1.52 ± 0.27	1.93 ± 0.43	6.21 ± 1.26	3/23
5	1.77 ± 0.29	1.88 ± 0.38	4.47 ± 0.89	3/24
10	1.64 ± 0.25	1.77 ± 0.31	3.34 ± 0.80	3/22

3.4 Modulation of Na transport

The previous experiments show that the luminal pH significantly changes urea transport. Stimulation was observed within the range of the physiological pH (> 5.8), and remarkable inhibition was demonstrated at lower pH (< 5.8). A low pH is known to disturb the transport properties and integrity of the rumen epithelium (57). Hence, the steep decline of urea transport at pH < 5.8 could represent a more general effect on transport physiology of the rumen epithelium. For this reason, we studied the well-known Na transport at luminal pH from 6.6 to 5.4 with and without luminal ammonia. Luminal ammonia did not change J_{ms} Na transport, but luminal pH caused a stepwise decrease of J_{ms} (Table 17 &18), an increase of J_{sm} Na, and hence, a dramatic decrease of J_{net} Na.

Table 17: Effect of luminal pH on Na transport of tissues of hay-fed sheep (luminal mean ± SD, buffer 2; N = number of sheep, n = number of tissues).

pH	J_{ms} Na ($\mu mol \cdot cm^{-2} \cdot h^{-1}$)	J_{sm} Na ($\mu mol \cdot cm^{-2} \cdot h^{-1}$)	J_{net} Na ($\mu mol \cdot cm^{-2} \cdot h^{-1}$)	G_t ($mS \cdot cm^{-2}$)	N/n
6.6	7.98 ± 2.18	1.75 ± 0.62	6.23 ± 1.73	3.35 ± 1.00	3/6
6.2	7.81 ± 1.99	1.95 ± 0.29	5.86 ± 1.75	4.15 ± 0.85	3/6
5.8	5.79^{a} ± 1.39	2.67^{b} ± 0.137	3.02^{c} ± 1.50	5.69^{d} ± 0.43	3/12
5.4	4.56^{a} ± 0.89	3.04^{b} ± 0.38	1.52^{c} ± 0.67	7.79^{d} ± 0.99	3/12

[a, b, c, d] Significant from pH 6.6: $p < 0.05$

The data of concentrate-fed sheep (Table 18) indicated a similar trend as in hay-fed sheep (Table 17). However, the Na transport rates in tissues of hay-fed sheep were much higher.

Table 18: Effect of luminal pH on Na transport of tissues of concentrate-fed sheep (mean ± SD, buffer 2; N = number of sheep, n = number of tissues).

pH	J_{ms} Na ($\mu mol \cdot cm^{-2} \cdot h^{-1}$)	J_{sm} Na ($\mu mol \cdot cm^{-2} \cdot h^{-1}$)	J_{net} Na ($\mu mol \cdot cm^{-2} \cdot h^{-1}$)	G_t ($mS \cdot cm^{-2}$)	N/n
6.2	15.19 ± 1.80	2.90 ± 0.61	12.29 ± 2.22	5.56 ± 1.32	2/8
5.8	13.15 ± 1.07	3.36^{b} ± 0.29	9.79^{c} ± 1.42	6.27^{d} ± 0.89	2/8
5.4	9.01^{a} ± 1.48	3.68^{b} ± 0.52	5.33^{c} ± 1.80	7.75^{d} ± 1.48	2/8

[a, b, c, d] Significant from pH 6.2: $p < 0.05$

The increase of J_{sm} Na was accompanied by a significant increase of tissue conductance, G_t (Table 17 and 18). A significant and linear correlation between J_{sm} and G_t (Figure 26 and 27) suggests that J_{sm} Na is passive and probably paracellular.

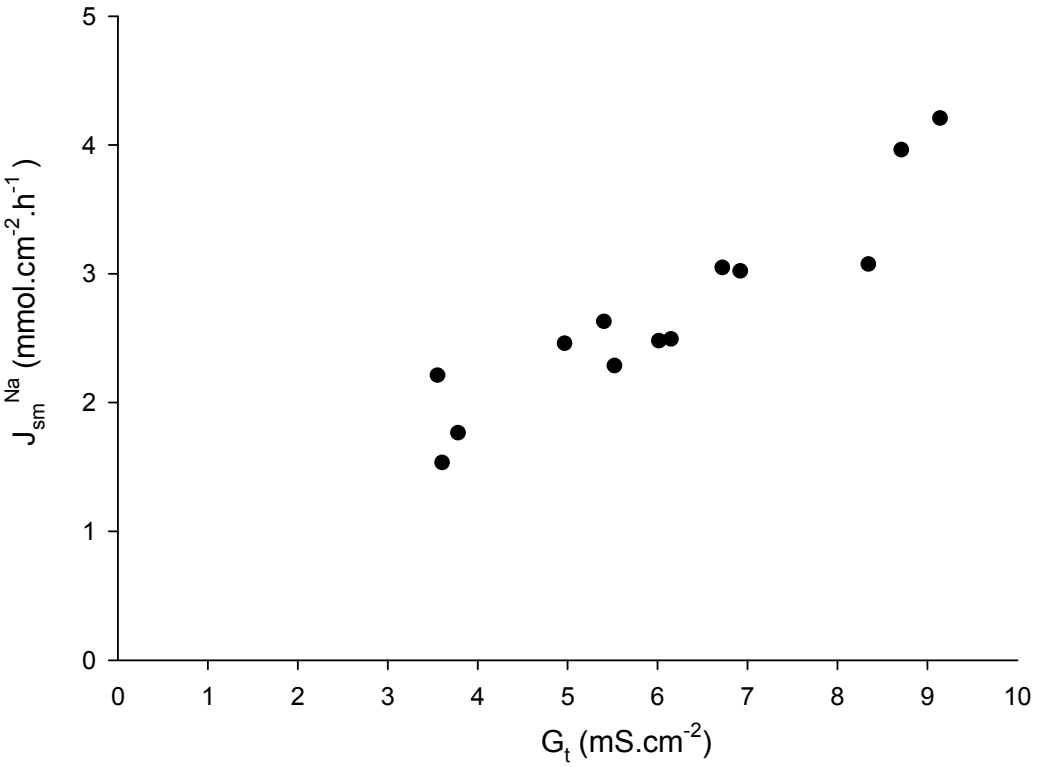

Figure 26: Linear and significant correlation between tissue conductance (G_t) and J_{sm} Na flux rates across the rumen epithelium in the presence of short chain fatty acids without luminal ammonia (y = 0.35x + 0.55, r^2 = 0.88; 4 sheep, 14 tissues)

