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Review Article

The role of rumen epithelial urea transport proteins in urea nitrogen salvage: A review

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ABSTRACT

The symbiotic relationship between the host and the rumen microbiome plays a crucial role in ruminant physiology. One of the most important processes enabling this relationship is urea nitrogen salvaging (UNS). This process is important for both maintaining ruminant nitrogen balance and supporting production of their major energy supply, bacterially-derived short chain fatty acids (SCFA). The key step in UNS is the trans-epithelial movement of urea across the ruminal wall and this is a highly regulated process. At the molecular level, the key transport route is via the facilitative urea transporter-B2, localized to ruminal papillae epithelial layers. Additional urea transport through aquaporins (AQP), such as AQP3, is now also viewed as important. Long-term regulation of these ruminal urea transport proteins appears to mainly involve dietary fermentable carbohydrates; whereas, transepithelial urea transport is finely regulated by local conditions, such as CO_2 levels, pH and SCFA concentration. Although the key principles of ruminal urea transport physiology are now understood, there remains much that is unknown regarding the regulatory pathways. One reason for this is the limited number of techniques currently used in many studies in the field. Therefore, future research in this area that combines a greater range of techniques could facilitate improvements to livestock efficiency, and potentially, reductions in the levels of waste nitrogen entering the environment.

1. Introduction

Nitrogen (N) is essential to life, with both mammals and their commensal gastrointestinal microbes frequently exposed to a limited supply. The availability of N is therefore a crucial factor shaping the evolution of animal digestive physiology and host–microbe relationships (Reese et al., 2018). Indeed, N availability is a fundamental driver of host–microbiome interactions (Holmes et al., 2017) and physiological strategies have evolved to conserve N, maximize its utilization and maintain these vital symbiotic relationships. One fascinating example of this is the urea N salvaging (UNS) process (Stewart and Smith 2005; Abdoun et al., 2006; Reynolds and Kristensen 2008).

In mammals, urea is produced in the liver to detoxify ammonia produced by protein catabolism. Due to a lack of urease, urea cannot be further metabolized and therefore represents an end-product in these animals. Interestingly, certain gastrointestinal bacteria can convert urea back to ammonia by secreting urease. The released ammonia can then be employed as a N source for microbial growth (Stewart and Smith, 2005), particularly for fibrolytic bacterial populations (Russell et al., 1992). Crucially, varying portions of plasma urea can be supplied from host to bacteria by shifting urea excretion from the kidney into the gastrointestinal tract (Fuller and Reeds, 1998). In turn, the derived bacterial products such as amino acids (AA), peptides and vitamins can be assimilated by host animals. Hence, in this process, the original urea N is salvaged and transformed into various forms which can be utilized by the host, sustaining this symbiotic relationship (Lapierre and Lobley, 2001). This co-evolutionary process, which benefits both host and gastrointestinal microbiome, has been referred to as

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the protein regeneration cycle (Houpt, 1959), N recycling (Fuller and Reeds, 1998; Lapierre and Lobley, 2001) or, most recently, urea nitrogen salvaging (Fig. 1; Stewart and Smith, 2005).

Urea N salvaging is key for ruminants, non-ruminants and humans (Stewart and Smith, 2005). However, due to the development of the forestomach prior to the small intestine, UNS is of particular physiological and nutritional importance in ruminants. In the last 2 decades, the main research progress of this field has been made in ruminants. Many dietary effects on UNS and urea transport proteins have been studied and new findings obtained. This review will first detail the importance of the UNS process in ruminants. It will then focus on the crucial role played by trans-epithelial urea transport across the ruminal wall, specifically the urea transport proteins known to be involved. Further focus will be given to the regulation of these transport mechanisms and the importance of utilizing appropriate techniques in their investigation. Therefore, the aim of this review is to identify the gaps in our knowledge regarding these ruminal transport proteins and to facilitate future studies in the research field.

2. Urea nitrogen salvage in ruminants

It has been well documented that domesticated ruminant species, such as cattle, sheep and goats, have greater ability to maximize the use of N in low N settings by shifting the urea secretion from the kidney to the rumen (Houpt, 1959; Houpt and Houpt, 1968; Harmeyer and Martens, 1980; Lapierre and Lobley, 2001; Stewart and Smith, 2005; Abdoun et al., 2006; Reynolds and Kristensen, 2008; Batista et al., 2017). This phenomenon has also been shown in wild ruminants, such as deer (Kay et al., 1980) and yaks (Zhou et al., 2017), and explains the high N utilization efficiency of highland animals (Jing et al. in this issue). Generally, 40% to 80% of liver-produced urea can be transferred into the gut (mainly rumen), of which 35% to 55% contributes to the anabolism of cattle and sheep (Lapierre and Lobley, 2001). Since the hepatic synthesis of urea can exceed apparent digestible N, without the salvage mechanisms, it would result in negative N balance in ruminants, even at high intakes (Lapierre and Lobley, 2001). UNS also supports the bacterial fermentation process that produces short chain fatty acids (SCFA), which are the main energy supply for host ruminants. Importantly, the breakdown of urea to ammonia by bacterial urease helps to buffer the acidic conditions formed by high SCFA concentrations (Lu et al., 2014). This is because the ammonia (NH_3) produced can bind the excess hydrogen ions (H^+) in the rumen (Lu et al., 2014). The NH_4^+ ions formed may then be readily reabsorbed across the rumen wall and the H^+ ions eventually excreted in the urine (Liebe et al., 2020). This second physiological role for the UNS process is therefore particularly important during high levels of SCFA production, for example in concentrate-fed ruminants (Simmons et al., 2009).

