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The renal interstitial matrix as a dynamic osmolyte reservoir: synthesis of classical hyaluronidase-glycosaminoglycan theory with contemporary aquaporin biology. A Comprehensive Narrative Review

Інтерстиціальний матрикс нирок як динамічний резервуар осмолітів: синтез класичної гіалуронідазно-глікозаміногліканової теорії з сучасною біологією аквапоринів. Комплексний наративний огляд

Prof. Anatoliy I. Gozhenko*, MD, PhD, DSc, Ukrainian Scientific Research Institute for Medicine of Transport, Odesa, Ukraine
Walery Zukow, MD, PhD, DSc, Assoc. Prof., Nicolaus Copernicus University, Toruń, Poland
Prof. Olena A. Gozhenko, MD, PhD, DSc, Ukrainian Scientific Research Institute for Medicine of Transport, Odesa, Ukraine
Prof. Dmytro D. Ivanov, MD, PhD, DSc, Bogomolets National Medical University, Kyiv, Ukraine

Anatoliy I. Gozhenko, Ukrainian Scientific Research Institute for Medicine of Transport, Odesa, Ukraine
*Corresponding author: mail: prof.gozhenko@gmail.com
ORCID: https://orcid.org/0000-0001-7413-4173
Walery Zukow, Nicolaus Copernicus University, Toruń, Poland
mail: w.zukow@wp.pl
ORCID: https://orcid.org/0000-0002-7675-6117
*Member of the Scientific Board
Olena A. Gozhenko, Ukrainian Scientific Research Institute for Medicine of Transport, Odesa, Ukraine
mail: olena.gozhenko@gmail.com
ORCID: https://orcid.org/0000-0002-4071-1304
Dmytro D. Ivanov, Bogomolets National Medical University, Kyiv, Ukraine
mail: ivanovdd@ukr.net
ORCID: https://orcid.org/0000-0003-2609-0051

Abstract

The mammalian kidney possesses a remarkable capacity to concentrate urine to osmolalities approximately four times that of plasma, representing a critical evolutionary adaptation for terrestrial existence. Contemporary nephrology has focused predominantly on epithelial transport mechanisms, particularly the aquaporin water channels discovered by Agre and colleagues, yet the mechanisms ensuring stability of the medullary osmotic gradient in the face of continuous vascular washout remain incompletely elucidated. This comprehensive narrative review synthesizes two historically disparate research traditions into an integrated conceptual framework.

The first tradition, developed by Soviet physiologists including Natochin and Ivanova during the 1960s through 1980s, proposed that the interstitial matrix rich in hyaluronan functions as a dynamic reservoir for osmolytes including sodium, chloride, and urea, with vasopressin activating hyaluronidases to release these bound solutes. The second tradition, emerging from the molecular revolution of the 1990s, established that vasopressin regulates collecting duct water permeability through trafficking of aquaporin-2 water channels.

We propose that vasopressin acts through two synergistic pathways: a rapid epithelial pathway involving aquaporin-2 translocation within five to fifteen minutes, and a slower matrix pathway involving hyaluronidase activation and osmolyte release over thirty to ninety minutes. Classical studies demonstrated that hyaluronidase inhibition reduces concentrating capacity by approximately forty percent, findings corroborated by the landmark study of Rowen and Law (1981) showing that antiserum against hyaluronidase blocked forty-three percent of vasopressin-induced water transport. Contemporary molecular evidence from conditional HAS2-knockout mice confirms comparable reductions in concentrating capacity without affecting aquaporin expression.

Biophysical analysis based on Manning's counterion condensation theory provides mechanistic explanation for sodium binding to the polyanionic hyaluronan matrix. Clinical implications include understanding concentrating

defects in chronic kidney disease as consequences of fibrotic matrix replacement, age-related decline as reflecting decreased hyaluronan synthase expression, and diabetic nephropathy as a biphasic process of initial hyaluronan accumulation followed by fibrotic depletion.

Keywords: hyaluronan, aquaporin-2, urine concentration, medullary interstitium, vasopressin, hyaluronidase, chronic kidney disease, counterion condensation, TonEBP/NFAT5, renal physiology, glycosaminoglycans, extracellular matrix

Abbreviations

Abbreviation	Full Term
AQP1	Aquaporin-1
AQP2	Aquaporin-2
AQP3	Aquaporin-3
AQP4	Aquaporin-4
cAMP	Cyclic adenosine monophosphate
CKD	Chronic kidney disease
CI	Confidence interval
ECM	Extracellular matrix
eGFR	Estimated glomerular filtration rate
HA	Hyaluronan (hyaluronic acid)
GAG	Glycosaminoglycan
HAS	Hyaluronan synthase
HAS1	Hyaluronan synthase 1
HAS2	Hyaluronan synthase 2
HAS3	Hyaluronan synthase 3
HYAL1	Hyaluronidase 1
HYAL2	Hyaluronidase 2
GPI	Glycosylphosphatidylinositol
HR	Hazard ratio
KO	Knockout
mRNA	Messenger ribonucleic acid
NFAT5	Nuclear factor of activated T-cells 5
NRI	Net reclassification improvement
PKA	Protein kinase A
TonEBP	Tonicity-responsive enhancer binding protein
ESRD	End-stage renal disease
UUO	Unilateral ureteral obstruction
V2R	Vasopressin V2 receptor

Key Terms and Definitions

Countercurrent multiplication system — The anatomical configuration of the loop of Henle and vasa recta that enables creation and maintenance of the osmotic gradient in the renal medulla through active solute transport in the ascending limb and passive water reabsorption in the descending limb.

Counterion condensation — A physicochemical phenomenon wherein cations become territorially bound around a highly charged polyanion when the electrostatic interaction energy exceeds thermal energy, as described by Manning's theory.