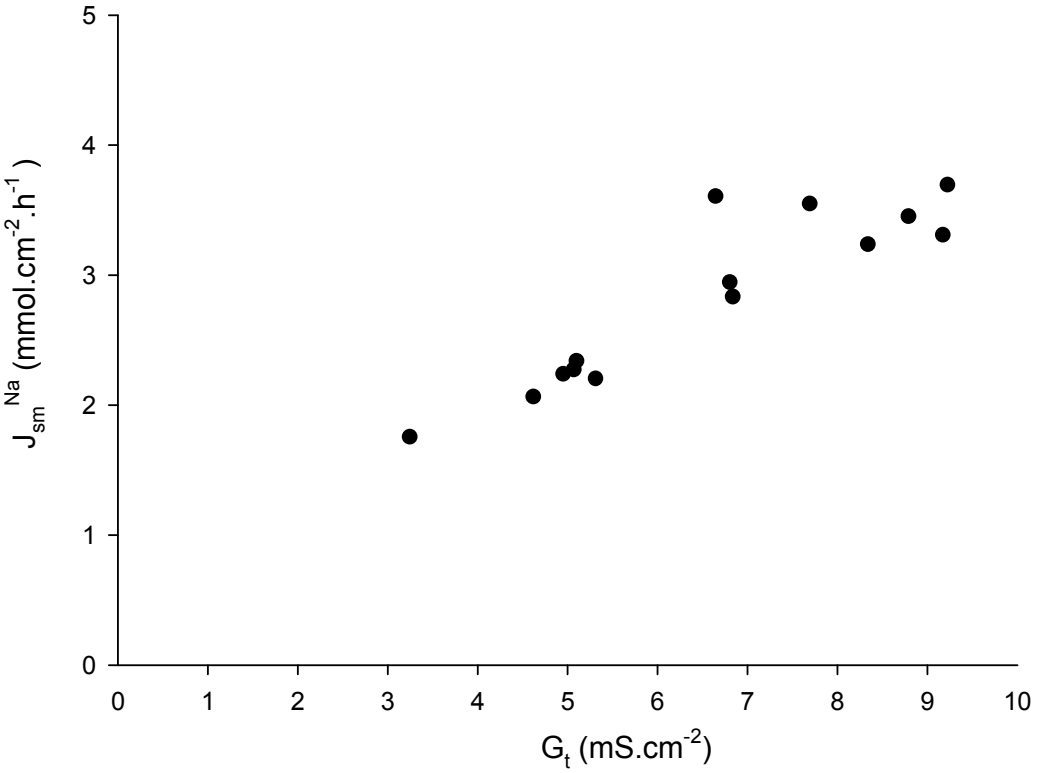

Figure 27: Linear and significant correlation between tissue conductance (G_t) and J_{sm} Na flux rates across the rumen epithelium in the presence of short chain fatty acids and 5 mmol·l^{-1} luminal ammonia (y = 0.32x + 0.72, r^2 = 0.84; 4 sheep, 14 tissues).

Chapter 4

Discussion and Conclusion

The aim of the current study was a better understanding of the mechanisms that lead to changes in urea transport across the ruminal epithelium in response to changes in dietary nitrogen intake.

Urea is the well-known end-product of nitrogen metabolism and is excreted in urine. In ruminants, the early observation of Simmonet et al. (152) led to the establishment of the concept that urea is recycled to the rumen and is hydrolyzed by bacterial urease to ammonia, which serves as an essential resource of microbial protein synthesis. Urea recycling in ruminants has attracted the attention of many scientists, and since the initial report of Simmonet et al. (152), several studies have been performed to elucidate urea recycling (66, 89, 135).

Urea entry rate is linearly correlated with nitrogen intake and accounts for ca. 65 % of nitrogen intake (66, 89). However, in contrast to the relatively constant urea entry rate, urea recycling and urea urinary excretion exhibit large variations. Reynolds and Kristensen (135) have shown, by a compilation of published data, that almost all urea (more than 90 %) is recycled into the gut at a low nitrogen intake. With a diet having a crude protein content of 18 %, the recycling rate is reduced to 25 %, and the renal excretion rate rises to 75 % (135).

Ammonia is a well known protein fermentation product in the rumen, and urea recycling is reduced with increasing ruminal ammonia concentration (Kennedy, 1980). Therefore, the negative correlation between crude protein intake and urea recycling rate suggests that urea recycling is modulated by the ruminal protein fermentation products ammonia, SCFA and CO_2. SCFA and CO_2 have been shown to stimulate urea transport (167), whereas ammonia has an inhibitory effect (79). The functional significance is clearly the regulation of the

supplement of ruminal nitrogen for microbial protein synthesis. However, the underlying mechanism behind this response remains unclear.

Based on the results of previous studies, the experimental design of these experiments had the intention to extend our knowledge of the interaction between ruminal ammonia and ruminal urea transport. The following questions were addressed:

1) Which form of ammonia inhibits ruminal urea transport, i.e., NH_3 or the protonated form, NH_4^+?
2) What is the effect of short chain fatty acids (SCFA) on ruminal urea transport and the interaction between ruminal fermentation products SCFA, CO_2 (stimulation), and ammonia (inhibition)?
3) What is the effect of luminal pH on urea transport?
4) Is the pathway of urea across rumen epithelium transcellular or paracellular?
5) What is the cDNA sequence of ruminal UT-B in sheep?
6) What are the possible mechanism(s) of ruminal urea transport regulation.

4.1 Effect of ammonia on urea transport: NH_3 or NH_4^+

The studies of Kennedy (79) indicated very early that increasing ruminal ammonia concentration caused an inhibitory effect on urea recycling *in vivo*. However, ammonia is present in two forms in solution, either as NH_3 or as NH_4^+. Which form is the effector on ruminal urea transport?