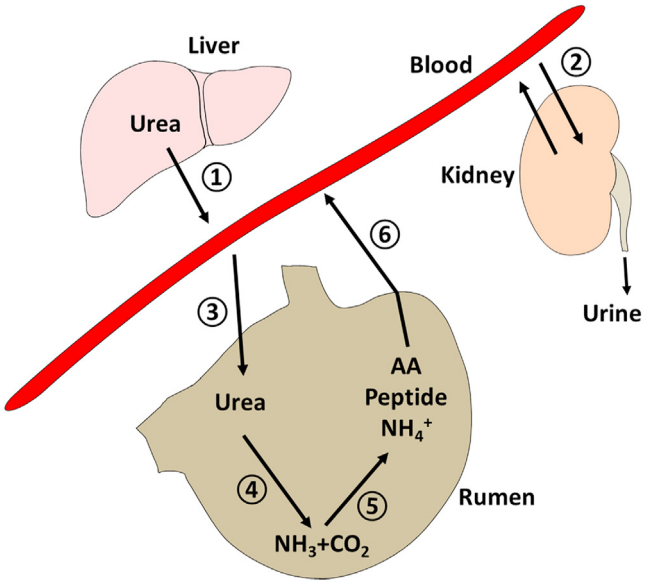

Fig. 1. A schematic diagram illustrating the urea nitrogen salvaging process (adapted from Stewart and Smith, 2005). ① Urea is produced in the liver, via the ornithine–urea cycle, and passed into the bloodstream. ② The blood is continuously filtered by the kidneys, with some urea being reabsorbed and some excreted in the urine. ③ The entry of urea into the rumen is primarily via diffusion across the rumen wall, but also occurs in the saliva. ④ Ruminal urea is rapidly hydrolysed by bacterial urease into ammonia and carbon dioxide. ⑤ This ammonia is then either used by bacterial populations for protein synthesis or bound with H^+ ions, which have been produced by the fermentation processes that produce short chain fatty acids, to form ammonium ions. ⑥ Finally, bacterial amino acids and peptides, as well as the ammonium ions, are absorbed by mammalian host, completing the salvaging of the urea nitrogen. (Note: This can occur either across the rumen wall, or later in the ruminant's gastrointestinal tract.)

The UNS process itself seems to be regulated in a complex manner, including systematic responses at both physiological and molecular levels. At the whole body level, the balance of urea N excretion between the kidney and rumen is a key regulatory factor and is highly diet dependent (Reynolds and Kristensen, 2008; Table 1). For example, one study has shown that a carbohydrate-rich diet with a very low N content stimulates 98% of urea to be excreted into the rumen, with minimal urea secretion into the urine (Wickersham et al., 2008). In direct contrast, 36 h of starvation reduces this value for rumen entry to almost zero (Harmeyer and Martens, 1980). At the molecular level, the rumen epithelium urea transport is believed to be the crucial control step for UNS, involving tissue alterations and precise cellular regulation of the abundance and function of ruminal urea transport proteins. In addition, the plasma urea N incorporation into microbes is also a key factor, which facilitates continual growth and hence the production of SCFA through bacterial fermentation processes. Better understanding of these regulation processes could potentially improve rumen homeostasis and N efficiency in ruminants and hence reduce potential loss of waste urea N into the environment.

3. Rumen epithelium urea transport

The rumen not only serves as a fermentation “tank”, but also allows selective absorption and secretion of many substances across the rumen wall, such as the SCFA themselves (Stumpff, 2018), ammonia (Liebe et al., 2020) and urea (Stewart et al., 2005). The mucosal surface of the rumen is lined with stratified squamous epithelium and is enlarged by leaf-like projections — called rumen papillae — which greatly increase the absorptive and secretive capacity, whilst also providing a niche for microbial populations, including the urease-producing bacterial species (Stewart and Smith, 2005). The epithelial cells of well-developed papillae have multiple layers that are classified into four regions: the stratum corneum, stratum granulosum, stratum spinosum and stratum basale (Graham and Simmons, 2005). The permeability barrier is believed to lie at the level of the stratum granulosum, as tight junction proteins, such as claudin-1 and zonula occludens-1 are mainly present in this layer, with decreasing density through the stratum spinosum to stratum basale and a total absence in the stratum corneum (Graham and Simmons, 2005).

Although urea is present in saliva and therefore represents an indirect influx of urea into the rumen, studies estimate that only 3% to 20% of ruminal urea entry can be attributed to saliva, with the majority to the direct transport across the rumen epithelium (Marini and Van Amburgh, 2003; Røjen et al., 2008 ; Zhou et al.,

Table 1
Factors and conditions affecting ruminal urea transfer/rumen epithelial urea permeability.

Factors	Effect on urea transport	Reference
Low protein intake	Gut urea clearance (mL/min) ↑ Kidney urea clearance (mL/min) ↓ Microbial N derived from plasma urea ↑ No change for urea-N entry to gut (in absolute term, g/d).	Marini and Van Amburgh (2003)
Low protein intake	Gut urea clearance (mL/min) ↑ Kidney urea clearance (mL/min) ↓ Gut urea-N entry (g/d) ↓	Marini et al. (2004)
Low protein intake	Rumen epithelial urea permeability ↑ (in vitro, Ussing chamber)	Muscher et al. (2010)
Low protein intake	Ruminal urea extraction from blood ↑	Kristensen et al. (2010)
Low protein intake	Rumen epithelial urea permeability ↑ (in vitro, Ussing chamber)	Doranalli et al. (2011)
Solid feed intake (milk-fed calves)	Urea-N transferred to gut (g/d) ↑	Berends et al. (2014)
Carbohydrate fermentability	Rumen epithelial urea permeability ↑ (in vitro, Ussing chamber)	Walpole et al. (2015)
Urea supplement	Urea-N entry to gut (g/d) ↑	de Oliveira et al. (2020)

↑ = increased; ↓ = decreased.

