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Topical Review

Facilitative Urea Transporters

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Introduction

Urea is the major end-product of amino acid deamination in mammals and consequently is often considered a waste product of metabolism. However, regulated movement of urea across plasma membranes underlies fundamental biological processes that include urinary concentration and urea nitrogen salvaging. Urea transporter (UT) proteins are central to modulating urea movement across biological membranes, and over the past decade major steps have been made in defining their molecular identity and basic functional characteristics. The subsequent generation of reagents, including specific nucleotide probes and antisera, has led to significant advances in understanding the molecular aspects of the role played by UTs in the kidney. It has also led to the discovery of UTs in tissues not previously associated with handling urea, such as brain, testis and heart. This review summarizes the progress made since the isolation of the first urea transporter cDNA. It describes the characteristics of UT genes, messenger RNAs, and proteins, and also summarizes what is currently known about UT regulation.

Previously, movement of urea across biological membranes was considered to occur by lipid phase diffusion. However, the urea permeabilities of erythrocyte membranes and of certain plasma membranes in the terminal part of the kidney collecting duct were found to be much higher than could be explained by passive lipid diffusion alone (for details, *see* review by Marsh & Knepper, 1992). This inconsistency prompted the notion that urea crosses biological membranes by a carrier-mediated mechanism. In the late 1980s, several studies pointed to the existence of specific urea transporter (UT) proteins distinct from those responsible for water transport (Chou & Knepper, 1989; Chou et al., 1990; Star, 1990).

Definitive proof of the existence of UT proteins soon followed with the isolation from kidney inner medulla of a cDNA encoding a UT protein, UT-A2 ((You et al., 1993) *see* Table). This breakthrough opened the door for homology-based screening that led to the characterization of a cDNA from human bone marrow which encoded a novel UT protein, UT-B1 (Table, Olives et al., 1994). Not surprisingly, UT-B1 turned out to have a high degree of homology to UT-A2, but was shown to be the product of a different gene (Promeneur et al., 1996). To date, all of the known facilitative UT proteins with characterized nucleotide sequences are the product of either one of two genes, termed UT-A and UT-B. In this review, to refer to the UT transcripts and proteins, we have used the systematic nomenclature proposed by Sands et al. (Sands, Timmer & Gunn, 1997).

The Urea Transporter Genes

The structure of the human UT-B gene has been resolved. We will therefore address this first and contrast it with what is known about the UT-A gene. The human UT-B gene consists of 11 exons spanning more than 30 kb (Lucien et al., 1998). Exons 4 to 11 contain the coding sequence of UT-B1 (Olives et al., 1994; Sidoux-

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Table. Summary of rat urea transporter proteins

Protein	Other names	Transcript size kb	Number of amino acids	Predicted M.W. kDa	Observed M.W kDa	Putative PKA sites	Putative PKC sites	Genbank Accession number	Reference
	UT1								
UT-A1	VRUT	4.0	929	102	97/117	7	7	U77971	Shayakul, Steel & Hediger, 1996
UT-A2	UT2	2.9	397	43	55	2	2	U09957	Smith et al., 1995a
UT-A3	UT4	2.1	460	50	44/67	2	4	AF041788	Karakashian et al., 1999
								AF031642	Terris et al., 2001
UT-A4		2.8?	413	43	46	3	3	AF042167	Karakashian et al., 1999
UT-A5		1.7? (1.5)	(323)	(35)	?	(0)	(2)	AF258602	Fenton et al., 2000
UT-B1	UT3 rUT11	3.8	384	42	41–54	0	2	U81518	Tsukaguchi et al., 1997
UT-B2	RNUT11	3.8	414	45	41–54	0	2	X98399	Couriaud, Ripoche & Rousselet, 1996

Other names refers to ad hoc names given prior to introduction of systematic names. *Number of amino acids and putative molecular weights* refer to values predicted from the open reading frame of cDNA. Rat UT-A5 has yet to be characterized, therefore statistics for mouse UT-A5 are shown in parentheses.

Walter et al., 1999). There is a high degree of sequence homology between exons 4–7, and 8–11, which suggests that duplication of an ancestral module may have taken place (Rousselet, Ripoche & Bailly, 1996; Lucien et al., 1998). This duplication is also a feature of the UT-A gene. Additionally, duplication of the whole gene may have taken place because the UT-A gene in rat and mouse has two promoters compared to UT-B's one (Cottingham et al., 1999; Nakayama et al., 1999). The most proximal promoter, UT-Aα, lies 5′ to exons encoding the NH-terminus of UT-A1 (*see* below). Another promoter, UT-Aβ, is present near the middle of the exons encoding UT-A1, and just before the first UT-A2 exon (Cottingham et al., 1999; Nakayama et al., 1999). It appears that at least three successive duplications have occurred to give six repeats of the ancestral module; two in UT-B and four in UT-A. One of these duplications, probably the last to take place, did not occur in UT-B, and thus occurred in UT-A after the UT-A/UT-B split. This hypothesis is supported by the fact that the sequence identity between urea transporter cDNAs encoded by the first and second half of the UT-A gene is higher than between the first or second half of the UT-A gene and UT-B1. A scheme of evolution of urea transporter genes in mammals is proposed in Fig. 1. From this analysis it can be speculated that UT-B may be the phylogenetically older of the two genes.

In the human genome both genes lie on chromosome 18 within the large region between q12–q21 (Olives et al., 1995; Olives et al., 1997). In mouse, we have finely mapped the UT-A and UT-B genes to chromosome 18 between the markers *D18Mit25* and *D18Mit186* (Fenton et al., 1999a), and shown that the genes are arranged in tandem. This is probably also a feature of the human genes, because they both map to approximately the same region, although finer mapping will be necessary to confirm this.

Based on northern analysis, the human UT-B gene gives rise to at least four transcripts (2.0, 2.5, 3.7 and 4.5–4.7 kb (Olives et al., 1994, 1996; Promeneur et al., 1996). These result from the use of alternative transcriptional start sites, differential splicing, and alternative polyadenylation signals (Lucien et al., 1998). Although several cDNAs have been characterized, there is a lack of correlation between cDNA size and the UT-B transcripts, as revealed by northern analysis. This suggests either that the cDNAs are truncated forms of the UT-B transcripts or that they represent low-copy transcripts which were not detected by northern analysis. Clearly, further work is needed to determine the molecular identity of all of the UT-B transcripts.

The number of UT-A transcripts in human has not been established. In rat, the UT-A gene gives rise, by use of alternative promoters, alternative transcriptional start sites and differential splicing (Smith et al., 1995; Shayakul, Steel & Hediger, 1996; Cottingham et al., 1999; Karakashian et al., 1999; Klein et al., 1999), to at least seven transcripts (1.7, 2.1, 2.8, 3.0, 3.1 kb, 3.7 kb and 4.0 kb (Smith et al., 1995a; Shayakul et al., 1996; Karakashian et al., 1999). The identities of some of the UT-A transcripts have been established (*see* Table).