Hyaluronan depolymerization — Enzymatic cleavage of high-molecular-weight hyaluronan (>2 MDa) to intermediate fragments (~20 kDa) by hyaluronidases, altering the physicochemical properties of the matrix.

Hydraulic permeability — A measure of the ease with which fluid can flow through a porous medium, defined by Darcy's law; a critical parameter for understanding osmolyte washout from the medullary interstitium.

Osmotic driving force — The difference in osmolality between two compartments that drives osmotic water flux across a semipermeable membrane, described by the equation: $Jw = Lp \times \Delta\pi$

Tubulointerstitial fibrosis — A pathological process wherein normal extracellular matrix is replaced by excessive collagen and other fibrotic proteins, leading to loss of renal function.

1. Introduction

1.1 The Physiological Challenge of Urine Concentration

The capacity of mammalian kidneys to produce urine ranging from extremely dilute to highly concentrated represents one of the most remarkable physiological adaptations enabling terrestrial life. This concentrating ability, which allows mammals to conserve water during periods of limited intake while excreting metabolic wastes,

depends upon the establishment and maintenance of a corticomedullary osmotic gradient within the renal medulla. The gradient increases progressively from approximately 300 mOsm/kg at the corticomedullary junction, equivalent to plasma osmolality, to 1200 mOsm/kg or higher at the papillary tip in humans, with some desert-adapted species achieving concentrations exceeding 5000 mOsm/kg (Bankir & de Rouffignac, 1985). The kangaroo rat, for example, can survive indefinitely without drinking water by producing urine concentrated to approximately 5500 mOsm/kg, extracting sufficient water from metabolic oxidation of dry seeds to maintain fluid balance (Schmidt-Nielsen, 1964).

The fundamental importance of concentrating ability for human health becomes apparent when this mechanism fails. Patients with diabetes insipidus, whether central (deficient vasopressin secretion) or nephrogenic (renal resistance to vasopressin), may excrete 15-20 liters of dilute urine daily, requiring constant fluid intake to prevent life-threatening dehydration (Sands & Bichet, 2006). More commonly, the gradual decline in concentrating ability that accompanies chronic kidney disease contributes to nocturia, polyuria, and susceptibility to volume depletion that significantly impacts quality of life and clinical outcomes (Rowe et al., 1976). Understanding the mechanisms that establish and maintain the medullary osmotic gradient is therefore essential for both basic physiology and clinical medicine.

1.2 Current Understanding: The Countercurrent System and Aquaporins

Contemporary understanding of renal concentrating mechanisms rests on two foundational concepts. The first is the countercurrent multiplication hypothesis, proposed by Kuhn and Ryffel (1942) and experimentally validated by Wirz et al. (1951), which explains how the hairpin configuration of the loop of Henle, combined with active sodium chloride transport in the water-impermeable thick ascending limb, generates the corticomedullary osmotic gradient. The molecular basis of this "single effect" was established with the identification of the Na-K-2Cl cotransporter NKCC2 (encoded by SLC12A1) in the thick ascending limb, mutations in which cause Bartter syndrome type 1 with characteristic salt wasting and concentrating defects (Simon et al., 1996).

The second foundational concept emerged from the discovery of aquaporin water channels by Preston and Agre (1991), work recognized with the 2003 Nobel Prize in Chemistry. The identification of aquaporin-2 (AQP2) in the renal collecting duct by Fushimi et al. (1993) and the demonstration by Nielsen et al. (1995) that vasopressin regulates AQP2 trafficking between intracellular vesicles and the apical plasma membrane provided the molecular explanation for vasopressin-regulated water permeability that had been sought for decades. Phosphorylation of AQP2 at serine 256 by protein kinase A, activated through the V2 receptor-cAMP signaling cascade, triggers exocytic insertion of AQP2 into the apical membrane, dramatically increasing water permeability and enabling osmotic equilibration between tubular fluid and the hypertonic medullary interstitium (Knepper et al., 2015).

1.3 The Unresolved Problem: Gradient Stability

Despite these advances, a fundamental question has received insufficient attention: how is the medullary osmotic gradient maintained in the face of continuous osmolyte washout by medullary blood flow? The vasa recta, the specialized capillary network supplying the medulla, are arranged in a countercurrent configuration that minimizes gradient dissipation, yet they nonetheless remove substantial quantities of solutes from the medullary interstitium (Pallone et al., 2003). Mathematical models of the concentrating mechanism consistently demonstrate that active solute transport by the thick ascending limb alone cannot fully account for gradient stability under physiological conditions of medullary blood flow (Layton & Layton, 2005). This discrepancy between theoretical predictions and observed gradient stability suggests the existence of additional mechanisms that have not been incorporated into current models.

The problem becomes particularly acute during sustained antidiuresis, when large volumes of water are reabsorbed from the collecting duct into the medullary interstitium. This water reabsorption dilutes the peri-tubular interstitium, reducing the local osmotic gradient and potentially compromising further concentration. Yet the kidney maintains stable concentrating ability over hours of antidiuresis, implying mechanisms that replenish the gradient as fast as it is dissipated.

1.4 The Overlooked Matrix Pathway

A parallel body of research, developed primarily by Soviet physiologists between the 1960s and 1980s but largely overlooked in Western literature due to language barriers and the subsequent dominance of molecular approaches, proposed that the interstitial matrix functions as a dynamic reservoir for osmolytes. According to this hyaluronidase-glycosaminoglycan theory, hyaluronan and associated glycosaminoglycans in the medullary interstitium electrostatically bind cations (primarily sodium) and trap urea within their hydration domains, with vasopressin activating interstitial hyaluronidases to release these bound osmolytes and replenish the gradient (Natochin, 1994).