The relative proportions of NH_3 and NH_4^+ depend on the pH according to the Henderson-Hasselbalch equation. The pK value of ammonia is 9.20, and hence at pH of 7.00, ca. only 1 % is in the form NH_3 and 99 % is in the protonated form (NH_4^+). Both forms of ammonia are transported across the apical membrane of ruminal epithelium, NH_3 probably by lipophilic diffusion and NH_4^+ through a putative cation channel (18). Despite the wide ratio

between the two forms of ammonia at pH 7.00, NH_3 is predominantly transported at that pH because of its much higher permeability (1). Because urea transport is not inhibited at pH 7.4 and, because NH_3 is mainly transported at that pH, this isoform does not interact with urea transport. The prevalent NH_3 uptake at pH 7.4 is underlined by the inhibition of Na^+/H^+ exchange indicating the protonation of NH_3 in the cytosol and an increase of pH_i (3).

The exclusion of NH_3 as a modulator of urea transport automatically suggests that NH_4^+ influences urea transport. This suggestion is supported by several independent observations. First, at a luminal pH of 6.4, 99.9 % of ammonia is NH_4^+, and this isoform induces a concentration-dependent increase of I_{sc} indicating the transport of this cation (1). Second, NH_4^+ dissociates in the cytosol and the released H^+ stimulates Na^+/H^+ exchange (Abdoun et al. 2005). Finally, manipulation of PD_a as the driving force for NH_4^+ uptake significantly changes urea transport consistent with the assumption that a depolarization of PD_a reduces NH_4^+ uptake and the inhibition of urea transport by NH_4^+ and vice versa. This conclusion is also supported by the observation of Bödeker et al. (20) who have demonstrated *in vitro* that alterations of PD_t and PD_a, as in the current study, change ammonia transport. The correlation between PD_a, NH_4^+ uptake, and urea transport is further corroborated by a plot between PD_a and urea transport (Figure 28). Lang and Martens (1999) found that, in sheep rumen epithelium, a linear correlation occurs between PD_t and PD_a: PD_a = 0.66 PD_t – 47.7 mV. The calculated PD_a at PD_t –25, 0, and +25 mV according to the equation of Lang and Martens (1999) significantly changes urea transport (table 4) in the presence of 2 $mmol \cdot l^{-1}$ luminal ammonia. Our experimental result supports the assumption of PD_a being the driving force for apical NH_4^+ uptake and exhibits a linear correlation between PD_a and urea transport in the presence of 2 $mmol \cdot l^{-1}$ luminal ammonia (Figure 28). We have

used this linear correlation for another approximation. The mean of urea transport of all control groups of this study is 168.75 nmol·cm^{-2}·h^{-1}. This urea transport rate, based on a linear correlation between PD_a and urea transport (Figure 28), represents a PD_a value of +28.33 mV, which reduces the driving force of luminal ammonia uptake (NH_4^+). At this PD_a and with 2 mmol·l^{-1} luminal ammonia, the calculated intracellular equilibrium concentration of NH_4^+ is ca. 0.6 mmol·l^{-1}, which according to this calculation would have no effect on urea transport (see below).

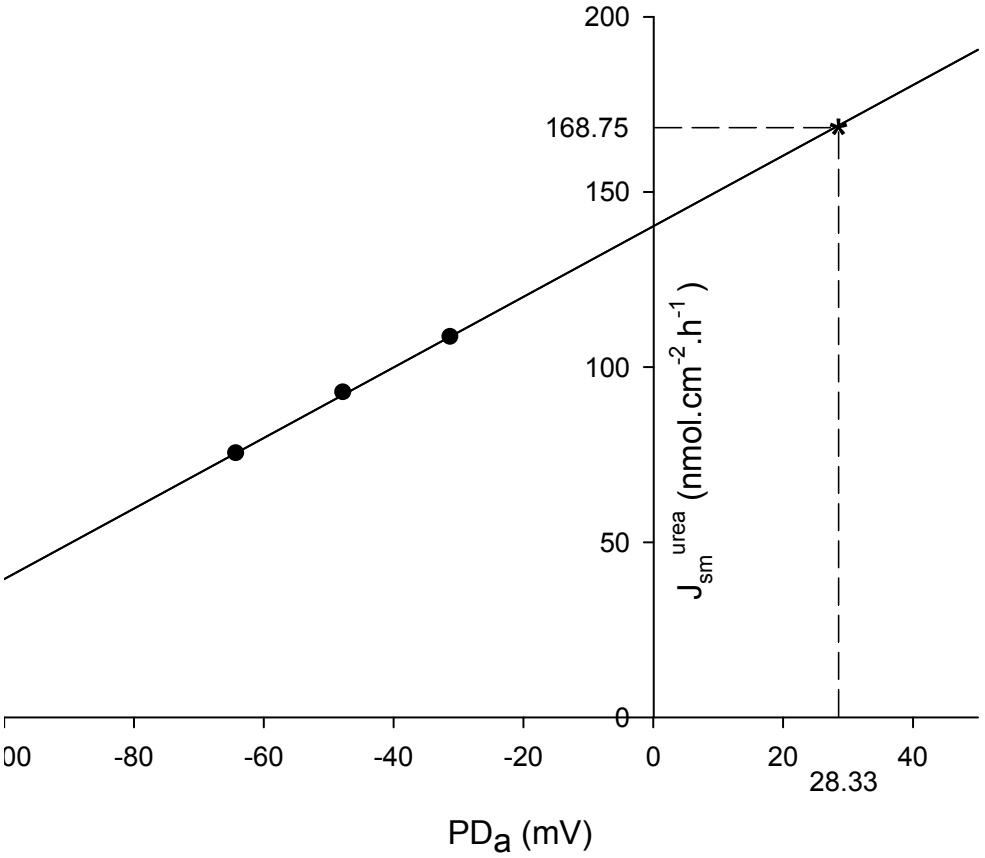

Figure 28: Effect of the potential difference of the apical membrane, PD_a, on urea flux rate in the presence of 2 mmol·l^{-1} luminal ammonia (urea concentration, 1 mmol·l^{-1}; data of table 8; y = 1.0073x + 140.21; y = J_{sm} urea, x = PD_a; R^2 = 0.9994). PD_a is calculated from the equation: $PD_a = 0.66PD_t - 47.7$ mV (Lang and Martens, 1999). The mean of J_{sm} urea of all control groups of this study is 168.75 nmol·cm^{-2}·h^{-1}, which was inserted into the 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^{-1} ammonia (for details see text).