Characteristics of Urea Transporter cDNAs and the Proteins They Encode

The relatively recent explosion in our knowledge of UT proteins is primarily due to the isolation and characterization of cDNAs. Several cDNAs encoding UT-A and UT-B proteins have been isolated from higher animals including rabbit (You et al., 1993), rat (Smith et al., 1995a), mouse (Fenton et al., 2000), human (Olives et al., 1994), shark (Smith & Wright, 1999), toadfish (Walsh et al., 2000) and frog (Couriaud et al., 1999).

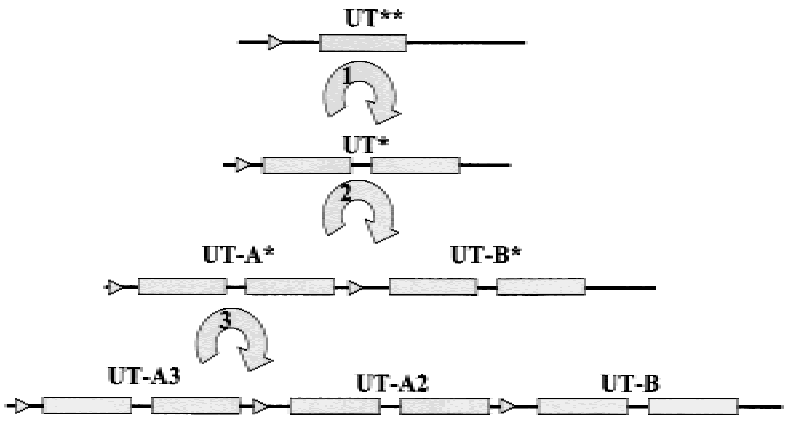

Fig. 1. Proposed sequence of duplication events leading to the formation of the UT-A and UT-B genes. Triangles represent promoters. Shaded blocks indicate groups of exons (not drawn to scale). The sequence begins with a primordial gene UT**. This is duplicated (1) to form a UT parent gene, UT*. Duplication (2) of UT* and subsequent divergence results in the formation of UT-A* and UT-B*. A further duplication (3) in the UT-A* gene, followed by some divergence in the duplicated cassettes results in the UT-A gene evident today.

The proteins they encode show a high degree of amino acid conservation. For example, human UT-A2 has >90% identity to rat UT-A2. Rat UT-A2 and rat UT-B1 share 75% amino acid identity. Shark and human urea transporters are the most distantly related sequences which have been identified; UT-A proteins from these organisms share 60% identity. The Table details the physical characteristics of rat UT cDNAs and Fig. 2 shows a schematic of the hypothetical open reading frames (ORF) for UT-A cDNAs.

Urea transporter proteins are structurally unique and constitute a closely related family of membrane proteins. They do not share significant sequence homology with any other family of proteins and have several distinctive structural characteristics. They contain a high proportion of hydrophobic residues organized into hydrophobic domains, which, it is suggested, span the plasma membrane. There are four of these domains in UT-A1, and two in all other urea transporters characterized to date (Fig. 2). The hydrophobic domains are joined by hydrophilic residues that are thought to project into the cytoplasm or extracellular space.

UT-A1 represents the largest member of the family. In rat it has a predicted ORF encoding a 929 amino acid protein (Table, Fig. 2 (Shayakul et al., 1996)). Contained within the UT-A1 coding sequence are cassettes that make up other UT-A splice variants called UT-A2, UT-A3, UT-A4 and UT-A5 (Fig. 2). UT-A1 is basically UT-A3 joined to UT-A2 by 73 mainly hydrophilic residues.

Urea transporters contain an above average number of proline residues. Proline residues are generally thought to disrupt secondary structure, and it is therefore not surprising that computer based structural prediction programs fail to agree on a putative structure for the UTs. Even such a fundamental question as whether the protein is comprised of alpha helices or beta sheets has not been established. Due to the unique characteristics of urea transporter proteins, the inadequacies of structural prediction algorithms, and the lack of direct empirically derived data, it is difficult to regard current topological models (You et al., 1993; Sands et al., 1997) as being representative of the native structure. The recent discovery of UT-A5 (Fenton et al., 2000), which topologically is a truncated form of UT-A3, would also refute these models because the primary sequence of UT-A5 is predicted to start at amino acid 139 of UT-A3 and therefore, based on the previously proposed models, UT-A5 lacks all or part of the first membrane spanning helix. In view of the lack of empirical data to support a defined topology for the UT proteins, in this review we have chosen simply for convenience to depict the basic repeating hydrophobic module as consisting of four membrane spanning alpha helices (Fig. 2).

The NH- and COOH-termini of urea transporter proteins are composed of hydrophilic residues and contain consensus motifs for PKA and/or PKC phosphorylation. The presence of these motifs has been taken as evidence that these regions project into the cytoplasm. Immunohistochemical studies using an antiserum raised against the COOH-terminal amino acids of UT-A1/UT-A2/UT-A4 predominantly labels the cytosolic side of renal inner medullary collecting duct (IMCD) membranes, adding weight to the notion that the COOH-terminus is intracellular (Nielsen et al., 1996).

Urea transporters are glycoproteins. This has been demonstrated using in vitro translation studies in the presence of pancreatic microsomes, or by deglycosylation of native proteins. The results of these experiments indicate that UT-A2 (Smith et al., 1995a), UT-B (Olives et al., 1995), UT-A3 and UT-A4 (Karakashian et al., 1999) are *N*-glycosylated at a single site, while UT-A1 is glycosylated at two sites (Shayakul et al., 1996). Computer analysis has been used to predict the site of these modifications, but experiments to verify these predictions are required. It would also be interesting to determine the effect glycosylation has on transporter function. Furthermore, it is not known whether other post-translational modifications, such as proteolytic cleavage, modify the UTs.