The pioneering work of Yuri Natochin, Lyudmila Ivanova, and their colleagues at the I.M. Sechenov Institute of Evolutionary Physiology and Biochemistry in Leningrad (now St. Petersburg) provided extensive experimental support for this concept. Their studies demonstrated that pharmacological inhibition of hyaluronidase reduces concentrating capacity by 35-45%, that exogenous hyaluronidase administration transiently enhances concentration, and that the time course of vasopressin-induced concentration exhibits biphasic kinetics consistent with sequential epithelial and matrix contributions (Ivanova & Natochin, 1972; Ivanova, 1985). These findings,

published primarily in Russian-language journals, have remained largely unknown to the international nephrology community.

1.5 Purpose and Scope of This Review

This comprehensive narrative review aims to synthesize these historically disparate research traditions—classical hyaluronidase-glycosaminoglycan theory and contemporary aquaporin biology—into an integrated conceptual framework. We propose that vasopressin regulates urine concentration through two synergistic pathways operating on complementary timescales:

The rapid epithelial pathway (5-15 minutes) involves the well-characterized cascade of V2 receptor activation, cAMP generation, protein kinase A activation, AQP2 phosphorylation, and translocation of AQP2 to the apical membrane of collecting duct principal cells, increasing transcellular water permeability.

The slower matrix pathway (30-90 minutes) involves vasopressin-induced activation of hyaluronidases, particularly HYAL2, through protein kinase A-mediated phosphorylation. Activated hyaluronidases cleave high-molecular-weight hyaluronan to smaller fragments, reducing the charge density of the polymer and releasing electrostatically bound sodium ions and trapped urea. These released osmolytes replenish the medullary gradient, sustaining the driving force for water reabsorption despite continuous washout.

We present evidence from classical physiological studies, contemporary molecular genetics, and biophysical theory to support this dual pathway model, and discuss its implications for understanding and treating disorders of water balance. The integration of these complementary perspectives offers a more complete understanding of renal concentrating mechanisms than either tradition provides alone.

2. Historical Foundations of Renal Concentrating Mechanisms

2.1 Early Recognition of Variable Urine Concentration

The recognition that mammalian kidneys can produce urine of variable concentration dates to the nineteenth century, when physiologists observed that urine osmolality varies with hydration status and dietary intake. However, the mechanisms underlying this variability remained mysterious until the mid-twentieth century. The kidney was known to filter large volumes of plasma at the glomerulus and reabsorb most of the filtrate along the tubules, but how the final urine could achieve concentrations far exceeding plasma was unclear.

Claude Bernard, in his concept of the milieu intérieur, emphasized the importance of maintaining constant internal conditions despite variable external circumstances (Bernard, 1878). The kidney's ability to vary urine concentration while maintaining plasma osmolality within narrow limits exemplifies this homeostatic principle. Yet the mechanisms enabling this regulation remained to be discovered.

2.2 The Countercurrent Multiplication Hypothesis

The conceptual breakthrough came with the countercurrent multiplication hypothesis, proposed independently by Werner Kuhn and colleagues in Switzerland. Kuhn, a physical chemist, recognized that the hairpin configuration of the loop of Henle resembled industrial countercurrent exchangers used for heat transfer and chemical separation. He proposed that this configuration, combined with active solute transport, could generate concentration gradients far exceeding what simple transport could achieve (Kuhn & Ryffel, 1942).

The principle of countercurrent multiplication can be understood through a simple thought experiment. Consider a tube bent into a hairpin, with fluid flowing down one limb and up the other. If a small concentration difference (the "single effect") is established between the two limbs at each level by active transport, the countercurrent flow arrangement multiplies this difference along the length of the tube, generating a large gradient between the bend and the open ends. Kuhn and Ryffel (1942) provided mathematical analysis demonstrating that multiplication factors of 10-fold or greater were theoretically achievable with modest single effects.

Hargitay and Kuhn (1951) extended this analysis specifically to the kidney, proposing that the loop of Henle functions as a countercurrent multiplier with active sodium chloride transport in the ascending limb providing the single effect. The water-impermeable ascending limb would become progressively more dilute as solute is removed, while the descending limb would equilibrate with the increasingly concentrated interstitium, delivering concentrated fluid to the bend of the loop.

2.3 Experimental Validation of the Countercurrent System

Experimental validation of the countercurrent hypothesis came from micropuncture and cryoscopic studies. Wirz et al. (1951) used freezing point depression to measure osmolality in different regions of the kidney, demonstrating that medullary tissue osmolality increases progressively from cortex to papilla, reaching values several times that of plasma. This corticomedullary gradient, predicted by the countercurrent hypothesis, provided strong support for the theory.

Gottschalk and Mylle (1959) extended these observations using micropuncture techniques to sample tubular fluid at different sites along the nephron. They demonstrated that fluid in the descending limb of the loop of Henle becomes progressively more concentrated as it descends into the medulla, while fluid in the ascending limb becomes progressively more dilute as it ascends toward the cortex. Fluid entering the distal tubule is hypotonic to plasma, having been diluted by solute removal in the ascending limb. In the presence of antidiuretic hormone, this

dilute fluid equilibrates with the hypertonic medullary interstitium as it passes through the collecting duct, producing concentrated urine.

These studies established the anatomical and functional basis of the countercurrent system but left open the molecular mechanisms of solute transport and water permeability regulation.

2.4 Discovery of Antidiuretic Hormone and Its Mechanism

The role of posterior pituitary extracts in regulating urine concentration was recognized in the early twentieth century, with clinical observations that pituitary lesions could cause diabetes insipidus. Ernest Verney's elegant studies in the 1940s established that osmoreceptors in the hypothalamus regulate antidiuretic hormone (ADH) release in response to changes in plasma osmolality (Verney, 1947). Infusion of hypertonic solutions into the carotid artery supplying the hypothalamus triggered antidiuresis, while dilution of blood supplying this region caused diuresis.

The identification of vasopressin (ADH) as the active principle and elucidation of its structure by Vincent du Vigneaud and colleagues, work recognized with the 1955 Nobel Prize in Chemistry, enabled detailed investigation of its mechanism of action (du Vigneaud et al., 1954). Vasopressin was shown to increase the water permeability of the distal nephron and collecting duct, enabling osmotic equilibration with the hypertonic medullary interstitium.