Ammonia at 1 mmol·l^{-1} inhibited urea transport by some 40 % (see Figure 14). The strong effect of this relative low ammonia concentration was surprising but might be explained by the equilibrium concentration. At as little as 1 mmol·l^{-1} ammonia, at pH 6.4, and at PD_t = 0 mV (or PD_a ~ -47 mV), the estimated intracellular concentration of NH_4^+ is ~ 6 mmol·l^{-1}, resulting in the marked reduction of urea transport.

No effects of manipulating PD_t and PD_a were observed at a luminal ammonia concentration of 5 mmol·l^{-1}. The effect of PD_a on NH_4^+ influx was probably too small on total NH_4^+ uptake at 5 mmol·l^{-1} ammonia, a level that was obviously high enough for inhibition of urea transport (see Table 8). Hence, the chemical gradient (5 mmol·l^{-1} ammonia) is the predominant driving force for NH_4^+ uptake, and the manipulation of PD_a (± 15 mV – see above) is probably too small to change the total driving force (chemical gradient + PD_a).

The inhibitory effect of NH_4^+ to urea transport can also be modulated by increasing the luminal K^+ concentration, which is well established to depolarize PD_a and to hyperpolarize PD_t (95, 107). We significantly enhanced urea transport from 86.61 ± 21.84 to 111.14 ± 19.81 nmol·cm^{-2}·h^{-1} (Table 9) by raising the luminal K^+ concentration from 5 to 65 mmol·l^{-1} in the presence of 2 mmol·l^{-1} luminal ammonia *under open circuit conditions*. This effect of K^+ was not observed under short-circuit conditions, because the depolarizing effect of luminal K^+ on PD_a is eliminated under these conditions.

The data of these two serious of experiments, namely a change of PD_a by altered PD_t or high luminal K^+ under open circuit conditions, clearly support the assumption of NH_4^+ uptake and its effect on urea transport. However, we should emphasize that the effect of K^+ was only observed at a low luminal ammonia concentration of 2 mmol·l^{-1}. Hence, this effect is probably of minor

importance under physiological *in vivo* conditions, because the ammonia concentration *in vivo* is significant higher under the current feeding condition (>> 2 mmol·l^{-1}).

Luminal NH_4^+ uptake is modulated not only by PD_a, but also by a K^+ channel blocker. Bödeker and Kemkowski (18) have reported that NH_4^+ uptake across the apical membrane of the rumen epithelium occurs most likely via a K^+ channel. They have also reported a reduction of the transepithelial ammonia fluxes in the presence of quinidine, an unspecific K^+ channel blocker, in the incubation solution. Hence, we performed experiments with putative cation channel blockers (Table 12, 13 & 14). A significant effect of verapamil (1 mmol·l^{-1}) on urea transport (increase) was observed in the presence of 5 mmol·l^{-1} ammonia. However, this unspecific blocker reduced urea transport in the absence of ammonia, indicating an unspecific effect on urea transport. Hence, the suggested effect of verapamil as a cation channel blocker is confounding by the decreased urea transport without luminal ammonia and does not allow a clear conclusion.

4.2 Dose-response curve of NH_4^+

The applied ammonia concentration in the first experiment (30 mmol·l^{-1}; table 1) was near the top of the values found physiologically. Subsequently, the effects of more physiological levels of ammonia between 1 and 15 mmol·l^{-1} were studied. The data show that urea transport is highly sensitive to luminal ammonia: 1 mmol·l^{-1} reduces J_{sm} urea by roughly 40 %. The calculated kinetic data reveal a half-maximal inhibition of urea transport by 1.1 mmol·l^{-1} ammonia, with no further inhibition of urea transport observed at > 5 mmol·l^{-1} ammonia. A similar value of 6 mmol·l^{-1} ammonia has been reported by Kennedy (79), who studied urea recycling at various ruminal ammonia concentrations *in vivo*. Maximum microbial growth is observed at about 3.6 mmol·l^{-1} (141). The

inhibition of urea recycling by 5 mmol·l^{-1} would thus appear to be optimal for the maintenance of conditions suitable for microbial growth, and the *in vitro* and *in vivo* data are in excellent agreement.

4.3 Short chain fatty acids and carbon dioxide

The effects of two other fermentation products, namely SCFA and CO_2, on urea transport have been studied *in vivo* and *in vitro*. Thorlacius et al. (167) enhanced ruminal urea transport by CO_2 (25%, 50%, and 100%) in the temporarily isolated and washed rumen of cows. Norton et al. (1981) infused Na-butyrate into sheep rumen and found an increase of urea recycling. The *in vitro* research of Abdoun et al. (2) clearly indicated a fourfold increase in urea flux by the combination of SCFA and CO_2. Our data in figure 4 are consistent with these previous studies. An increase in the luminal SCFA concentration (0, 40, and 80 mmol·l^{-1}) leads to a linear rise of urea transport. The exact mechanism of the SCFA and CO_2 stimulation of urea transport is still unknown. However, hints for intra-epithelial effects of these fermentation products have been deduced from studies by stimulating Na transport across the rumen epithelium (29, 57, 108). SCFA and CO_2 increase the intracellular availability of H^+ and decrease intracellular pH (pH_i). This process activates the Na^+/H^+ exchanger (NHE) and hence Na transport. The decrease of pH_i was suggested as a possible modulator of ruminal urea transport by SCFA and CO_2 (2).