A repeated motif known as the “LP box” has been suggested to be a unique feature common amongst urea

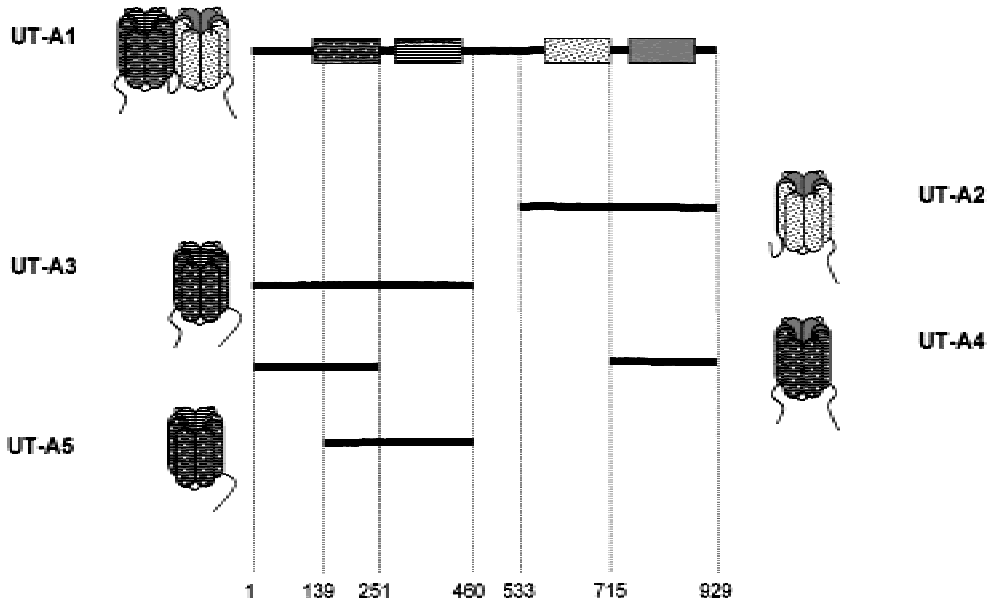

Fig. 2. The UT-A urea transporter family. Alternative splicing of the UT-A gene and alternative promoter activity results in at least five isoforms. Horizontal lines represent composition of protein relative to unfolded UT-A1 (*top*) showing the four hydrophobic domains (shaded boxes). Numbers refer to UT-A1 amino acids. The topologies of these models are hypothetical.

transporters (Rousselet et al., 1996). This eight amino-acid sequence (L/VPXXTXXF) occurs twice in UT-A2, UT-A3, UT-A4 and UT-A5 and four times in UT-A1. This sequence is also found in E-cadherin (Genbank Acc #Z35402), but the motif is not duplicated in these proteins. The structural or functional importance of the LP box remains to be determined.

Functional Characteristics of Urea Transporters

The gene products of both UT-A and UT-B are facilitative urea transporters and as such are unable to transport urea against a urea gradient. Movement of urea is not coupled to movement of Na^+ or Cl^-, nor is it electrogenic (You et al., 1993; Olives et al., 1994). Transport of urea is inhibited by millimolar concentrations of the aglycon phloretin and by some urea analogues (You et al., 1993). The kinetic variables K_m and V_{max} for cloned urea transporters have not been established. To some extent this is due to the technical difficulties associated with the high rate of transport these proteins exhibit. However, derivation of these values should be possible if a suitable expression system is found. Estimates based on urea fluxes across erythrocyte membranes have been determined. Values for maximum rates of transport in erythrocytes range from 0.8×10^{-7} mol $\cdot$ cm$^{-2} \cdot$ s^{-1} at 25°C (Brahm, 1983) to 2.5×10^{-7} mol $\cdot$ cm$^{-2} \cdot$ s^{-1} at 22–26°C (Mayrand & Levitt, 1983). Estimates of the number of copies of urea transporter molecules per red cell range from 14,000/cell (Masouredis et al., 1980) to <32,000/cell (Mannuzzu, Moronne, Macey, 1993). Averaging these estimates, and assuming the erythrocyte membrane area to be 137 μm^2 (Mayrand & Levitt, 1983), gives a value of 5×10^6 urea molecules per UT-B transporter per second. This is considerably higher than reported for other facilitated transporters which typically transport 10^2–10^3 molecules per second (Nishimura et al., 1993).

Using a sensitive ELISA method to measure the number of protein molecules in microdissected IMCDs in conjunction with the in vitro perfused renal tubule technique to measure corresponding urea fluxes, Kishore and coworkers (Kishore et al., 1997) calculated the maximum turnover number of IMCD UT-A proteins to be $\geqslant 0.3$–1×10^5 urea molecules per transporter per second. Compared to the average estimate for the turnover number of UT-B this is fifty-fold smaller. Whether this represents a true difference between UT-A and UT-B proteins is not clear. However, it must be taken into account that the antiserum used in the ELISA assay was raised to the COOH-terminus of UT-A1 and consequently would recognize any molecules possessing this epitope (UT-A1, UT-A2, and UT-A4). In addition, the assay probably could not differentiate between active and inactive forms of the protein. If these factors are considered, then the turnover number estimated by Kishore et al. is likely to be an underestimation of the true value. Nevertheless, it is higher than would be expected if urea was translocated in a classical carrier-mediated fashion. Thus it has

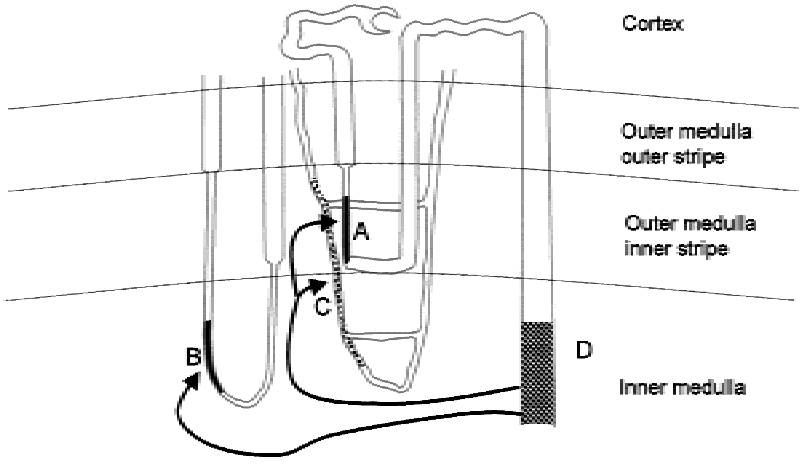

Fig. 3. Localization of urea transporters in mammalian nephron. UT-A2 is located in short type I (*A*) and long type III (*B*) thin descending limbs of the loop of Henle. UT-B is located in descending vasa recta (*C*). UT-A1 and UT-A3 are located in mid and terminal inner medullary collecting ducts (*D*). Arrows indicate routes of urea movement mediated by urea transporters. Urea is recycled by urea entering the nephron mediated by UTs at *A* and *B* and travels along the nephron until it re-enters the medullary interstitium in the IMCD via UTs (*D*).

been suggested that urea transporters may be mechanistically more like ion channels which typically handle millions of molecules per second (Kishore et al., 1997).