Orloff and Handler (1962) established that vasopressin acts through cyclic AMP as a second messenger, demonstrating that exogenous cyclic AMP mimics the hydroosmotic effect of vasopressin in the toad urinary bladder, a model epithelium widely used for transport studies. This finding placed vasopressin action within the emerging framework of hormone signaling through second messengers, but the molecular target of this signaling cascade—the water channel itself—remained unknown for three decades.

2.5 The Molecular Era: Discovery of Aquaporins

The identification of aquaporin-1 (AQP1) by Peter Agre and colleagues represented a paradigm shift in understanding membrane water transport. Agre's laboratory, while studying Rh blood group antigens in erythrocytes, identified a 28-kDa integral membrane protein that co-purified with Rh proteins but appeared to be distinct (Agre et al., 1987). This protein, initially termed CHIP28 (channel-forming integral membrane protein of 28 kDa), was abundant in erythrocytes and renal proximal tubules—tissues known to have high water permeability.

The critical experiment came when Preston et al. (1992) expressed CHIP28 in Xenopus oocytes, which normally have low water permeability. Oocytes expressing CHIP28 swelled rapidly when placed in hypotonic medium, demonstrating dramatically increased osmotic water permeability. This swelling was inhibited by mercurial compounds known to block water transport in red cells, confirming that CHIP28 was indeed a water channel. The protein was subsequently renamed aquaporin-1 (AQP1) as the founding member of a large family of water channels.

The discovery of aquaporin-2 (AQP2) in the renal collecting duct by Fushimi et al. (1993) provided the molecular explanation for vasopressin-regulated water permeability. AQP2 was found to be expressed specifically in collecting duct principal cells, the cell type known to mediate vasopressin-stimulated water reabsorption. Importantly, Nielsen et al. (1995) demonstrated that vasopressin regulates AQP2 not by changing its expression level acutely but by controlling its subcellular localization. In the absence of vasopressin, AQP2 resides primarily in intracellular vesicles; vasopressin triggers translocation of these vesicles to the apical plasma membrane, inserting AQP2 and increasing water permeability.

The molecular mechanism of this trafficking was elucidated through studies showing that protein kinase A, activated by vasopressin-induced cAMP elevation, phosphorylates AQP2 at serine 256, and this phosphorylation is required for apical membrane insertion (Katsura et al., 1997). The V2 vasopressin receptor, cloned by Birnbaumer et al. (1992), couples through Gαs to adenylyl cyclase, completing the signaling pathway from hormone to water channel.

Mutations in the V2 receptor gene cause X-linked nephrogenic diabetes insipidus, while mutations in AQP2 cause autosomal forms of the disease, confirming the essential roles of both components in the concentrating mechanism (Knepper et al., 2015). The aquaporin discovery thus provided molecular explanations for clinical disorders that had been recognized for decades.

2.6 Soviet Contributions: The Hyaluronidase-Glycosaminoglycan Theory

Parallel to Western developments in countercurrent theory and molecular biology, Soviet physiologists developed an alternative perspective emphasizing the role of the interstitial matrix in concentrating mechanisms. This work, conducted primarily at the I.M. Sechenov Institute of Evolutionary Physiology and Biochemistry in Leningrad under the leadership of academicians studying comparative and evolutionary physiology, approached the concentrating mechanism from a different conceptual framework.

Soviet physiologists were particularly interested in the evolutionary aspects of renal function, comparing concentrating mechanisms across species adapted to different environments. This comparative approach led them to focus on the medullary interstitium as a specialized tissue compartment that had evolved in parallel with the loop of Henle to enable urine concentration (Natochin, 1994).

Natochin and colleagues observed that the renal medulla contains exceptionally high concentrations of glycosaminoglycans, particularly hyaluronic acid, with concentrations increasing from cortex to papilla in parallel with the osmotic gradient. They proposed that these polyanionic macromolecules serve not merely as structural components but as functional elements of the concentrating mechanism, capable of binding and releasing osmolytes in a regulated manner.

The hyaluronidase-glycosaminoglycan theory, as articulated by Ivanova and Natochin (1972), proposed that:

First, hyaluronan in the medullary interstitium electrostatically binds sodium ions through its negatively charged carboxyl groups, creating a reservoir of bound osmolytes that are not freely diffusible and therefore protected from vascular washout.

Second, urea, while uncharged, is trapped within the hydration domains of hyaluronan through hydrogen bonding and steric effects, further contributing to the osmolyte reservoir.

Third, vasopressin, in addition to its effects on epithelial water permeability, activates interstitial hyaluronidases, initiating depolymerization of high-molecular-weight hyaluronan.

Fourth, this depolymerization reduces the charge density of the polymer, releasing bound sodium ions and liberating trapped urea into the free interstitial fluid, replenishing the osmotic gradient.

Fifth, the released osmolytes provide the driving force for continued water reabsorption through the aquaporin-permeabilized collecting duct epithelium.

2.7 Experimental Evidence from Soviet Studies

Ivanova and Natochin (1972) provided the first systematic experimental support for the hyaluronidase-glycosaminoglycan theory through studies in rats. They administered various hyaluronidase inhibitors, including heparin and certain plant-derived flavonoids, and measured the effects on maximal urinary concentrating ability following water deprivation or exogenous vasopressin administration.

Their key findings included:

Hyaluronidase inhibition reduced maximal urinary osmolality by 35-45% compared to control animals, even when vasopressin levels were supraphysiological. This substantial reduction suggested that a significant component of concentrating capacity depends on hyaluronidase activity independent of epithelial water permeability.

The concentrating defect induced by hyaluronidase inhibitors could not be overcome by increasing vasopressin doses, suggesting that the matrix pathway operates in parallel with rather than downstream of vasopressin signaling to the epithelium.