Increasing SCFA concentrations linearly enhanced urea transport in the current study, in agreement with knowledge about the interaction between SCFA and urea transport. A linear correlation between SCFA and urea transport was also found in the presence of 5 mmol·l^{-1} ammonia. However, the slope of the regression was significant lower, and the relative inhibition (ca. 63 %) remained unchanged at both SCFA concentrations. A possible explanation for the relative constant inhibitory effect of ammonia despite

increasing concentrations of SCFA (stimulation of urea transport) can be proposed based on the observations of Bödeker et al. (19, 20) and Remond et al. (133) that SCFA and CO_2 enhance ammonia absorption in the rumen. Hence, the possible stimulation of urea transport by SCFA and CO_2 is diminished by enhanced ammonia absorption at the same time. The interaction between SCFA, CO_2, activity of carbonic anhydrase (CA), and ammonia transport has been described in detail in the literature and need not be repeated here. The positive effect of SCFA, CO_2, and CA on ammonia absorption is proposed as a fine-tuning mechanism of urea transport. Production of SCFA and CO_2 indicates fermentation and thus a requirement of nitrogen for microbial protein synthesis. Stimulation of urea transport by these fermentation product fits into the general understanding of ruminal biology. If the production of SCFA and CO_2 is, however, accompanied by the production of sufficient ammonia, then increased urea transport (recycling) is not necessary, and the enhancing effect of SCFA and CO_2 on urea transport should be diminished. A surplus of ammonia cannot be used by bacteria under these circumstances, is absorbed, and must be detoxified in the liver (cost of energy). Furthermore, we should mention that, in agreement with the proposed model of the interaction between SCFA, CO_2, and ammonia, the last-mentioned does not inhibit urea transport in the absence of the two other fermentation products (SCFA and CO_2).

SCFA enhances ruminal ammonia absorption (18). SCFA and ammonia stimulate Na transport via NHE (3, 110), and NHE activity increases SCFA absorption in sheep omasum (4). Therefore, ruminal ammonia has been suggested to stimulate SCFA transport via altered NHE activity. However, our data in table 13 shows that luminal ammonia (0, 5, and 10 $mmol \cdot l^{-1}$) does not change SCFA transport.

4.4 Effect of luminal pH

As is well known, urea transport across the rumen epithelium is modulated by SCFA (118) and CO_2 (41, 167). However, the effect of these fermentation products (SCFA, CO_2) has clearly been demonstrated to depend on the luminal pH. During most of the experiments of Thorlacius et al. (167), the ruminal pH was between 6.0 and 6.5, and a variable fall of pH was observed during CO_2 administration. The data of Abdoun et al. (2) showed, for the first time, that at pH 7.4, no significant effect of SCFA on the magnitude of the urea flux rates can be found. This is obviously also the case for the effect of ammonia. Our result indicates that even a high concentration of 30 $mmol \cdot l^{-1}$ ammonia does not inhibit urea transport at a luminal pH of 7.4 (see Table 5), a finding that again supports the conclusion that NH_4^+ and not NH_3 is modulating urea transport (see above). A reduction of the pH to 6.4 (hence within the physiological range) stimulates urea transport in control tissues, as observed in previous studies (2). This stimulation was significantly reduced (47.7 %) in the presence of luminal ammonia (Figure 14).

The effect of luminal pH has been studied in more detail by a stepwise decrease of pH from 7.4 to 5.4. In both groups of tissues (ammonia free and 5 $mmol \cdot l^{-1}$ luminal ammonia), the urea transport exhibits a bell-shape curve. The peak of both curves is at pH 5.8. At pH 7.4 and 7.0, 5 $mmol \cdot l^{-1}$ luminal ammonia does not change the urea fluxes. However, urea transport rates are significant lower in the ammonia group at pH 6.6, 6.2, 5.8, and 5.4 (Figure 18). The luminal pH-dependent inhibition of ammonia on urea fluxes can be explained by the pH-dependent uptake of ammonia. As mentioned above, NH_3 is the predominant form of ammonia that is taken up at pH 7.4 and 7.0. At pH 7.4, the uptake of NH_3 exceeds that of NH_4^+ by a factor of approximately four (1). Conversely, at pH < 7.0, NH_4^+ is primarily absorbed, and Abdoun et al. (1) have calculated that, at pH 6.4, about 70% of ammonia is absorbed in the form of

NH_4^+. Nevertheless, it should be mentioned that the dominant factor determining urea transport is the pH value at high and low pH (2) and, according to the results of the present study, ammonia. The underlying mechanism involving pH and ammonia are discussed below.

4.5 The pathway of urea across the rumen epithelium

The data of Abdoun (2) revealed no significant correlation between transepithelial conductance (G_t) and serosal to mucosal urea flux, and no correlation was found between urea and mannitol fluxes as a marker for paracellular transport. Therefore, these results suggest that urea is transported across the rumen epithelium probably and solely via a transcellular pathway. Tissue conductance, G_t, was again tested as a possible parameter of changed urea transport. Urea flux did not show a correlation with G_t (Figure 18) in tissue without luminal ammonia. This was also the case in the presence of 5 $mmol \cdot l^{-1}$ luminal ammonia (Figure 19). Hence, our data support Abdoun's result that urea is transported across the rumen epithelium via a transcellular pathway. The transcellular pathway of urea includes transport across the various layers of the rumen epithelium (stratum basale, stratum spinosum, stratum granulosum, stratum corneum). Hence, urea has to pass the corresponding membranes of these cell layers, and the suggested urea transporter, UT-B, has been demonstrated in all layers of the rumen epithelium by immunostaining (153). The structural preconditions of transcellular urea transport are therefore present.

4.6 cDNA sequence of ruminal UT-B in sheep

According to previous studies, urea is transported by passive diffusion across the rumen epithelium via urea transporters that have been detected in the rumen epithelium (139, 157). Stewart et al. (162) have obtained the full length fragment of UT-B cDNA from bovine rumen epithelium. The bovine UT-B

coding sequence (GenBank: AY624602) was used for the design of PCR primer pairs to obtain the full length sequence of UT-B in ovine rumen epithelium (GenBank: GQ118969). The alignment with bovine urea transporter showed 95.9% identity (Figure 11 and Figure 12). The coded protein structure (Figure 29) was simulated by on-line software SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html).