The question of whether urea transporters are water-filled pores, and thus transport water as well as urea, has been the topic of much debate. One study reported that rat UT-B1 contains a water pathway when expressed in *Xenopus* oocytes (Yang & Verkman, 1998), but subsequent findings have refuted this claim and ascribe the observation to overexpression of the protein (Sidoux-Walter et al., 1999). Human UT-B1 (Martial et al., 1996; Sidoux-Walter et al., 1999), rat UT-B2 (our unpublished observations) and rat UT-A2 when expressed in *Xenopus* oocytes do not increase plasma membrane water permeability when analyzed under conditions where urea is efficiently transported (Martial et al., 1996; Yang & Verkman, 1998). Therefore, the consensus is that urea transporters do not transport water under physiological conditions.

Tissue Distribution of UT Proteins

UT-A

In all mammalian species so far analyzed the kidney has been found to contain large amounts of UT-A mRNAs and proteins. The renal distribution of UT-A proteins has previously been reviewed in a number of articles (e.g., Trinh-Trang-Tan & Bankir, 1998; Sands, 2000); here we present an overview and update of this topic. The two most abundant transcripts in rat kidney are UT-A1 and UT-A2. The exact nephron location of these transcripts has been resolved using the reverse transcription-polymerase chain reaction technique (RT-PCR; Shayakul et al., 1997). Specific primer sets were used to detect the UT-A1 or UT-A2 mRNA in microdissected nephron segments. Under normal conditions (i.e., normal hydration and dietary protein) UT-A1 transcripts were detected in the most distal segments of the collecting duct (middle and terminal IMCD; Fig. 3). In contrast, UT-A2 mRNA was present in the outer medullary, short thin type I descending limbs of the loop of Henle and in inner medullary, long thin type III descending limbs of the loop of Henle (Fig. 3). Although the exact nephron location of these transcripts has not been determined in other species, northern analysis of isolated kidney regions has revealed some species differences in their distribution (You et al., 1993; Smith et al., 1995a).

In rat, UT-A3 is also present in the kidney inner medulla (Doran et al., 1998; Karakashian et al., 1999; Fenton et al., 2000), although the nephron location of this transcript remains to be determined. Recently, Terris and colleagues (Terris, Knepper & Wade, 2001) have reported results using an antiserum raised to the fourteen COOH-terminal amino acids of rat UT-A3. This antiserum, antiserum Q, recognized 44- and 67-kDa proteins in kidney inner medulla. Deglycosylation experiments using peptide: *N*-glycosidase F reduced these bands to a single band at 40 kDa, indicating that the 44 and 67 kDa proteins were UT-A3 in different glycosylation states. Interestingly, although thirteen out of the fourteen amino acids making up the immunizing peptide are common to UT-A1 and UT-A3, the antiserum did not detect UT-A1, as might be expected. The authors suggest that either the terminal amino acid of UT-A3, which is not present in UT-A1, may be the source of this ambiguity or that the epitope is somehow masked in UT-A1.

Using antiserum Q, Terris and colleagues (Terris, Knepper & Wade, 2001) localized UT-A3 to the cytosol of the middle and terminal segments of IMCD. They clearly showed that UT-A3 was not present in the plasma membrane, which implies that under normal circumstances this isoform is not involved in the transcellular movement of urea. However, it may represent a pool of urea transporters with the capacity to be inserted in the

plasma membrane, similar to what has been described for Aquaporin 2 (AQP2, Nielsen et al., 1995), to bring about a rapid increase in urea permeability.

Relatively little is known about UT-A4. UT-A4 mRNA has been detected in heart, however, northern analysis was unable to reveal its renal location (Doran et al., 1998). The failure to detect UT-A4 in kidney may reflect the very low abundance of this mRNA species, and effective detection may depend upon the use of more sensitive RT-PCR based methodologies like those used to isolate the first UT-A4 cDNA (Karakashian et al., 1999).

The most recent addition to the UT-A family, UT-A5, appears to be restricted to testes (Fenton et al., 2000). Northern analysis of several mouse tissues, including brain, heart, lung, liver, spleen, kidney, testes, epididymus, intestine, and skeletal muscle, revealed strong expression of the 1.5 kb UT-A5 mRNA only in testes. This suggests that UT-A5 may be unique amongst the UT-As in that it is not expressed in the kidney. In the testis UT-A5 has been localized by *in situ* hybridization to cells surrounding the seminiferous tubules. Although the subcellular location of the UT-A5 protein is not yet known, this isoform may mediate movement of urea into or out of the seminiferous tubules (Fenton et al., 2000). The rat equivalent of UT-A5 is probably the 1.7 kb transcript detected only in testis by Karakashian and coworkers (Karakashian et al., 1999).

Studies employing peptide-specific antisera targeted to parts of urea transporter proteins have proved very useful in discerning the nephron and cellular location of UT-A proteins. However, the results of these studies require careful interpretation, because several UT-A isoforms have common epitopes. To overcome this problem, Knepper and colleagues have championed the use of several polyclonal antisera raised to different parts of the UT-A1 protein. Antiserum L448 was raised to amino acids 500–522 of UT-A1 (Terris et al., 1998) and antiserum L194 to the COOH-terminal 19 amino acids of UT-A1 (Nielsen et al., 1996; Terris et al., 1998; Wade et al., 2000).

Western analysis using antiserum L448 or L194/L403 detects 97 kDa and 117 kDa bands in inner medulla (Terris et al., 1998) and both bands are thought to represent UT-A1. Studies to determine the glycosylation state of native urea transporters have shown that treatment of IMCD proteins with the deglycosylating agent *N*-glycosidase F reduces the 97 kDa and 117 kDa bands to a single 84 kDa band, indicating that they probably represent UT-A1 in different glycosylation states (Rouillard et al., 1998). Immunostaining using L194 labeled apical membranes and cytoplasm of IMCD principle cells (e.g., Nielsen et al., 1996).

Antiserum L194 also recognizes a 55 kDa protein on western blots of kidney medulla. When used for immunocytochemistry this antiserum detects proteins in type I and type III thin descending limbs of the loop of Henle (Wade et al., 2000). The 55 kDa protein is thought to be UT-A2 for a number of reasons. First, its size is approximately that expected for a 397 amino acid protein if glycosylation is taken into account. Second, it colocalizes with UT-A2 mRNAs in thin descending limbs (Shayakul et al., 1997). Finally, it is not recognized by an antiserum (L446) raised to amino acids 56-78 of rat UT-A1 (Wade et al., 2000).

Western analysis of kidney using UT-A antisera also reveals several other UT-A proteins, the molecular identity of which remains uncertain. For example, in addition to UT-A2 antiserum L194 recognizes a 46 kDa protein and a 33 kDa protein in kidney outer medulla. The 46 kDa protein is thought to represent UT-A4 and the 33 kDa protein UT-A2 in its unglycosylated form (Wade et al., 2000) although the authors highlight the need for further data to support these conjectures.