2017). Whilst this is not a complete estimate, since saliva secretion is mainly determined by the physical composition of diets (Kennedy and Milligan, 1980) and urea entry into the rumen is mainly affected by ruminal local conditions (pH, SCFA etc.; Abdoun et al., 2010), it is reasonable to assume that the changes of ruminal urea entry are largely from the alterations of direct transport of urea across rumen epithelium. The fact that the rumen epithelium is permeable to urea is well documented (Ritzhaupt et al., 1997). Theoretically, multiple factors can affect the delivery of urea into the rumen (Fig. 2). These include the following: (1) blood flow supplying the rumen epithelium; (2) plasma urea concentration, which provides the source of urea and the concentration gradient favourable for diffusion; (3) epithelial surface area that can be enhanced by ruminal papillae growth; (4) transepithelial urea permeability of ruminal epithelium, which is mediated by urea transport proteins; and (5) bacterial urease activity which serves as driving force and maintains the urea concentration gradient in the direction towards the rumen (Cheng et al., 1979; Cheng and Wallace 1979; Wallace et al., 1979).

4. Facilitative urea transport proteins

The membrane proteins encoded by the solute carrier 14 (*SLC14*) gene family all facilitate rapid and passive movement of urea across cell membranes, down a concentration gradient. These specialized urea transport proteins are hence referred to as facilitative urea transporters (UT). The first urea transporter (UT-A2) was cloned nearly 30 years ago from rabbit renal medulla (You et al., 1993).

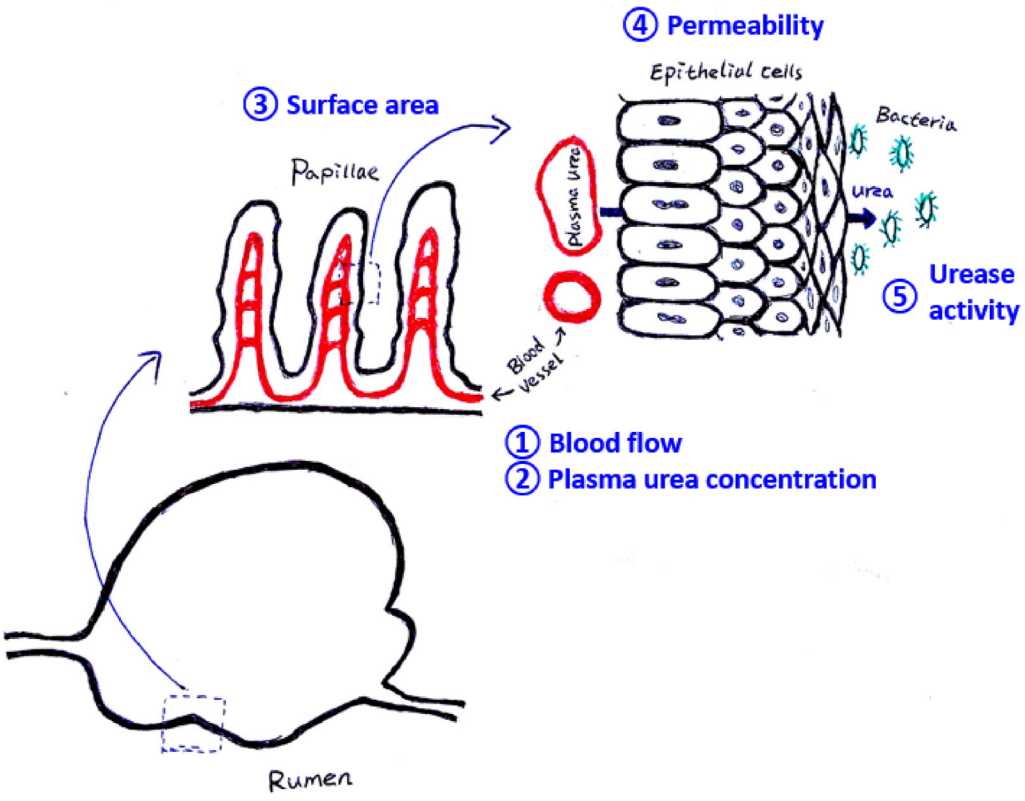

Fig. 2. Diagram illustrating the key factors affecting total urea transfer from the bloodstream into the rumen. Although all these factors significantly contribute to the process, it is believed that the major point of control is the transcellular transport across ruminal epithelial layers, mainly via urea transport proteins.

Since then, the knowledge of these proteins and their physiological roles in various organs has been greatly explored (reviewed in Stewart, 2011; Yu et al., 2019). Two closely related but distinct subfamilies have been characterized in various mammalian species: the *SLC14A2* gene encoding UT-A and the *SLC14A1* gene encoding UT-B. The *SLC14A2* gene gives rise to multiple transcripts and protein isoforms as a result of differential transcription and translation processes (Smith and Fenton, 2006). Six protein isoforms have been characterized and classified as UT-A1 to UT-A6. The *SLC14A1* gene has 2 protein products fully characterized to date, termed as UT-B1 and UT-B2; though there is evidence of additional isoforms (Walpole et al., 2014). UT-A transporters are mainly found in the kidney, except for UT-A5 and UT-A6, which are found in the testes and colon, respectively (Stewart, 2011). Most UT-A isoforms are acutely regulated via phosphorylation and trafficking of the glycosylated transporters to the plasma membranes, which is induced by the antidiuretic hormone vasopressin (Stewart, 2011). In contrast, although they are also highly glycosylated, UT-B proteins are more widespread in tissue location and appear to be chronically regulated (Yu et al., 2019). Crucially, rumen epithelium highly expresses UT-B transporters, whilst UT-A transporters are absent (Stewart et al., 2005).

Apart from the facilitative urea transporters, in vitro evidence suggests that some other membrane proteins are also permeable to urea. For example, when expressed in *Xenopus* oocytes, a subgroup of the aquaporin (AQP) water channel family, classified as aquaglyceroporins (AQGP), are not only permeable to water and glycerol, but also to urea. This group includes AQP3 (Echevarria et al., 1994; Ishibashi et al., 1994), AQP7 (Ishibashi et al., 1997), AQP9 (Ishibashi et al., 1997) and AQP10 (Ishibashi et al., 2002). It has been reported that AQP7 has the highest urea permeability, which is in the same order of magnitude as UT-A and UT-B proteins (Ishibashi et al., 1997). Although AQP3 and AQP9 also induce increased cell membrane urea permeability, this is by one order of magnitude lower than UT-B (Mannuzzu et al., 1993). The tissue distributions and proposed physiological roles of these AQGP have been extensively reviewed previously (Rojek et al., 2008; Zeuthen et al., 2009; Bollag et al., 2020). As such, AQP3 has been shown to have a wide tissue distribution (e.g. kidney, skin, gastrointestinal tract), whereas AQP7 is mainly found in adipose tissue, AQP9 in the liver and AQP10 in the small intestine.