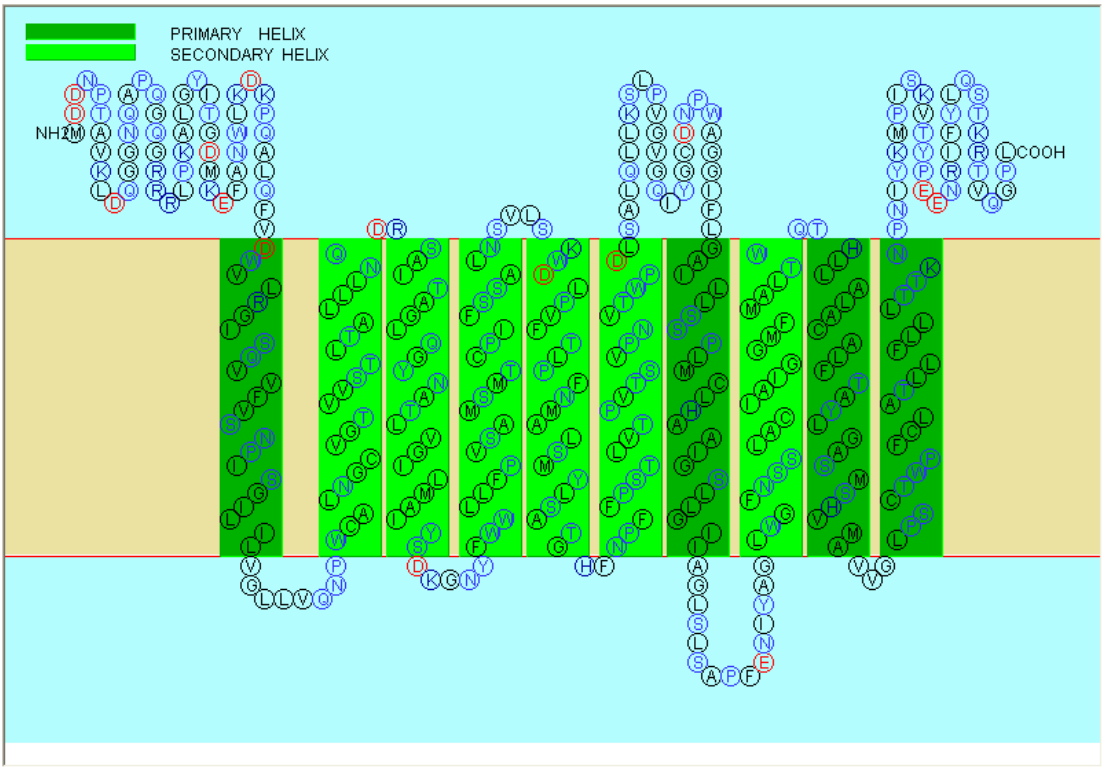

Figure 29: The simulated structure of UT-B from ovine rumen epithelium. (Upwards is the intracellular side.)

4.7 Uptake of NH_4^+ and intracellular pH

From the very start of scientific investigations into ruminal nitrogen transport, considerable controversy has surrounded the issue of whether ammonium ions (NH_4^+) can be absorbed through the ruminal wall.

Undoubtedly, at extremely alkaline pH, the ruminal absorption of ammonia increases markedly, most likely via the lipid diffusion of NH_3. However, ammonia transport *in vivo* does not decline with pH as is predicted from the Henderson-Hasselbalch equation and remains robust *in vivo* at low pH levels,

leading to high losses of ammonia from the rumen (21). *In vitro* studies suggest that, at low pH, NH_4^+ primarily diffuses through a putative cation channel (3, 18). In parallel experiments with microelectrodes (responsible investigator F. Stumpff), the possible effects of NH_4^+ uptake was studied. The experiments with microelectrodes support the hypothesis of NH_4^+ uptake, 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 7.4, no significant change in intracellular pH could be detected. A previous study has shown that, at pH 7.4, Na^+/H^+ exchange is inhibited by the addition of ammonia. Considerable amounts of ammonia appear to enter the cell as NH_3 at this pH, so that acids entering the tissue or produced via metabolism are buffered, with Na^+/H^+ being inhibited by just enough to preserve the physiological pH of the intracellular milieu.

At pH 6.4, a marked and statistically significant intracellular acidification was observed. Most likely, a fraction of the ammonium 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 effects ammonia on ruminal Na^+/H^+ exchange, i.e., data showing a stimulation of proton extrusion via this pathway at pH 6.4 (3). However, concentrations of ammonia of < 5 $mmol \cdot l^{-1}$ lead to a highly significant reduction of urea transport, whereas any acidification is discrete, arguing against an ammonia-induced acidification as the primary and sole signaling event for the inhibitory effects of ammonia.

4.8 Effects of protons and ammonia on urea transport: acid sensing or channel gating?

The data of the present and previous studies show that manipulations that result in changes of pH_i and of intracellular ammonium concentration modulate

urea transport. Unfortunately, at the present time the mechanism of modulated urea transport cannot be precisely identified. There is no doubt that the fluxes of ammonia, urea, and SCFA are independently regulated in response to dietary intake , arguing against a model that transepithelial fluxes are depending only on the rate of blood perfusion. Interestingly, acid-sensitive receptors within the ruminal epithelium appear to reduce rumen motility during bouts of acidosis (37, 64); these receptors might therefore have an effect on transport too.

However, a simpler model is possible. The results of the current experiments firmly establish that urea permeates the membrane through channel-like pores which conduct non-ionic solutes. The urea transporter B (UT-B) has been demonstrated in the rumen epithelium (162), and immunostaining has shown that this transporter is inserted in all membranes of the multilayered epithelium mediating uptake into and release out of the epithelium and passage from cell to cell within the epithelium (Simmons et al., 2009). The sequence of bovine UT-B has been published (GenBank: AY624602), and ovine UT-B exhibits 95.9 % identity with it. The effects of diet composition on UT-B expression is controversial (116) and does not explain the rapid changes of urea transport. Hence, altered transport rates of urea are likely to be caused by acute modulation of UT-B transport activity. Like classical ion channels, these urea pores can undergo conformational changes in response to various stimuli (86). Renal urea transport is thus regulated by vasopression (= ADH); this regulation occurs not only by increasing the number of UT-A proteins in the membrane of the inner medullary collecting duct (= longer term effect), but also by rapidly enhancing the urea permeability of pre-existing UT-A proteins via cAMP/protein-kinase-A-mediated phosphorylation of serine residues within the channel pore . However, in a cell model (MDCK), Tickle et al. (2009) expressed bovine UT-B and applied the classic modulators of altered UT-A activity such

as cAMP and Ca^{++}. No effect on urea transport was observed. Tickle et al. (2009) concluded that UT-B are not regulated in a manner similar to that of renal UT-A, and that further work is required to determine exactly how UT-B activity is controlled.

Hence, alternative mechanisms of NH_4^+, beside the possible effect of pH_i on UT-B activity, must be included for further discussion. For example, ammonium ions might also bind to the cytosolic side of urea transporters, changing the conformation in a manner that inhibits urea flux. This mechanism of ammonium-gated channels is thought to contribute to the toxic effects of ammonia in the central nervous system but is highly speculative regarding possible interactions between NH_4^+ and UT-B.