As mentioned above, urea transporter mRNAs and proteins have been discovered in some extra-renal tissues. UT-A transcripts are present in heart (Doran et al., 1998) and testis (Karakashian et al., 1999; Fenton et al., 2000). In addition, UT-A isoforms have been detected in colon (You et al., 1993) and liver (Klein et al., 1999). The possible physiological roles these transporters may play are discussed below.

UT-B

Northern analysis of both human and rat tissues has revealed that UT-B mRNA is widely distributed. UT-B transcripts have been detected in human heart, skeletal muscle, colon, small intestine (transcript sizes 2 kb and 3.6 kb), and in brain, thymus, prostate, liver and ovary (transcript sizes 2.0 kb and 4.5 kb) (Olives et al., 1996). In rat a similar distribution has been reported, except that only a 3.8 kb transcript was detected (Promeneur et al., 1996; Tsukaguchi et al., 1997). Because mammalian erythrocytes do not contain mRNA, it is not possible to perform northern analysis. However, UT-B transcripts have been detected in human spleen (2.5 kb and 4.7 kb, Olives et al., 1996) adult spleen erythroblasts (2.5 kb and 4.7 kb) and the erythroleukemic cell line HEL (2.0 kb and 4.3 kb, Olives et al., 1994). These are thought to reflect the expression of the UT-B gene in differentiating reticulocytes.

UT-B protein is strongly expressed in red blood cells (Olives et al., 1995). Western analysis of human erythrocytes using an antiserum targeting the NH_2-terminal amino acids (residues 16–31 of UT-B) labels a broad band between 46–60 kDa (Olives et al., 1995). Other groups have observed very similar results using an antiserum raised to the COOH-terminus (residues 373–391) of UT-B (Timmer et al., 1998a). Similarly, immunopre-

cipitation of red cell proteins using a UT-B antiserum yields a broad band of 46–60 kDa which is reduced to a single band of 36 kDa on treatment with *N*-glycanase (Olives et al., 1995). Together, these results suggest that a single isoform of UT-B is expressed in erythrocytes and it is subject to varying degrees of glycosylation.

The abundance of UT-B mRNA in brain has attracted some interest because the striking level of expression implies that this transporter may serve an important role. UT-B mRNA is strongly expressed in astrocytes throughout the brain (Couriaud, Ripoche & Rousselet, 1996; Promeneur et al., 1996; Tsukaguchi et al., 1997) and also in a subgroup of neurons in the midbrain (Berger, Tsukaguchi & Hediger, 1998). Urea is formed in the brain (Buniatian & Davtian, 1962) and although the physiological role of UT-B in this tissue remains to be established, these proteins may serve to dissipate urea gradients which would otherwise form.

UT-B is also expressed in kidney in the descending vasa recta (Promeneur et al., 1996; Tsukaguchi et al., 1997; Xu et al., 1997) which are the blood vessels that perfuse the kidney medulla. Because these vessels make up only a small fraction of the renal mass, UT-B mRNA represents a small proportion of the RNA in whole kidney homogenates, and northern analysis of whole kidney consequently reveals only a very faint signal corresponding to UT-B (Couriaud et al., 1996).

Western analysis of rat kidney using an antiserum directed to the COOH-terminal amino acids of UT-B detects a broad band between 41–54 kDa in kidney inner and outer medulla, but not in kidney cortex (Timmer et al., 1998a). Treatment with *N*-glycosidase F reduced this diffuse band to a single sharp band, and, as observed for UT-B expressed in erythrocytes, suggests that UT-B in kidney medulla is glycosylated to varying degrees. In both humans and rat, this signal represents UT-B expressed in endothelial cells forming the blood supply to the renal medulla (descending vasa recta, Figure 3, Promeneur et al., 1996; Tsukaguchi et al., 1997; Xu et al., 1997; Timmer et al., 1998a). Consistent with this localization, Pallone (1994) showed that urea movement across rat vasa recta was inhibited by phloretin.

In the testes UT-B mRNA has been detected in Sertoli cells associated with the early stages of spermatocyte development (Tsukaguchi et al., 1997). The cellular localization of UT-B in other tissues where it has been found has yet to be established.

The Kidd Antigen

The UT-B protein expressed in red blood cells has been shown to carry the Kidd blood group polymorphism (Olives et al., 1995). This polymorphism consists of 2 functional alleles (Jk^a and Jk^b), leading to three common phenotypes Jk(a+b−), Jk(a−b+), and Jk(a+b+) and a rare null phenotype Jk_{null}. It was first linked to urea transport when it was discovered that Jk_{null} erythrocytes do not lyse in 2 M urea solutions, leading to aberrant results in automated blood cell counters (Heaton & McKloughlin, 1982). Direct measurements of the membrane permeability of these cells revealed that they were considerably less permeable to urea and thiourea than erythrocytes from unaffected individuals, but displayed normal values for water permeability (Frohlich et al., 1991). Knowledge of the human UT-B gene has facilitated the identification of the molecular bases of the phenotypes (Lucien et al., 1998). The Jk^a or Jk^b alleles correspond to a single point mutation (G to A) at base pair 838 in UT-B1 leading to an Asp to Asn substitution at position 280 (Olives et al., 1997). The Jk_{null} phenotype is caused by mutations in UT-B splice sites, resulting in the absence of UT-B protein expression (Lucien et al., 1998). Individuals with a Jk_{null} phenotype are genetically devoid of functional UT-B protein. Despite this, they appear virtually asymptomatic. A disease phenotype is only evident when Jk_{null} individuals are challenged by thirsting, and this manifests as a weak urinary concentrating defect (Sands et al., 1992). This suggests that under normal circumstances UT-B is not essential for homeostasis, although further analysis is required to determine if compensatory changes occur. The fact that the absence of UT-B does not appear to disrupt homeostatic balance may not be true for all species, because interspecies differences in sensitivity to gene disruption or removal are evident in the case of other transporter proteins. For example, humans genetically lacking the water channel aquaporin 1 (AQP1) do not present a pathological phenotype (Preston et al., 1994), whereas AQP1 knock-out mice have a profound urinary concentrating defect (Ma et al., 1998). From this viewpoint, the engineering and analysis of UT-B-deficient mice will be of substantial interest and may reveal roles of UT-B that are hidden in humans due to compensatory mechanisms.