5. UT-B urea transporter/channel

The mammalian *SLC14A1* (UT-B) gene has been characterized in several species, including rat, mouse, human and cow. However, compared to the *SLC14A2* (UT-A) gene (Smith and Fenton, 2006; Shayakul et al., 2013), only minimal information is available regarding the transcriptional mechanisms regulating *SLC14A1* gene expression. For example, the human *SLC14A1* gene has 11 exons, 13 transcripts and 12 predicted amino acid sequences; the bovine *SLC14A1* gene has 11 exons, 8 transcripts and 3 predicted amino acid sequences (www.ncbi.nlm.nih.gov).

Two distinct protein isoforms, UT-B1 and UT-B2, have been characterized in mammals. UT-B1 was first cloned from bone marrow (Olives et al., 1994) and is encoded by 8 exons, with an amino acid length of 385 AA in most species. Importantly, UT-B2 was originally characterized in bovine rumen (Stewart et al., 2005) and appears to be a rumen-specific protein (Stewart, 2011). An extra exon is incorporated in UT-B2, compared to UT-B1, which adds an additional 55 AA to the amino terminus (Stewart et al., 2005). Equivalent transporters to bovine UT-B2 have also been reported in the rumen of other species, including sheep (Lu et al., 2014) and deer (Zhong et al., 2022). On western blots, the predicted sizes of these 2 isoforms are approximately 40 kDa for UT-B1 and approximately 50 kDa for UT-B2, respectively. However, as can be seen in Table 2, the actual UT-B protein sizes detected in a variety of tissues generally do not match these predictions. Whilst various theories have been proposed for this discrepancy (Walpole et al., 2014), no conclusive explanation has been determined.

UT-B1 is abundantly expressed in erythrocytes, kidney, ureter and bladder, as well as in other tissues, including bone marrow, brain and gastrointestinal tract (Yu et al., 2019). It is important for several physiological reasons. For example, UT-B1 mediated rapid urea diffusion across erythrocyte membranes allows erythrocytes to cope with the large osmotic changes they experience when passing through the renal medulla (Bagnasco, 2006). In the kidney, UT-B1 is located in the descending vasa recta blood vessels (Timmer et al., 2001) and is part of the renal urea recycling process, during which it mediates passage of interstitium urea into the vascular system in the inner medulla (Yang and Bankir, 2005). In contrast, as previously stated, it has been consistently reported that UT-B2 is the major ruminal isoform (Stewart et al., 2005; Coyle et al., 2016; Zhong et al., 2020). UT-B2 mediates the transfer of plasma urea into the rumen, supplying N to the microbes (Stewart and Smith, 2005) and buffering the pH changes produced by bacterial SCFA production (Abdoun et al., 2010; Lu et al., 2014).

Interestingly, all UT-B and UT-A isoforms share significant homology at the nucleotide and amino acid level. Like UT-A2, the primary structure of UT-B predicts 10 trans-membrane domains, of which the first five and last five domains share significant homology to each other (Levin et al., 2012). An extracellular loop connects the 2 homologous halves and carries an asparagine-linked glycosylation site (N211) (Lucien et al., 2002), with both amino and carboxy termini oriented into the cytoplasm. Unlike UT-A2, the consensus sites for phosphorylation of protein kinase A and protein kinase C are not found in UT-B (Olives et al., 1994).

The crystal structures of bovine UT-B1 protein (Levin et al., 2012) and the bacterial homolog of mammalian urea transporters, *Desulfovibrio vulgaris* UT (dvUT) (Levin et al., 2009), have both been resolved. The structural basis proposed for urea transport, permeation mechanisms and selectivity have been elegantly discussed (Knepper and Mindell, 2009). The bovine UT-B and dvUT are highly similar in structure, as both proteins form trimers with parallel orientation in the cell membrane (Levin et al., 2009, 2012). Each subunit (i.e. single protein) contains 2 homologous halves with opposite orientations in the membrane, forming a membrane-spanning pore with a narrow selectivity filter that operates by a channel-like mechanism (Levin et al., 2009, 2012). This finding is consistent with previous studies that UT-B proteins were capable of much greater levels of urea transport than UT-A proteins (Mannuzzu et al., 1993; Maciver et al., 2008). It can therefore be argued that it is more physiologically accurate to use the term “UT-B channels” rather than the traditional term “UT-B transporters”. Finally, the trimer structure reported for bovine UT-B1 (Levin et al., 2012) has also been evident in studies investigating UT-B in the human bladder (Walpole et al., 2014).

Direct evidence for the acute regulation of UT-B proteins in response to specific signalling pathways remains scarce. Mouse and rat UT-B1 do have a consensus sequence for kinase phosphorylation (Tsukaguchi et al., 1997; Yang et al., 2002), but human UT-B1 appears to lack this sequence (Olives et al., 1995). However, it has been shown that deletion of the first 59 AA of the human amino terminus, or the mutation of Cys-25 and Cys-30, both prevented UT-B1 membrane localization (Lucien et al., 2002), suggesting this region plays a crucial role in membrane trafficking processes. In contrast, several studies have addressed the chronic regulation of UT-B1 expression in the kidney and gastrointestinal tract of non-ruminant animals (Yu et al., 2019). In rats, the chronic administration of vasopressin induced a significant decrease of UT-B1

Table 2
Apparent molecular weights of UT-B protein on Western blot.