Significantly more information is available concerning the gating of channels by protons. Acid-sensing ion channels (ASIC) instantly change their conformation in response to an acidic stimulus, resulting in an immediate stinging sensation on damaged skin. Likewise, urea channels are gated by protons in many bacterial species, in which 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 (12). The influx of urea into the periplasmic compartment follows, where it is degraded by urease to form buffering NH_3. The precisely regulated influx of urea thus enables *Helicobacter pylori* to maintain its internal pH within the extremely acidic environment of the mammalian glandular stomach. A similar proton-gated urea channel would certainly enhance the ability of ruminal epithelial cells to respond to fluctuations in ruminal pH.

Interestingly, 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 current study for urea . Meanwhile, protonation of AQP 3 has been established to underlie this effect . 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 around 6.4 and reducing again at lower pH . Although notably absent in the erythrocytes of ruminants , AQP 3 conducts urea and has recently been identified in the rumen of cows (137), making it an interesting candidate for further studies, in addition to UT-B.

4.9 Urea recycling and pollution of the environment

Cows are kept for milk production, and milk production has been increased during the last few decades. According to the statistics of the Arbeitsgemeinschaft Deutscher Rinderzüchter (ADR), milk production per lactation (305 d) has increased from some 4000 kg to 8000 kg durng the last fifty years. Mean values of dairy herds far above these values are well known. This increase of milk production is only possible by feeding regimes according to the requirement for protein, energy, minerals etc. The efficiency of nitrogen metabolism for milk production is relative low and accounts for approximately 25 % of nitrogen intake. A dairy cow with a daily milk production of 40 kg has a nitrogen requirement (as protein) of approximately 600 g nitrogen/d. Assuming an urea entry rate of ca. 65 % of nitrogen intake, 390 g urea nitrogen are produced. The crude protein concentration of a typical ration of a dairy cow is 16 – 18 % of dry matter, and according to Figure 7, some 25 % is recycled, and the rest (293 g urea nitrogen) is excreted in urine. Urea is hydrolysed to CO_2 and ammonia in the slurry, and some ammonia is lost by evaporation and is released into the atmosphere contributing to greenhouse gases. Furthermore, a small amount of ammonia is metabolized in the soil to NO_2, which is some 300 times more effective than CO_2 as a greenhouse gas (17). Hence, the

current milk production is accompanied by significant challenges to the environment. Reduction of nitrogen excretion via urine is possible by changing the fate of urea: recycling versus excretion in the urine. The current feeding regime of cows opposes corresponding changes, because the high intake of energy and protein cause a low pH and high ammonia (> 5 $mmol \cdot l^{-1}$) in the rumen; according to the data of the current study, this clearly reduces urea recycling. Hence, our results cannot offer a solution to the problem of the pollution of the environment by nitrogen excretion but help to explain the underlying mechanisms. A better understanding of these mechanisms could help with the development of alternatives to the current production system.

4.10 Conclusion

In conclusion, the data of the present study are in general agreement with the known *in vivo* observation that urea recycling is modulated by the protein fermentation products ammonia, SCFA, and CO_2. Ammonia reduces and SCFA/CO_2 stimulates urea recycling. However, new and important observations have been made. First, the effect of ammonia is related to NH_4^+ uptake and depends on the pH in the rumen (see Figure 20), and second, a low ammonia concentration (< 1 $mmol \cdot l^{-1}$) even enhances urea influx. Third, the effect of ammonia on urea transport (enhancing or reducing) is not clear and could be mediated by changes of pH_i and, hence, the protonation of UT-B or indirectly by inducing an acid-sensing signal cascade. However, experimental data is still lacking concerning ammonia modulating intracellular pH over the range of ammonia concentrations applied in this study and an interaction of NH_4^+ with UT-B cannot be excluded. Fourth, the urea entry rate is relatively high and accounts for some 65 % of nitrogen intake (66, 89), and only a small part of UER is recycled to the rumen under the current feeding conditions (135). Most of UER is excreted via urine, and this loss of nitrogen is a major concern with regard to pollution of the environment (17). The data of the present study

offer a better understanding of the shift of urea from recycling to urinary excretion (135). The clear effects of ammonia on the one hand and the pronounced effect of ruminal pH on the other hand might lead to feeding strategies with improved urea recycling.

Chapter 5

Summary

It has been well established from previous *in vivo* studies that urea is recycled to rumen across rumen epithelium via trans-cellular passive diffusion (urea concentration gradient between blood and rumen acts as main driving force) and urea recycling is modulated by crude protein in the diet. According to previous researches, the urea transport from blood to rumen is modulated by the luminal protein fermentation products SCFA, CO_2 and ammonia. SCFA and CO_2 stimulate urea recycling while ammonia has an inhibitory effect.

It was therefore, the intention of this study to investigate the mechanism of urea transport regulation by ammonia, luminal pH, and SCFA/CO_2. Using conventional Ussing chamber technique, *in vitro*, urea transport across the rumen epithelium was carried out to study these effects on ruminal urea transport. The following results were obtained:

1. Urea transport rate is inhibited by luminal ammonia in both directions (blood to rumen and rumen to blood). The negative effect of ammonia is enhanced with increasing luminal ammonia concentration up to 5 $mmol \cdot l^{-1}$. Above 5 $mmol \cdot l^{-1}$, ammonia does not exert further inhibition of urea transport. Low concentration of ammonia (0.1 $mmol \cdot l^{-1}$) stimulates urea transport rate.
2. Two forms of ammonia are present in the ruminal fluid at physiologic pH: NH_3 and NH_4^+ according the Henderson-Hasselbalch-Equation. It is concluded that the effect of ammonia is mediated by NH_4^+. Alterations of the potential difference of the apical membrane, PD_a, and hence the driving force for luminal uptake of NH_4^+ into the epithelial cells caused corresponding changes of urea transport: Depolarization of PD_a (= reduced uptake of NH_4^+) causes enhanced urea transport rates and hyperpolarization the opposite effect.
3. Urea transport rate is linearly stimulated by increasing luminal SCFA

concentration. This stimulation is significantly reduced by luminal ammonia and the relative degree of inhibition by ammonia is the same at all SCFA concentrations. Hence, increasing SCFA concentrations, which stimulate urea transport, does not overcome the inhibitory effect of ammonia.