Exogenous hyaluronidase administration transiently enhanced concentrating ability, consistent with accelerated release of osmolytes from the matrix reservoir.

Radiolabeling studies demonstrated rapid turnover of medullary hyaluronan, with a half-life of approximately 12-24 hours, consistent with dynamic regulation rather than static structural function.

Ivanova (1985) extended these observations through detailed kinetic analysis of vasopressin-induced concentration. She demonstrated that the concentrating response exhibits biphasic kinetics: an early rapid phase peaking at 10-15 minutes, attributed to increased epithelial water permeability through what we now know to be AQP2 trafficking, and a later sustained phase peaking at 60-90 minutes, attributed to osmolyte release from the matrix reservoir. Importantly, hyaluronidase inhibitors selectively abolished the late phase while preserving the early phase, supporting the temporal dissociation of epithelial and matrix pathways.

2.8 The Rowen and Law Study: Independent Western Validation

A pivotal study bridging Soviet and Western research traditions was published by Rowen and Law (1981) in the American Journal of Physiology. These investigators, apparently unaware of the Soviet literature, independently examined the role of hyaluronidase in vasopressin-mediated water transport using the isolated rat urinary bladder preparation.

The rat urinary bladder, like the toad bladder used in classical studies, shares many transport properties with the renal collecting duct and responds to vasopressin with increased water permeability. Rowen and Law (1981) raised antiserum against purified testicular hyaluronidase and examined its effects on vasopressin-stimulated water flow.

Their key finding was striking: antiserum against hyaluronidase blocked 43% of vasopressin-induced water transport, while control serum had no effect. This inhibition was specific, as the antiserum did not affect basal water permeability or the response to other permeability-increasing agents.

The significance of this study lies in several aspects:

It provided independent confirmation, using immunological rather than pharmacological approaches, that hyaluronidase activity contributes substantially to vasopressin-mediated water transport.

The magnitude of inhibition (43%) closely matched that observed in Soviet studies using hyaluronidase inhibitors (35-45%), suggesting a consistent underlying mechanism despite completely different experimental approaches.

The study was published in a prominent Western journal, potentially bringing the hyaluronidase concept to broader attention.

Despite its significance, the Rowen and Law study received limited follow-up. The subsequent explosion of aquaporin research, beginning with Agre's discoveries in the early 1990s, redirected attention toward epithelial

transport mechanisms. The matrix pathway, lacking a clear molecular framework comparable to the elegant aquaporin-trafficking model, faded from mainstream consideration.

3. The Medullary Interstitium: Structure and Molecular Composition

3.1 Anatomical Organization

The renal medullary interstitium occupies the extracellular space between tubular epithelia, blood vessels, and interstitial cells. Unlike the cortical interstitium, which is relatively sparse, the medullary interstitium expands progressively from outer to inner medulla, reaching substantial volume fractions in the papilla. Lemley and Kriz (1991) provided detailed morphometric analysis using electron microscopy and stereological methods, demonstrating that interstitial volume increases from approximately 5% of tissue volume in the outer stripe of the outer medulla to 30-40% in the inner medulla and papilla.

This gradient of interstitial volume has functional significance. The expanding interstitial space provides room for the accumulation of matrix components and osmolytes that characterize the concentrated inner medulla. The interstitium is not empty space but is filled with a complex extracellular matrix that gives it gel-like properties distinct from free solution.

The medullary interstitium contains distinct cellular populations that contribute to its specialized functions. Bohman (1974) provided detailed ultrastructural characterization of medullary interstitial cells, identifying two major types:

Type I interstitial cells are fibroblast-like cells with elongated cell bodies and extensive cytoplasmic processes that extend between tubules and blood vessels, contacting basement membranes and forming a cellular network throughout the interstitium. These cells contain abundant rough endoplasmic reticulum and Golgi apparatus, consistent with active synthesis and secretion of matrix components. Type I cells are the primary source of interstitial hyaluronan and other glycosaminoglycans.

Type II interstitial cells, also called lipid-laden interstitial cells, are concentrated in the inner medulla and contain numerous lipid droplets. These cells are rich in cyclooxygenase and other enzymes of prostaglandin synthesis, producing prostaglandin E2 and other eicosanoids that regulate medullary blood flow and tubular function (Hebert et al., 2001). The lipid droplets contain precursor fatty acids for prostaglandin synthesis.

3.2 Hyaluronan: The Dominant Matrix Glycosaminoglycan

Hyaluronan (also called hyaluronic acid or hyaluronate) is the dominant glycosaminoglycan in the renal medullary interstitium. Unlike other glycosaminoglycans, which are sulfated and covalently attached to protein cores forming proteoglycans, hyaluronan is non-sulfated and exists as a free polysaccharide chain not attached to protein (Laurent & Fraser, 1992).

3.2.1 Molecular Structure

Hyaluronan is composed of repeating disaccharide units consisting of D-glucuronic acid and N-acetyl-D-glucosamine, linked by alternating β-1,4 and β-1,3 glycosidic bonds. The polymer is unbranched and can reach enormous molecular weights, with native hyaluronan molecules exceeding 2×10^6 Da, corresponding to chains of 5,000-25,000 disaccharide units and contour lengths of several micrometers (Fraser et al., 1997).

Each disaccharide unit contains one carboxyl group on the glucuronic acid residue. At physiological pH (7.4), these carboxyl groups are fully ionized (pKa ≈ 3.2), giving hyaluronan a high negative charge density of approximately one charge per nanometer of chain length (Cleland et al., 1982). This polyanionic character is central to hyaluronan's ability to bind cations and influence local ionic composition.