Molecular Physiology of Urea Transporters

Role in the Kidney

Acute control of urea permeability is an integral part of the urinary concentrating mechanism. In mammals, the differential water and urea permeability of collecting ducts, brought about by spatially distinct expression of transporter proteins, and coordinated increases in collecting duct permeability triggered by arginine vasopressin (AVP), are central to the urinary concentrating machinery. The main features of this mechanism are as follows. An increased level of AVP stimulates an increase in cortical collecting duct (CCD) water permeability, but not urea permeability. This causes water reabsorption

from the tubular fluid passing down the CCD. Urea is concentrated in the tubular lumen and a urea-rich solution flows into the IMCD. In the middle and terminal part of the IMCD, AVP activated UT proteins mediate movement of urea down its concentration gradient, across the tubular epithelium and out of the tubule into the medullary interstitium. This in turn generates a transepithelial osmotic gradient, which drives further water reabsorption through AQPs.

Movement of urea across the apical membrane of the IMCD is the rate-limiting step in the transcellular movement of urea into the medullary interstitium (Star, 1990). Within one minute of exposure to AVP the urea permeability of the apical membrane increases. This effect is mediated by V_2 vasopressin receptors coupled to cAMP (Grantham & Burg, 1966; Star et al., 1988) and lasts for 10 minutes. It is followed by a slower rate of increase spread over the next 50 minutes.

Vasopressin-stimulated trafficking of AQP2-bearing vesicles to the apical membrane mediates the accompanying increase in collecting duct water transport (Nielsen et al., 1995). The increased IMCD urea permeability is the result of an increase in the number of functional transporting UTs (i.e., increase in V_{max}) in the apical membrane (Chou & Knepper, 1989). Although UT-A1 is present in intracellular vesicles (Nielsen et al., 1996), the amount of UT-A1 protein in the apical membrane does not increase in response to AVP (Inoue et al., 1999). Therefore, the observed increase in urea permeability in the IMCD is not due to shuttling of UT-A1 to the apical membrane. Urea transport by UT-A1 expressed in *Xenopus* oocytes is increased after treatment with cAMP (Shayakul et al., 1996) indicating that the AVP-induced increase in IMCD urea permeability is probably due at least in part to a cAMP-dependent change in activity of UT-A1 proteins already residing in the apical membrane (Inoue et al., 1999). Alternatively or additionally, it could be due to shuttling to the apical membrane of another UT isoform which was not detected by the antiserum used in the above experiment, but which is expressed in the IMCD, for example UT-A3 (Terris et al., 2001).

The above mechanism of activation probably explains the initial rapid increase in IMCD urea permeability, but is it responsible for the second phase of the response? Although this requires testing, it is possible that two complementary mechanisms may be operating: an acute direct activation of dormant urea transporters and a gradual recruitment of transporter proteins to the apical membrane from intracellular pools. If this hypothesis is true, then it would follow that UTs sequestered in intracellular vesicles may be involved in the second phase of the AVP-induced response.

Urea permeability in the IMCD is also acutely stimulated by hyperosmolality. Exposing IMCD to increased tonicity by addition of NaCl, mannitol or raffinose, but not urea, brings about an increase in the V_{max}, but not the K_m, of urea transport, leading to an acute increase in urea permeability (Gillin & Sands, 1992). Vasopressin and hyperosmolality stimulate urea permeability via different intracellular mechanisms because the hyperosmotic effect is independent of activators of adenylate cyclase (Gillin, Star & Sands, 1993). In addition, the two responses are additive and stimulation by one does not negate stimulation by the other. Interestingly, stimulation of IMCD cells by hyperosmolality induces an increase in intracellular calcium suggesting that this second messenger may also modulate UT function.

We have focused on the collecting ducts, but UT-A2 is also expressed in the thin descending limbs of Henle and plays an important role in urinary concentration. It is thought that these transporters are involved in renal urea recycling (*see* Fig. 3) by restricting loss of urea from the medulla. In addition, UT-B expressed in the descending vasa recta is suggested to contain the corticomedullary urea gradient by driving urea, which would otherwise diffuse throughout the cortex, into the inner medulla.

UT-B expressed in erythrocytes is also thought to augment this action by preventing dissipation of the cortico-medullary urea gradient because erythrocytes that traverse the kidney medulla would otherwise tend to carry urea out of the medulla and into the general circulation. This feature is favored by the fact that UT-B has asymmetric transport properties, which can be likened to rectification in ion channels (Levitt & Mlekoday, 1983). The affinity for transport out of erythrocytes is more or less double that for movement into these cells. The observation that Jk_{null} individuals actually present a mild urinary concentrating defect (Sands et al., 1992) supports this role. It has also been suggested that UT-B expressed in erythrocytes prevents cells from detrimental shrinkage and swelling as they traverse the kidney medulla (Macey & Yousef, 1988).

We have only addressed what can be termed short-term, acute changes in UT function. Like the majority of other proteins, the amount of UT mRNA and protein are regulated in response to longer term alterations in homeostasis. Studies looking at sustained antidiuresis induced over several days by restricting water or infusing AVP, have revealed that UT mRNA and protein levels are modulated. Thirsting for 1 to 3 days causes heterogeneous changes in urea permeability in rat IMCD subsegments (Kato et al., 1998). Thirsting for 3 days induces an increase in UT-A2 mRNA (Smith et al., 1995a; Promeneur et al., 1996) and protein in inner and outer medulla thin descending limbs (Wade et al., 2000). Thirsting has no effect on UT-A2 mRNA levels in Brattleboro rats, which congenitally lack AVP, whereas chronic AVP treatment induces a large increase in

UT-A2 mRNA. Both of these observations suggest that AVP is necessary for the response to thirsting (Smith et al., 1995b; Shayakul et al., 2000).

Receptors with the pharmacological characteristics of vasopressin V_{1a} receptors have been detected in the thin descending limbs of short loops and these may mediate modulation of UT-A2 levels (Arpin-Bott et al., 1999). However, to date no vasopressin receptors have been detected in long-looped nephrons. In these segments an indirect mechanism for modulating UT-A2 levels has been proposed. It has been suggested that increased medullary NaCl and urea content brought about by the countercurrent exchanger act as stimuli for increased UT-A expression. This hypothesis was demonstrated in vitro and in vivo (Leroy et al., 2000). Interestingly, the NaCl and urea appear to act synergistically in this regulation. In mouse and rat the UT-Aβ promoter contains the consensus sequence for osmotic response elements, raising the possibility that modulation of UT-A gene transcription by NaCl may be involved (Ferraris et al., 1996; Fenton et al., 1999b; Nakayama et al., 1999). The effect of urea would appear different from that of NaCl and could reside in post-transcriptional events that remain to be identified.