Tissue	Organism	MW, kDa (Glycosylated/Un-glycosylated)	Reference
Red Blood Cells	Cattle	35-50/32	Simmons et al. (2009)
Kidney	Cattle	35-50/32	Simmons et al. (2009)
Kidney	Sheep	40-55/36	Marini et al. (2004)
Rumen	Cattle	36-55/36	Simmons et al. (2009)
Rumen	Cattle	30, 32	Røjen et al. (2011)
Rumen	Sheep	47/32	Ludden et al. (2009)
Rumen	Goat	50/30	Lu et al. (2015)
Rumen	Cattle	50	Coyle et al. (2016)
Salivary gland	Cattle	40, 32-34/30	Dix et al. (2013)

UT-B = urea transporter-B; MW = molecular weight.

protein abundance in the inner medulla of kidney (Trinh-Trang-Tan et al., 2002). Additionally, a low protein diet induced a decrease of UT-B1 expression in the colon, but an increase in the outer medulla of the kidney (Inoue et al., 2005). In the human colon, there is a 35 kDa glycosylated UT-B1 protein with a higher abundance in the ascending colon compared to the descending colon, matching the pattern of measured trans-epithelial urea transport (Collins et al., 2010). Since microbial populations are higher in the ascending colon, these findings indicate that microbial activity could be involved in regulating human colonic UT-B function (Collins et al., 2010). This supports the idea that great similarities exist between UNS in the ruminal and colonic tissues of various mammalian species (Stumpff, 2018).

6. Historical evidence for ruminal urea transport proteins

The attempts to identify ruminal urea transporters/channels date back to the late 1990s. For example, using RT-PCR primers for human erythrocyte UT (later designated UT-B1), a cDNA fragment most homologous to rat kidney UT-B1 was amplified from sheep rumen (Ritzhaupt et al., 1998). These preliminary findings were further investigated using multiple techniques. Most significantly, it was shown that specific UT-B isoforms exist in the bovine rumen (Stewart et al., 2005). Two RNA transcripts were identified — UT-B1 (3.5 kb) and UT-B2 (3.7 kb), derived from alternative splicing. The UT-B2 transcript was predominant in the rumen, whilst UT-B1 was predominant in the kidney (Stewart et al., 2005; Coyle et al., 2016; Zhong et al., 2020). Glycosylated UT-B2 proteins are detected in the rumen and located on the plasma membranes of all epithelial cell layers except for stratum corneum (Stewart et al., 2005; Simmons et al., 2009; Coyle et al., 2016. The involvement of UT-B in mediating the urea transport across the rumen epithelium was shown by the observations that the urea flux was bidirectional and significantly inhibited by phloretin (Ritzhaupt et al., 1997; Stewart et al., 2005), thiourea (Ritzhaupt et al., 1997) and acetamide (Thorlacius et al., 1971) — all known inhibitors of facilitative urea transporters (Yang and Verkman, 2002; Zhao et al., 2007; Tickle et al., 2009).

It was originally confirmed that the bovine UT-B isoforms were indeed functional using an in vitro oocyte expression system (Stewart et al., 2005). Namely, the expression of bovine UT-B1 and UT-B2 cRNA in *Xenopus* oocytes induced significant increases in urea permeability compared with water-injected controls (Stewart et al., 2005). Further studies addressed the acute regulation of bovine UT-B function using a MDCK (Madin–Darby canine kidney) cell line transfected with ruminal UT-B2 (Tickle et al., 2009). Unlike many renal UT-A urea transporters cloned into the same cell line (Fröhlich et al., 2004; Stewart et al., 2009), UT-B2 function and protein abundance were not affected by short-term exposure to antidiuretic hormone vasopressin, intracellular cyclic adenosine monophosphate (cAMP), calcium, or protein kinase activity (Tickle et al., 2009). These findings were consistent with the original description of the UT-B gene that had no evidence of cAMP response elements in the promoter region (Lucien et al., 1998). It was also found that UT-B2 protein was absent from the cytoplasm, which seemed to rule out regulation through trafficking proteins to the cell membranes (Tickle et al., 2009). This apparent lack of acute regulation, along with the very high trans-epithelial urea transport rate observed, indicated that ruminal UT-B2 was constitutively activated (Tickle et al., 2009). Due to its role in UNS, it is possible that ruminants require chronic dietary regulation of UT-B2 protein abundance, coupled with very rapid, acute regulation of UT-B2 proteins already present in the cell membrane.

Finally, the idea that ruminal urea transport also involves AQGP was originally proposed based on the observation that urea transport across rumen epithelium was not completely suppressed by the known UT-B inhibitors phloretin and thionicotinamide (Stewart et al., 2005). This was further supported by the following findings: (1) a significant portion of urea flux across isolated rumen epithelium was inhibited by $NiCl_2$ (an inhibitor of AQGP) regardless of phloretin sensitive urea transport (Walpole et al., 2015); (2) many AQGP messenger ribonucleic acid (mRNA) have been detected in rumen epithelium (Røjen et al., 2011; Walpole et al., 2015; Zhong et al., 2020), as well as AQP3 transporter protein in various ruminal epithelial layers (Zhong et al., 2020); and (3) ruminal urea transport can be regulated by luminal pH in the presence of SCFA and CO_2 with a bell-shaped pattern (Abdoun et al., 2010; Lu et al., 2014) and the transport properties of AQP3 are known to be modulated by pH in a similar manner (Zou et al., 2000). Therefore, the future research of ruminal urea transport should consider both UT-B and AQGP proteins.