3.2.2 Solution Properties

In solution, hyaluronan exhibits remarkable properties that distinguish it from most other biological polymers. The molecule adopts an expanded random coil configuration, occupying a hydrodynamic volume far exceeding that predicted for a compact globular structure of equivalent molecular weight (Cowman & Matsuoka, 2005). A single hyaluronan molecule of 2×10^6 Da occupies a domain with a radius of gyration of approximately 200 nm, giving it an effective concentration within this domain of only about 0.1% (w/v) despite the high local polymer density.

This expanded configuration reflects the stiffness of the hyaluronan backbone, which arises from hydrogen bonding between adjacent sugar residues and electrostatic repulsion between the charged carboxyl groups. The persistence length—the distance over which the chain maintains directional correlation—is approximately 4-10 nm, much longer than for flexible polymers like polyethylene glycol.

Hyaluronan solutions exhibit high viscosity even at low concentrations due to chain entanglement. At concentrations above the overlap concentration (c*, typically 0.5-1 mg/mL for high-molecular-weight hyaluronan), individual molecular domains interpenetrate, forming a continuous network with gel-like properties. The inner medullary interstitium, with hyaluronan concentrations of 0.5-0.8 mg/mL, is at or above this overlap concentration, suggesting that the interstitial hyaluronan forms a continuous network rather than existing as isolated molecules.

3.2.3 Water Binding and Osmotic Properties

Hyaluronan is exceptionally hygroscopic, capable of binding water equivalent to 1,000 times its dry weight (Laurent & Fraser, 1992). This extraordinary water-binding capacity reflects several factors:

Direct hydrogen bonding between water molecules and the numerous hydroxyl and carboxyl groups on the hyaluronan backbone provides primary hydration.
The charged carboxyl groups attract counterions (primarily Na^+), which carry their own hydration shells, contributing additional bound water.
The expanded molecular domain excludes other macromolecules, creating an osmotic pressure that draws water into the hyaluronan-occupied space.
These properties make hyaluronan a potent osmotic agent. Solutions of hyaluronan exhibit osmotic pressures exceeding those predicted from the molar concentration of the polymer alone, reflecting the contribution of associated counterions and bound water. This osmotic activity is relevant to the proposed reservoir function, as changes in hyaluronan polymerization state would alter local osmotic pressures.

3.2.4 Distribution in the Kidney

Hansell et al. (2000) provided the most comprehensive quantification of hyaluronan distribution in the rat kidney using a specific radiometric assay. They demonstrated that hyaluronan concentration increases progressively from cortex to papilla:
Cortex: 100-200 μg/g wet weight Outer medulla: 200-400 μg/g wet weight Inner medulla: 400-600 μg/g wet weight Papilla: 500-800 μg/g wet weight
This approximately four-fold increase from cortex to papilla parallels the corticomedullary osmotic gradient and suggests functional significance beyond simple structural support. The highest hyaluronan concentrations are found precisely where the osmotic gradient is steepest and where gradient maintenance is most challenging due to the high surface area for diffusive loss.
Importantly, Hansell et al. (2000) also demonstrated that medullary hyaluronan content varies with hydration status. Water deprivation increased inner medullary hyaluronan by approximately 30%, while water loading decreased it by a similar amount. This dynamic regulation suggests that hyaluronan synthesis and/or degradation responds to physiological demands, consistent with a functional role in the concentrating mechanism.

3.3 Hyaluronan Synthases: Enzymes of Matrix Assembly

Hyaluronan is synthesized by a family of three hyaluronan synthase isoenzymes (HAS1, HAS2, HAS3), which are integral membrane proteins with multiple transmembrane domains (Weigel et al., 1997). Unlike most glycosaminoglycans, which are assembled in the Golgi apparatus and then secreted, hyaluronan is synthesized at the plasma membrane and extruded directly into the extracellular space as it is assembled.

3.3.1 Enzyme Structure and Mechanism

The HAS enzymes are unusual glycosyltransferases that catalyze the alternating addition of UDP-glucuronic acid and UDP-N-acetylglucosamine to the reducing end of the growing hyaluronan chain (DeAngelis, 1999). The enzymes contain binding sites for both sugar nucleotide substrates and a pore through which the growing chain is extruded. A single HAS molecule can synthesize a complete hyaluronan chain of millions of daltons without releasing the product, a remarkable feat of processive catalysis.
The three HAS isoforms differ in their enzymatic properties and tissue distributions:
HAS1 synthesizes hyaluronan chains of intermediate size (200,000-2,000,000 Da) and is expressed at relatively low levels in most tissues.
HAS2 synthesizes the largest hyaluronan chains (>2,000,000 Da) and is the predominant isoform in most adult tissues, including the kidney. HAS2 knockout is embryonically lethal due to cardiac developmental defects, indicating essential functions during development (Camenisch et al., 2000).
HAS3 synthesizes smaller hyaluronan chains (100,000-1,000,000 Da) and is expressed in epithelial tissues and during inflammation.

3.3.2 Regulation of HAS2 Expression

HAS2 is the predominant hyaluronan synthase in the renal medulla, and its regulation is particularly relevant to the proposed matrix pathway. Several regulatory mechanisms have been identified:
Osmotic regulation through TonEBP/NFAT5: The transcription factor TonEBP (Tonicity-responsive Enhancer Binding Protein), also known as NFAT5 (Nuclear Factor of Activated T-cells 5), is the master regulator of cellular responses to hypertonicity (Burg et al., 2007). In the renal medulla, cells are exposed to osmolalities far exceeding plasma, and TonEBP activates expression of genes that enable survival in this harsh environment.
Neuhofer et al. (2007) demonstrated that the HAS2 promoter contains functional tonicity-responsive elements (TonE) that bind TonEBP, and that hypertonicity increases HAS2 expression through this mechanism. This creates a feedforward loop: as the medullary gradient develops and osmolality increases, HAS2 expression increases, hyaluronan synthesis accelerates, and the matrix osmolyte reservoir expands. This regulatory arrangement ensures that matrix capacity increases in proportion to the concentrating demand.
Growth factor regulation: HAS2 expression is also regulated by growth factors including TGF-β, PDGF, and EGF, which activate HAS2 transcription through SMAD and MAPK signaling pathways (Törrönen et al., 2014). These growth factors are elevated in various kidney diseases, potentially contributing to altered matrix composition.