Thirsting or treatment with AVP also causes a small reduction in the amount of UT-A1 mRNA in the inner medulla (Smith et al., 1995a, 1995b; Shayakul et al., 2000). These treatments have no detectable effect on expression of the 97 kDa UT-A protein in Brattleboro or normal rats, but treatment with AVP reduces the amount of the 117 kDa UT-A protein in inner medulla (Terris et al., 1998). It has not been established whether the reduction in UT-A1 mRNA causes this decrease in UT protein, or what the physiological significance of the response is.

Like thirsting, diuresis has been shown to affect urea permeability and UT-A mRNA and protein expression in a nephron segment-dependent manner (Smith et al., 1995a; Kato & Sands, 1998a; Terris et al., 1998). Interestingly, furosemide-induced diuresis has been found to stimulate a sodium-urea antiporter in the initial IMCD and to inhibit active urea secretion into the terminal IMCD (Kato & Sands, 1998a; Kato et al., 1998).

In addition to being responsive to hydration state, urea transporter expression in the kidney is also sensitive to the amount of protein consumed in the diet. It is well established that a reduced protein diet causes changes in renal urea handling, with the net result that urea reabsorption increases (Schmidt-Nielsen, 1958; Peil, Stolte & Schmidt-Nielsen, 1990). The IMCD is regarded as the primary nephron site of these adaptive changes. Several studies have measured IMCD urea permeability in response to a low protein diet (LPD) and documented increases in urea permeability that differ depending on the duration of LPD (Isozaki, Verlander & Sands, 1993; Ashkar et al., 1995; Isozaki et al., 1995). These alterations are accompanied by time-dependent fluctuations in urea transporter expression that are exemplified by the following observations: One week after rats were changed from a diet containing 18% protein (normal diet) to a LPD containing 8% protein, the amount of UT-A2 mRNA in the initial part of the inner medulla (neighbouring the inner stripe of the outer medulla) increased. The amount of UT-A1 mRNA was unchanged (Isozaki et al., 1995), and there was no increase in UT-A1 protein in the IMCD (Kishore et al., 1997). The increase in UT-A2 mRNA presumably occurs in the thin descending limbs of the loops of Henle. AVP is implicated in the response, because UT-A2 mRNA levels did not increase in Brattleboro rats treated in the same fashion. Instead, this strain showed a decrease in UT-A1 mRNA in the inner medullary tip (Hu, Bankir & Trinh-Trang-Tan, 1999). After two weeks of LPD, the level of UT-A2 mRNA in AVP-replete normal rats remains elevated in the initial inner medulla. There is also an increase in AVP-sensitive urea transport in the initial IMCD (Isozaki et al., 1995), which is suggestive of the activation and/or increase in expression of UT-A1. However, there is no discernible increase in the amount of UT-A1 mRNA in the initial inner medulla (Isozaki et al., 1995) or UT-A protein in the IMCD (Kishore et al., 1997). Therefore, the increase in permeability is due to one of the following: (i) activation of UT-A1 which is already present, but below the limits of detection of standard northern and western blotting; (ii) another UT-A isoform such as UT-A3, or (iii) a novel UT protein. After four weeks of exposure to LPD, UT-A2 mRNA levels are no different from control levels. However, UT-A1 mRNA levels are greatly increased (Smith et al., 1995a). Corroborating this finding, Terris et al. (1998) reported that feeding rats a 4% protein diet for three weeks causes an increase in the 117 kD UT-A1 protein in the renal papilla.

These changes underlie alterations in renal function that are assumed to reduce the amount of urea excreted and maintain plasma urea concentrations. Why this should be necessary is open to speculation. Perhaps urea is conserved in the event that it is required for urinary concentration should water become limiting. An alternative suggestion is that animals on a low protein diet are effectively consuming less nitrogen, and that one way of conserving nitrogen is to conserve urea. This would then explain why urea transporters are expressed in the large intestine. In colon, UTs are thought to play a role in nitrogen salvaging (Meakins & Jackson, 1996). Urea circulating in the blood diffuses into the colon and is hydrolyzed by resident microfloral urease, liberating CO_2 and ammonia. The nitrogen moieties are then utilized by both the microflora and host to synthesize amino acids. In this way the urea nitrogen is recycled. Know-

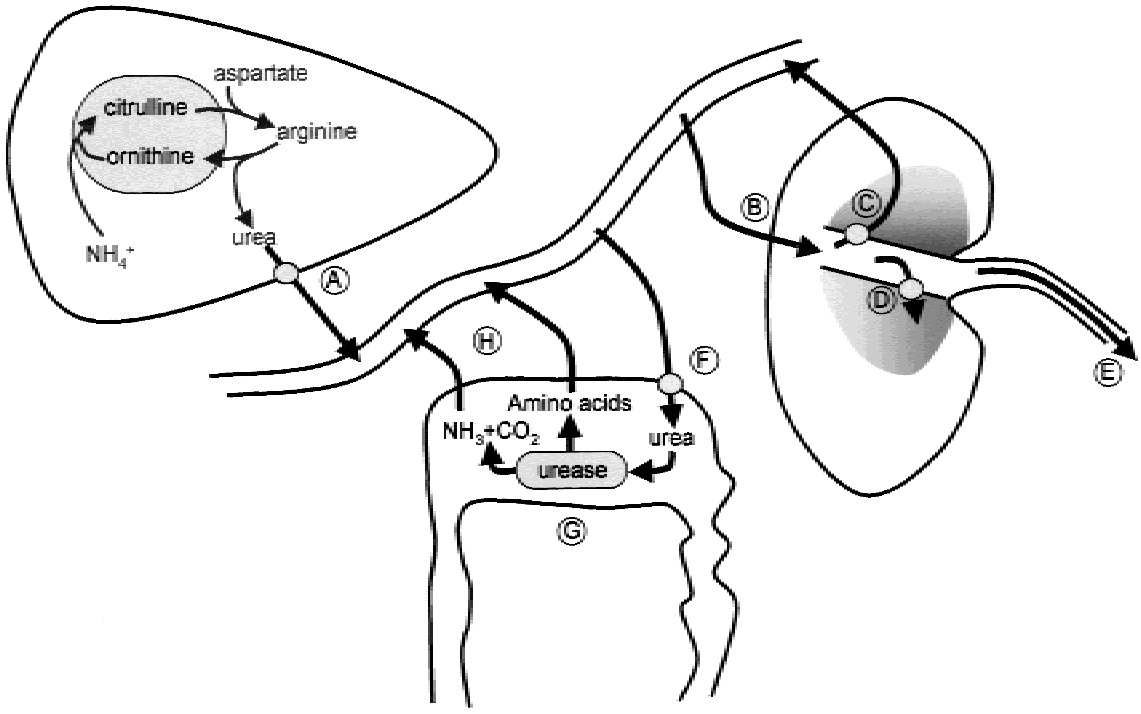

Fig. 4. Routes of urea movement in mammals. Urea is the major end-product of protein catabolism and plays several important roles in body homeostasis. It is synthesized almost exclusively in the liver by the ornithine-urea cycle, passes out into the circulation by a carrier-mediated process (*A*) and circulates in human plasma at concentrations between 4–10 mmol l^{-1}. It is freely filtered by the kidney (*B*) and depending on a person's nutritional and diuretic state, between 50% and 70% of the urea filtered at the glomerulus is reabsorbed (*C*). Some urea contributes to the hypertonic renal medulla which is central to water reabsorption (*D*). The remainder passes out in the urine (*E*). Urea also diffuses into the colon (*F*) by what is thought to be a urea transporter-mediated process. It is utilized by the resident microflora (*G*) which breaks it down into CO_2 and NH_3. The latter being used for synthesis of amino acids. There is evidence to support reabsorption and utilization of this urea nitrogen by the host (*H*).

ing the cellular location of the UT proteins in the large intestine would be beneficial (Fig. 4).