7. Long-term regulation of ruminal urea transport proteins

The response of ruminal UT-B and AQP3 to dietary intake have been studied intensively in recent years. Contrary to predictions, UT-B expression has seemed relatively unresponsive to dietary N intake under isoenergetic feeding conditions (Muscher et al., 2010; Røjen et al., 2011). For example, Ludden et al. (2009) examined UT-B expression along the gastrointestinal tract of lambs and no response was found to changing dietary protein levels. Instead, UT-B protein abundance was greater in lambs supplemented with rumen degradable protein (Ludden et al., 2009). Indeed, the link between quantitative urea transfer or rumen epithelial urea permeability and the expression of UT-B and AQPs seems generally lacking. In lambs fed low protein diets, the gastrointestinal urea entry rate decreased, whilst the UT-B protein remained unchanged (Marini et al., 2004). In contrast, similar experiments with dairy heifers demonstrate that urea entry rate remained unchanged, whilst the UT-B protein was down-regulated (Marini and Van Amburgh, 2003). In addition, ruminal extraction of arterial urea was greater in dairy cows fed low protein diets, but UT-B mRNA and

protein abundance remained unchanged (Kristensen et al., 2010). Furthermore, mRNA expression of *AQP3*, *AQP7* and *AQP10* were all down-regulated by low protein intake (Røjen et al., 2011). However, it should be noted that these in vivo measurements of urea entry rate or ruminal vein-arterial urea concentration difference may not be good parameters to link correlations between urea transport and the expression of urea transport proteins. This is because plasma urea concentration and urease activity varies greatly with N intake, and the changes of blood flow and the surface area (e.g. changes in ruminal papillae) were not considered (Kristensen et al., 2010; Marini et al., 2004; Marini and Van Amburgh, 2003; Muscher et al., 2010). These cofactors may counterbalance the effects of potential molecular changes. Instead, the urea flux or urea transport rate across ex vivo rumen epithelium, which can exclude other co-factors, seem to be a good indicator of epithelial urea permeability. For example, utilising the Ussing chamber technique, Muscher et al. (2010) and Doranalli et al. (2011) demonstrated that the rumen epithelium of animals fed low N diet have significantly higher urea permeability (in nmol/cm^2 per h). The *UT-B* mRNA abundance, however, did not correspond to the permeability differences (Muscher et al., 2010). For certain, it would be more informative to see whether protein abundance or glycosylation state corresponded to this change, but combined ex vivo urea flux measurements and protein abundance under low N intake data are currently lacking.

Additionally, 2 recent studies have addressed the effect of dietary urea supplement on the expression of urea transporters in cattle. Interestingly, the *UT-B* mRNA was unaltered (Saccà et al., 2018) or down-regulated (de Oliveira et al., 2020). The *AQP3* mRNA expression was also depressed, whilst *AQP7* remained unchanged (de Oliveira et al., 2020; Saccà et al., 2018). One suggestion is that the increased ruminal ammonia concentration, derived from increased urea hydrolysis, might inhibit the expression of both UT-B and AQP3.

In contrast to the relative indifference to the dietary N intake, UT-B and AQP3 expression appears to be far more responsive to ruminal fermentable carbohydrate content. Compared with silage-fed animals, concentrate-fed steers had higher expression of UT-B at both mRNA and protein level, along with more widespread protein localization across rumen epithelial layers (Simmons et al., 2009). Similarly, *UT-B* and *AQP3* mRNA increased linearly during the period steer calves were subjected to a diet change from hay-based to medium grain content diets (Walpole et al., 2015). In lactating dairy cows, *UT-B* mRNA was found to increase when the diet changed from high-straw/low-energy to low-straw/high-energy content (Minuti et al., 2015). In lambs, *AQP3* mRNA was significantly higher when the diet had a higher concentrate-to-forage ratio, whilst *UT-B* mRNA was not (Scott et al., 2020). In goats, *UT-B* mRNA and protein were upregulated by diets rich in non-fibre carbohydrate regardless of N content (Lu et al., 2015). Furthermore, increasing dietary non-fibre carbohydrate content not only increased SCFA production, microbial diversity, microbial protein synthesis and UT-B expression; it also increased G protein-coupled receptor (GPR) 41 and GPR43 expression (Lu et al., 2015), indicating that these are key receptors in the regulatory process. All these data indicate that easily fermentable substrates, especially highly fermentable non-fibre carbohydrate, are the dietary factors most affecting UT-B and AQP3 expression.

The importance of fermentable carbohydrate content to ruminal expression levels of UT-B or AQP3 has been further illustrated in several studies investigating rumen development. Instead of age per se, events such as solid feed intake and weaning seem to be the apparent stimulus of the expression of urea transporters. For example, Berends et al. (2014) quantified that UNS contributes to 19% of N retention during the transition from pre-ruminants (milk-fed calves) to ruminants (solid-fed milk-based calves). In line with this, mRNA expression of *UT-B* and *AQP3* were increased with solid feed intake (Berends et al., 2014). The UT-B presented a quadratic increase pattern, namely about a 4-fold increase with the initial provision, then only slightly further upregulated when the amount of solid feed intake increased (Berends et al., 2014). AQP3 exhibited a linear increase from the initiation to gradually increased level of solid feed intake, whilst *AQP7* mRNA was weakly detected and remained unaltered (Berends et al., 2014). In another study, the *UT-B* mRNA expression in post-weaning calves (10 weeks) was markedly higher than pre-weaning calves (5 weeks; Naeem et al., 2012), suggesting the transition from milk-based to solid feed-based diets has a dramatic effect on the expression of UT-B. Additionally, our recent studies have shown that UT-B protein abundance in the calf rumen significantly increases with age in the first three months of life, in response to dietary changes and the development of rumen function, whilst AQP3 remained at low levels (Zhong unpublished data). These data suggest that UT-B transporters/channels are the primary mechanism for ruminal urea transport, at least in calves. Overall, since the provision of solid feed to calves stimulates the development of the rumen function and fermentation ability, the direct factors affecting urea transporters could be the fermentation products themselves, such as SCFA. Therefore, it appears that the role of ruminal UT-B transporters/channels in buffering the effects of increased ruminal SCFA production may best explain their long-term regulation (Lu et al., 2014).

Finally, the regulatory impact of changes at the tissue level cannot be ignored. For example, a previous study detailed, as expected, significant differences in the overall UT-B protein abundance between ventral and dorsal sac regions of the bovine rumen (Coyle et al., 2016). Importantly, there was more UT-B in the ventral sac, as predicted by the presumption that more would be needed to facilitate greater urea secretion to supply ruminal bacteria in this region. However, this difference was not in cellular UT-B abundance or localization—which were the same in both regions—but simply by the fact that the ruminal papillae were much larger in the ventral sac (Coyle et al., 2016). It remains a significant difficulty to the rumen physiology field that many studies fail to report the basic characteristics of the tissue being studied. For example, on-going studies into deer rumen suggest that regulation of urea transport can occur in many different ways—at both a cellular level, such as UT-B protein abundance (e.g. biological sex), but also at a tissue level, such as changes in rumen size (e.g. biological sex), ruminal papillae length (e.g. ageing) or ruminal papillae density (e.g. dietary changes; Zhong et al., unpublished studies).