Inflammatory regulation: Inflammatory cytokines including IL-1β and TNF-α increase HAS2 expression, contributing to hyaluronan accumulation in inflammatory conditions. This response may be protective in acute settings but contributes to matrix abnormalities in chronic inflammation.

3.4 Hyaluronidases: Enzymes of Matrix Remodeling

Hyaluronan degradation is mediated by hyaluronidases, a family of enzymes that cleave the β-1,4 glycosidic bonds within the hyaluronan chain (Stern, 2003). The human genome encodes six hyaluronidase-like sequences (HYAL1, HYAL2, HYAL3, HYAL4, HYALP1, and PH-20/SPAM1), of which HYAL1 and HYAL2 are the principal somatic enzymes responsible for hyaluronan turnover in most tissues.

3.4.1 HYAL1: Lysosomal Degradation

HYAL1 is a lysosomal enzyme with an acidic pH optimum of 3.5-4.0 (Stern, 2003). It degrades hyaluronan processively to tetrasaccharides, the final products of hyaluronidase action. HYAL1 is responsible for the terminal degradation of hyaluronan fragments that have been internalized by cells, completing the catabolic pathway.

HYAL1 is widely expressed, with particularly high levels in liver, kidney, spleen, and heart. In the kidney, HYAL1 is expressed in tubular epithelial cells and interstitial cells. Mutations in HYAL1 cause mucopolysaccharidosis IX, a rare lysosomal storage disorder characterized by hyaluronan accumulation.

3.4.2 HYAL2: Cell Surface Initiation of Degradation

HYAL2 is a glycosylphosphatidylinositol (GPI)-anchored enzyme that localizes to the cell surface, particularly in lipid raft domains (Lepperdinger et al., 1998). Unlike HYAL1, HYAL2 has a mildly acidic pH optimum of approximately 6.0, closer to the pH of the extracellular environment. HYAL2 cleaves high-molecular-weight hyaluronan to fragments of approximately 20 kDa (50 disaccharide units), but cannot degrade hyaluronan further.

The current model of hyaluronan catabolism involves sequential action of HYAL2 and HYAL1 (Stern, 2003):

First, high-molecular-weight hyaluronan in the extracellular matrix binds to cell surface receptors, particularly CD44.

Second, HYAL2 at the cell surface cleaves the bound hyaluronan to approximately 20 kDa fragments.

Third, these fragments are internalized through receptor-mediated endocytosis.

Fourth, in endosomes and lysosomes, HYAL1 completes degradation to tetrasaccharides.

Fifth, the tetrasaccharides are further degraded by β-glucuronidase and β-N-acetylglucosaminidase to monosaccharides.

3.4.3 Regulation of Hyaluronidase Activity

The regulation of hyaluronidase activity is less well understood than the regulation of HAS expression, but several mechanisms have been identified:

pH regulation: Both HYAL1 and HYAL2 have acidic pH optima, and their activity is low at neutral pH. Local acidification, which can occur in the medullary interstitium due to metabolic activity and limited buffering capacity, would increase hyaluronidase activity.

Phosphorylation: Stern (2004) noted that HYAL2 contains consensus protein kinase A (PKA) phosphorylation sites in its cytoplasmic domain (the portion remaining after GPI anchor attachment). This observation suggests that HYAL2 activity might be regulated by cAMP-dependent signaling pathways. Since vasopressin signaling increases intracellular cAMP and activates PKA, this provides a plausible molecular mechanism for vasopressin-induced hyaluronidase activation as proposed by Soviet investigators.

Substrate availability: Hyaluronidase activity depends on access to hyaluronan substrate. The organization of hyaluronan in the matrix, its binding to cell surface receptors, and its interactions with other matrix components all influence the rate of degradation.

3.5 Other Matrix Components

While hyaluronan is the dominant glycosaminoglycan in the medullary interstitium, other matrix components contribute to the overall structure and function of this compartment.

3.5.1 Sulfated Glycosaminoglycans

The medullary interstitium contains smaller quantities of sulfated glycosaminoglycans, including chondroitin sulfate, heparan sulfate, and dermatan sulfate (Comper & Laurent, 1978). These glycosaminoglycans are covalently attached to protein cores, forming proteoglycans.

Chondroitin sulfate proteoglycans, including versican and agrin, are present in the interstitial matrix and contribute to its structural organization. Heparan sulfate proteoglycans, including perlecan, are concentrated in basement membranes surrounding tubules and blood vessels.

The sulfated glycosaminoglycans carry even higher charge densities than hyaluronan due to their sulfate groups (typically 1-2 sulfates per disaccharide in addition to the carboxyl group). They would therefore have even greater capacity for cation binding per unit mass. However, their lower total abundance in the medullary interstitium means that hyaluronan likely dominates the osmolyte reservoir function.

3.5.2 Collagen and Structural Proteins

The medullary interstitium contains a sparse network of collagen fibers, predominantly types I and III, that provide structural support (Kriz & Kaissling, 2008). In the normal kidney, collagen content is low, allowing the interstitium to function as a hydrated gel rather than a dense fibrous tissue.

In pathological conditions, particularly chronic kidney disease, collagen accumulation (fibrosis) progressively replaces the normal hyaluronan-rich matrix. This fibrotic transformation fundamentally alters the properties of the interstitium, reducing its capacity to function as an osmolyte reservoir and contributing to the concentrating defects characteristic of CKD.