Other factors have been found to affect renal urea handling and/or UT expression. For example, glucocorticoids increase fractional urea excretion (Knepper et al., 1975), and, conversely, adrenalectomy reduces urea excretion and increases urea permeability in the terminal IMCD (Naruse et al., 1997). These responses are thought to involve modulation of UT-A1 protein levels. Glucagon significantly increases the fractional excretion of urea in rats (Ahloulay et al., 1992). The mechanism of action mediating this effect does not appear to involve changes in IMCD urea permeability, but modulation of urea transporter function in other nephron segments has not been investigated (Isozaki et al., 1995). Several other factors have been found to affect urea handling by the kidney (*see* Sands, 2000).

Other Tissues

The role UTs play in other tissue is very much open to speculation. Because urea is synthesized in the liver, it is not surprising that UT-A and UT-B are present in this tissue. Although it remains to be determined which cells they are expressed in, it can be speculated that urea transporters allow urea to pass from the liver into the circulation (Klein et al., 1999). Interestingly, a cDNA encoding a novel AQP water channel, AQP 9, has recently been found in liver (Tsukaguchi et al., 1999). This protein transports water, urea and other solutes and it may contribute to urea transport in this organ, but until the cellular location of this protein and the UT proteins are known it is difficult to formulate models of hepatic urea handling.

In rat testis, Tsukaguchi et al. (1997) showed that UT-B expressed in Sertoli cells was upregulated in the early stages of spermatocyte development. This suggests that UT-B expression may be linked in some way to spermatocyte maturation.

UT-B is also expressed in bone. In cultured human trabecular bone explants UT-B is downregulated during the switch to adipogenesis (Prichett et al., 2000). This is significant, because the decrease in bone volume associated with osteoporosis and age-related osteopenia is accompanied by increased bone marrow adipose tissue for-

mation. The authors suggest that UT-B gene expression may therefore be a marker of adipogenesis in osteoblasts (Prichett et al., 2000).

The identification of UTs in tissues not previously associated with urea metabolism such as testis, bone, heart and brain, has fueled speculation as to possible alternative roles for these proteins other than urea transport. A provocative hypothesis might be drawn from the independent cloning of human UT-B as a gene complementing a fission yeast (*Schizosaccharomyces pombe*) cell cycle checkpoint mutation (Davey & Beach, 1995). The authors screened a human glioblastoma library for genes which could complement the *rad1-1* mutation. Through this screen, Davey & Beach isolated a gene called RACH2, which is identical to UT-B. The UT-B protein was able to restore the delay in mitotic entry after UV irradiation that is lost in the mutant. Curiously, when over-expressed in a human cell line, UT-B induced apoptosis (Davey & Beach, 1995). It is difficult to explain these results strictly in terms of urea transport by UT-B, which suggests that UT-B may have a distinct function that is independent of urea transport.

Active Urea Transport

The concept of ion-coupled urea transporters capable of moving urea against a concentration gradient is not new (Smith, 1931; Schmidt-Nielsen, 1958). However, compelling evidence indicating their presence in mammals has only recently come to light. Here we present only a brief overview; for a more in-depth treatise readers should refer to the recent review by Sands (2000). Two studies have shown that rats fed an 8% protein diet for three to four weeks express a Na^+-dependent urea cotransporter in the initial IMCD (Isozaki et al., 1994; Sands, Martial & Isozaki, 1996). This transport activity is not inhibited by phloretin and is not sensitive to AVP. In contrast, feeding rats a diet consisting of 10% glucose in water causes diuresis and induces expression of a Na^+-urea antiporter in the terminal IMCD (Kato & Sands, 1998b). This transport activity is inhibited by phloretin and stimulated by AVP (Kato & Sands, 1998b). Interestingly, this activity is inhibited in rats treated with the diuretic furosemide, but induced in the basolateral membrane of the initial part of the IMCD adjacent to the outer medulla (Kato & Sands, 1998a). Although the physiological significance of these proposed active urea transporters has yet to be confirmed, they are potentially of great importance to renal urea handling. As for the facilitated urea transporters, there is no doubt that knowledge of the molecular structures of these proteins will be of great benefit in the quest to understand the role they play in both physiology and pathophysiology.

Conclusion

Over the past decade, the application of molecular biology to the study of urea transport has revolutionized the field. The cloning of urea transporter cDNAs and the subsequent generation of reagents, including specific nucleotide probes and antisera, have led to significant advances in our understanding of the molecular aspects of urea transporters. There are, however, still some large gaps in our knowledge. For example, the functional and kinetic characteristics of UT proteins and how these relate to the structure of the UT isoforms is an area that needs attention.

Molecular data alone are also insufficient to explain the role played by urea transporters in the diverse array of tissues where they are now known to be present. In the kidney, although a picture is emerging of differential expression of UT-A and UT-B, it is currently not possible to directly relate changes in urea permeability at the level of the nephron to altered expression of UT proteins. This is because most studies have looked at global changes in either mRNA or protein levels. A more refined approach is required which measures the change in expression in individual nephron subsegments and correlates this to changes in urea permeability.

Localization and physiological experiments are also required to understand the role of urea transporters in tissues that were not previously associated with urea metabolism, such as testes, bone, brain and heart. In the case of UT-A this will demand immunostaining with a battery of antisera chosen so as to differentiate between the numerous UT-A isoforms. With regard to UT-B, knowledge of the nucleotide sequence and proteins encoded by the various transcripts is required, followed by the development of transcript-specific probes and, if novel UT-B proteins are discovered, isoform-specific antisera. In addition, incorporation of data from *in situ* hybridization and RT-PCR studies will be of benefit. It is only through an integrated approach incorporating an array of molecular, biochemical and physiological techniques that future breakthroughs in this important field will be made.

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