8. Short-term regulation of ruminal urea transport proteins

As discussed above, there is a growing body of evidence that UT-B and AQP3 expression are responsive to easily fermentable diets. This strongly suggests that the microbial metabolites and the change of local chemical conditions such as pH, the concentrations of SCFA, CO_2 and ammonia may be the key stimuli for the long-term regulation of urea transporters. Is there any evidence of these factors contributing to short-term regulation? In a series of carefully performed studies using isolated sheep rumen epithelium, Abdoun et al. (2010) and Lu et al. (2014) examined the short-term effects of pH, SCFA, CO_2 and ammonia on urea transport, with both interdependent and synergetic effects being found. For example, in the presence of SCFA or CO_2/HCO_3^-, the pH (7.4 to 5.4) exhibited a bell-shaped relationship with the urea flux rate (i.e. the urea flux rate was low at pH 7.4, increased as pH acidified to 6.4, but then decreased again as pH further acidified to 5.4). The point of maximum urea flux rate seemed to vary across studies on sheep, reported as pH 6.2 by Abdoun et al. (2010) but pH 5.8 by Lu et al.

(2014). Interestingly, this latter peak value of 5.8 was shifted to 6.2, when the sheep were switched from hay-fed to concentrate-fed (Lu et al., 2014). The effect of SCFA and CO_2/HCO_3^- increased as the concentration increased, and were additive (Abdoun et al., 2010; Lu et al., 2014). No effects were observed at slight alkaline/neutral pH in the presence of SCFA or CO_2/HCO_3^- or lowering the ruminal pH in buffers without SCFA or CO_2/HCO_3^- (Abdoun et al., 2010). It was further demonstrated that the effects were likely caused by changes of apical microclimate and the resulting changes of intracellular H^+ concentration, based on the evidence that SCFA increased the acidification effect of luminal pH on the cytosolic pH, and inhibiting Na^+/H^+ exchange increased urea flux rate at pH 7.4, but reduced it at pH 6.4 (Abdoun et al., 2010).

The effect of ruminal ammonia was dependent on both pH and ammonia concentration. At extremely low concentration (in the form of NH_4Cl, < 1 mmol/L), it had a stimulating effect on urea transport rate, whilst at high concentration (>1 mmol/L) it had an inhibitory effect (Lu et al., 2014). At the high concentration and in the presence of SCFA, the effects were pH dependent, resulting in a similar bell-shaped relationship to the effect of SCFA alone, but with reduced amplitude (Lu et al., 2014). The direct and indirect changes of the transepithelial potential difference (known to affect the uptake of NH_4^+) and inhibiting cation channels significantly affected the inhibitory effect of ammonia on urea flux rate, suggesting the association with the uptake of NH_4^+ into the cytosol through cation channel proteins (Lu et al., 2014). These data did not support the effects being attributable to competition with the pore of a transporter with affinity for both urea and NH_3, as altering the NH_3 concentration did not change the effects (Lu et al., 2014).

Overall, it appears that the in vivo and in vitro observations are in good agreement. The pH dependent short-term regulation of SCFA, CO_2 and ammonia effects on urea transport are believed to be mediated by transcellular pathways involving urea transport proteins, subject to acute regulation that alters intracellular pH through the influx of H^+ and NH_4^+ into cytosol (Lu et al., 2014). No such effects were found on the flux rate of mannitol (Abdoun et al., 2010; Lu et al., 2014), suggesting that it cannot be explained by paracellular transport pathways. The most likely candidates are the UT-B2 and AQP3 proteins extensively discussed in the current review. The simple, initial hypothesis was that cytosolic H^+ and/or NH_4^+ altered the function of urea transport proteins through changing the configuration of the protein structure (Abdoun et al., 2010; Lu et al., 2014). However, this was not sufficient to explain the upregulation of these transporters' in vivo during adaptation to readily fermentable diets. A second hypothesis is therefore possible; as described in the previous section, that similar conditions also affected the density or localization patterns of these transporting proteins on the rumen epithelium. To test this hypothesis, Lu et al. (2015) further investigated the effect of SCFA, ammonia and urea on the UT-B expression using primary rumen epithelial cell cultures. Exposure of cells for 24 h to an acid pH at 6.8, but not 6.4, elevated the mRNA and protein expression of UT-B (compared with the treatment of pH 7.4; Lu et al., 2015). The effects of SCFA were pH and concentration dependent, being inhibitory at pH 7.4 for both 20 mmol/L and 40 mmol/L SCFA, whilst inhibitory at pH 6.8 for 20 mmol/L and stimulating at pH 6.8 for 40 mmol/L SCFA (Lu et al., 2015). NH_4Cl (1.25 mmol/L, pH 6.8) significantly decreased the expression of UT-B mRNA and protein, whilst urea (4 mmol/L, pH 7.4) had the opposite effect (Lu et al., 2015). Whether these changes are sufficient to explain the acute effect (20 to 40 min) on urea transport observed in vitro, or if it also involves configuration related functional changes to urea transport proteins, now needs further investigation.