4. Urea transport rate is modulated by luminal pH in the presence and absence of luminal ammonia. The range of luminal pH from 7.4 to 5.4 induced a bell-shaped change of urea transport with significant lower flux rates below pH 6.6 in the presence of ammonia.
5. No correlation exists between urea transport rate and tissue conductance indicating predominantly transcellular urea transport.
6. Urea transporter B has been detected in sheep rumen epithelium. The alignment with *Bos taurus* urea transporter showed 95.9% identity.

It is concluded that ruminal transport rate is stimulated by SCFA/CO_2 and inhibited by luminal ammonia in the form of NH_4^+. The negative effect of ammonia is dependent on the presence of SCFA and luminal pH. The mechanism of acute modulation of urea transport rateby ammonia is still unclear. There is no doubt that SCFA, CO_2 and ammonia modulate ruminal transport rate by changing intracellular pH, but alterations of pH_i does not explain sufficiently the obtained data. It is suggested that ammonia (NH_4^+) interacts with the urea transporter. However, more experiments are needed to prove this hypothesis.

ZUSAMMENFASSUNG

Es ist aus vielen *in vivo* Untersuchungen bekannt, dass Harnstoff durch das Pansenepithel transportiert wird. Der Konzentrationsgradient zwischen Blut und Pansenflüssigkeit ermöglicht diesen passiven Transport in den Pansen, der auch als Harnstoffrecycling bezeichnet wird. Der Rohproteingehalt des Futters korreliert negativ mit der Transportrate (Recycling) und es ist aus Untersuchungen bekannt, dass der ruminale Harnstofftransport durch die Fermentationsprodukte SCFA, CO_2 und Ammoniak beeinflusst wird. SCFA und CO_2 stimulieren und Ammoniak hemmt den Harnstofftransport.

Es war daher die Absicht der vorliegenden Untersuchungen, die Mechanismen näher zu charakterisieren, die die Modulation des Harnstofftransports durch Ammoniak, den lunimalen pH und SCFA/CO_2 verursachen. Die Untersuchungen wurden *in-vitro* mit isolierten Epithelien des Pansens von Schafen mit der konventionellen Ussing Kammer Methode durchgeführt. Folgende Versuchsergebnisse wurden ermittelt:

1. Der unidirektionale Transport von Harnstoff (Transport von der Lumen- zur Blutseite und umgekehrt) wird durch die luminale Ammoniakkonzentration gehemmt. Eine maximale Hemmung wurde bei einer Konzentration von 5 $mmol \cdot l^{-1}$ beobachtet. Höhere Konzentrationen verursachten keine weitere Reduzierung. Sehr niedrige Ammoniakkonzentrationen (0.1 $mmol \cdot l^{-1}$) stimulierten – wenn auch in geringem Umfang - signifikant den Harnstofftransport.
2. Ammoniak liegt in dem pH Bereich der Pansenflüssigkeit (5.4 – 7.4) in zwei Formen vor entsprechend der Henderson-Hasselbalch-Gleichung: NH_3 and NH_4^+. Der Effekt von Ammoniak auf den Harnstofftransport wird durch NH_4^+ vermittelt. Veränderungen der Potentialdifferenz der apikalen Membran (PD_a) und somit der Triebkraft für die passive Aufnahme von NH_4^+ in die Epithelzelle verursachen entsprechende Zu- bzw. Abnahmen der

Transportraten von Harnstoff. Eine Depolarisation von PD_a (= Reduzierung der NH_4^+ Aufnahme) erhöht den Transport von Harnstoff und eine Hyperpolarisation bewirkt den umgekehrten Effekt.

3. Der Transport von Harnstoff wird mit steigender Konzentration von SCFA linear und signifikant stimuliert. Diese Stimulierung wird signifikant durch Ammoniak gehemmt. Die relative Hemmung des Harnstofftransportes veränderte sich jedoch nicht, d. h. dass steigende SCFA Konzentrationen die hemmende Wirkung von Ammoniak nicht kompensieren.
4. Der Transport von Harnstoff wird maßgeblich durch den luminalen pH-Wert bestimmt. pH-Werte im Bereich von 5.4 – 7.4 induzieren eine glockenförmige Zu- und Abnahme des Harnstofftransports. Ammoniak (5 $mmol \cdot l^{-1}$) verursacht eine signifikante Abnahme des Transportes ohne Veränderung der Form der Zu- und Abnahmen. Mit und ohne Ammoniak wurden maximale Transportraten im pH-Bereich von 5.8 – 6.2 beobachtet.
5. Der Transport von Harnstoff korreliert nicht mit der Gewebeleitfähigkeit. Diese Beobachtung unterstützt die Annahme eines transzellulären Transportes.
6. Der Urea Transporter B (UT-B) wurde im Pansenepithel nachgewiesen und weist eine Übereinstimmung mit dem bovinen UT-B von 95.9 % auf.

Die erhaltenen Ergebnisse lassen die Schlussfolgerung zu, dass der Transport von Harnstoff durch $SCFA/CO_2$ stimuliert und durch Ammoniak gehemmt wird. In beiden Fällen wird die Wirkung der Fermentationsprodukte maßgeblich durch den luminalen pH-Wert bestimmt. Es besteht kein Zweifel, dass die Effekte von CO_2 und SCFA mit einer Abnahme des intrazellulären pH-Wertes, pH_i, korrelieren, der die Aktivität des UT-B modulieren könnte. Ferner wird die Hemmung des Harnstofftransportes durch Ammoniak nur beobachtet, wenn $SCFA/CO_2$ vorhanden sind und der luminale pH-Wert < 7.0 beträgt. Der Effekt von Ammoniak muss jedoch weiterhin als nicht geklärt angesehen werden.

Eine Interaktion von NH_4^+ mit dem UT-B wird als mögliche Erklärung zur Diskussion gestellt.

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Selbständigkeitserklärung zur Dissertation

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Ferner erkläre ich, dass die Arbeit bisher in keinem anderen Promotionsverfahren angenommen oder abgelehnt worden ist.

Zhongyan Lu Berlin, den 24.7.2013