Lastly, the effects of osmotic changes in the external environment on urea transport mechanisms cannot be discounted. For example, increased SCFA production in the rumen will increase

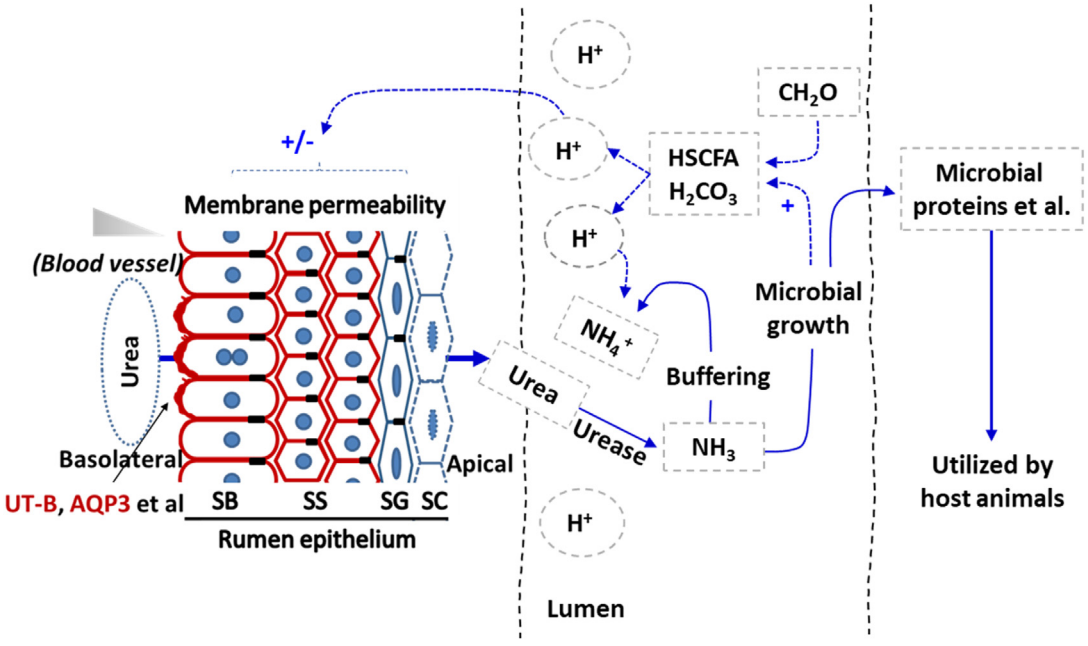

Fig. 3. Diagram illustrating the four layers of rumen epithelium, the locations of urea transport proteins, and the physiological roles and proposed regulation mechanisms of urea transport across rumen epithelium. The urea transport proteins, such as urea transporter (UT)-B and aquaporin (AQP)-3, facilitate urea movement across the rumen epithelium down a concentration gradient. The urea transferred is broken down by urease into NH_3, which serves 2 roles: buffering the acid conditions in the lumen and providing a nitrogen source for bacterial populations. Evidence suggests that the urea permeability of rumen epithelial cells is regulated by the fermentation products, such as SCFA, CO_2 and NH_3, and their resulting H^+ changes in the microclimate of rumen epithelium. SB = stratum basale, SS = stratum spinosum, SG = stratum granulosum, SC = stratum corneum, HSCFA = short chain fatty acids, CH_2O = carbohydrate, especially easily fermentable carbohydrate. The red colour indicates the locations of urea transport proteins, e.g. UT-B and AQP3, with the intensity increasing near blood vessels (source of urea). The solid blue arrows represent the urea nitrogen flow, while the dotted arrows represent proposed regulation routes.

ruminal fluid osmolality (e.g. from 300 to 400 mOsm) and external osmolality changes are known to rapidly alter bovine UT-B transporter/channel function (Levin et al., 2012). While it is known that increased external osmolality can also alter UT-B protein abundance in bladder (Farrell and Stewart, 2019) and localization in the brain (Huang et al., 2021), no such studies have yet been performed in ruminal tissues.

9. Conclusion

Promoting the transfer of urea into rumen is a potential strategy to improve ruminal homeostasis and N utilization efficiency. Functional studies have strongly suggested that the urea permeability of rumen epithelium is the key step in the regulatory process, with UT-B transporters/channels and aquaglyceroporins believed to be very important (Fig. 3). These urea transport proteins are abundant in the rumen epithelium, particularly the rumen-specific UT-B2 protein, with the major location being in cell membranes of the stratum basale layer. The expression and abundance of the urea transporters/channels was originally believed to be regulated primarily by dietary N intake, but minimal correlation has been found. Instead, a growing body of evidence now suggests that these urea transport proteins are more responsive to highly fermentable carbohydrates. This is due to the changes they produce in ruminal fermentation product levels, such as SCFA and CO_2, as well as ammonia and pH (Fig. 3), in both the short- and long-term.

One crucial factor potentially preventing further understanding of ruminal UNS is the limited number of techniques currently used in many studies. This is because regulation of urea transport occurs at microbial, molecular, tissue and/or animal levels, so all aspects ideally need to be investigated in any given study (Fig. 2). The authors of this review suggest that the following data should be obtained: size of rumen, papillae size, papillae density, transporter RNA expression, transporter protein abundance and localization, transporter glycosylation state, and (if feasible) functional transepithelial urea transport. Additional studies investigating whole animal physiology and the rumen microbiome will also greatly aid better understanding. Therefore, it appears obvious that more multi-disciplinary research teams are now required to advance the rumen physiology field.

Some key questions remain to be answered regarding the regulation of the ruminal UNS process via urea transport proteins. These include, but are not limited to: Do the relative contributions of UT-B and AQP protein vary with either species or dietary intake? Does UT-B2 and AQP3 abundance or glycosylation state vary with dietary N intake? Would changes to such things be enough to explain rapid 20- to 40-min functional changes in rumen urea transport, or are configuration changes of proteins already in the membrane also involved? Does a basic increase in external osmolality affect rumen urea transport proteins in a functionally significant manner? Fundamentally, improved understanding of the regulation and function of the UNS process would facilitate the development of strategies to improve its efficiency. This may identify novel strategies to improve both animal health and N efficiency, which will have substantial benefits to both the livestock industry and the environment.

Author contributions

Chongliang Zhong: Writing-Original draft preparation, Funding. **Ruijun Long:** Writing-Review and editing, Supervision, Funding. **Gavin Stewart:** Writing-Original draft, review and editing, Supervision, Resource.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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