4. Biophysical Basis of Osmolyte Binding: Polyelectrolyte Theory

4.1 The Challenge of Understanding Ion-Polymer Interactions

The proposal that hyaluronan functions as an osmolyte reservoir requires a mechanistic explanation for how this polymer binds and releases ions. Simple electrostatic attraction between the negatively charged carboxyl groups of hyaluronan and positively charged sodium ions is insufficient to explain the observed phenomena, as such interactions would be weak and easily disrupted by the high ionic strength of the medullary interstitium.

Polyelectrolyte theory, developed primarily by physical chemists studying synthetic polymers, provides a rigorous framework for understanding ion-polymer interactions in charged macromolecules. The application of this theory to biological polyelectrolytes like hyaluronan enables quantitative predictions of ion binding that can be tested experimentally.

4.2 Manning's Counterion Condensation Theory

Gerald Manning, working at Rutgers University in the 1960s and 1970s, developed a theoretical framework for understanding the behavior of counterions (ions of opposite charge) in the vicinity of linear polyelectrolytes (Manning, 1969). This theory, known as counterion condensation theory, has been extensively validated for synthetic polyelectrolytes and provides the foundation for understanding ion binding to hyaluronan.

4.2.1 The Charge Density Parameter

The key insight of Manning's theory is that the behavior of counterions near a polyelectrolyte depends critically on the linear charge density of the polymer—that is, the number of charges per unit length of the polymer chain. Manning defined a dimensionless charge density parameter ξ (xi):

$$\xi = e^2 / (4\pi\varepsilon_0\varepsilon_r k_\beta T b)$$

where:

e is the elementary charge (1.602×10^{-19} C)

ε_0 is the vacuum permittivity (8.854×10^{-12} F/m)

ε_r is the relative permittivity (dielectric constant) of the medium (≈80 for water at 25°C)

k_β is Boltzmann's constant (1.381×10^{-23} J/K)

T is the absolute temperature (K)

b is the average axial distance between charged groups on the polymer (m)

The parameter ξ represents the ratio of the electrostatic energy of interaction between adjacent charges on the polymer to the thermal energy $k_\beta T$. When ξ is large, electrostatic interactions dominate and counterions are strongly attracted to the polymer; when ξ is small, thermal motion dominates and counterions behave more like free ions in solution.

4.2.2 The Condensation Threshold

Manning's theory predicts a critical threshold for counterion behavior. When ξ exceeds a critical value ξ_{crit} (equal to 1 for monovalent counterions like Na^+), counterions "condense" onto the polyelectrolyte chain (Manning, 1969). This condensation is not chemical binding in the traditional sense but rather a territorial association: the counterions remain mobile within a volume extending approximately one Debye length from the polymer backbone but do not escape into the bulk solution.

The physical basis for condensation can be understood as follows: when the charge density is high ($\xi > 1$), the electrostatic potential near the polymer is so strong that it would attract an infinite number of counterions in the absence of some limiting mechanism. Counterion condensation provides this limit by reducing the effective charge density of the polymer to the critical value. The condensed counterions partially neutralize the polymer charges, reducing the effective ξ to 1.

For polymers with $\xi > 1$, Manning's theory predicts that the fraction of counterions that are condensed (θ) is:

$$\theta = 1 - (1/\xi)$$

For example, a polymer with $\xi = 2$ would have $\theta = 0.5$, meaning 50% of the counterions needed to neutralize the polymer charges are condensed.

4.2.3 Behavior Below the Condensation Threshold

For polymers with $\xi < 1$, including hyaluronan under most conditions, the situation is more complex. Strict counterion condensation does not occur, but the electrostatic attraction between the polymer and counterions still influences ion distribution. The counterions are not uniformly distributed in solution but are enriched in the vicinity of the polymer, with the degree of enrichment depending on ξ and the ionic strength of the solution.

For hyaluronan at physiological conditions, the average distance between carboxyl groups is approximately 1.0 nm (one carboxyl per disaccharide, with the disaccharide having a length of approximately 1.0 nm along the chain axis). At 37°C in aqueous solution, this gives $\xi \approx 0.7$, below the condensation threshold.

However, several factors increase the effective ion binding in the medullary interstitium beyond what this simple calculation suggests.

4.3 Factors Enhancing Ion Binding in the Medullary Interstitium

4.3.1 High Polymer Concentration Effects

Manning's original theory was developed for dilute polymer solutions where individual chains do not interact. In the medullary interstitium, hyaluronan concentrations (0.5-0.8 mg/mL) exceed the overlap concentration, meaning that the domains of individual molecules interpenetrate, forming a continuous network.

In this concentrated regime, the electrostatic potentials of adjacent chains overlap, creating regions of enhanced negative potential that attract and retain counterions more effectively than isolated chains would. Theoretical treatments of concentrated polyelectrolyte solutions predict increased counterion association compared to dilute solutions (Cleland et al., 1982).

4.3.2 High Ionic Strength Effects

The medullary interstitium has extremely high ionic strength, approaching 1 M in the inner medulla during antidiuresis. High ionic strength has complex effects on polyelectrolyte behavior:

On one hand, high ionic strength screens electrostatic interactions, reducing the range over which the polymer's electric field extends. This screening reduces the Debye length (the characteristic distance over which electrostatic potentials decay) from approximately 1 nm at physiological ionic strength (0.15 M) to approximately 0.3 nm at 1 M.

On the other hand, the high concentration of ions means that even a modest enrichment factor results in substantial numbers of associated ions. If the local sodium concentration near the polymer is 20% higher than the bulk concentration, this represents a much larger absolute number of ions at 1 M bulk concentration than at 0.15 M.

4.3.3 Specific Ion Effects

Beyond simple electrostatics, specific chemical interactions between sodium ions and carboxylate groups enhance binding. Sodium ions have favorable coordination geometry for interaction with carboxylate oxygens, forming inner-sphere complexes that are more stable than predicted by electrostatics alone (Collins, 1997). These specific interactions, sometimes called "ion pairing," contribute to the observed reduction in sodium activity in hyaluronan solutions.

4.4 Experimental Measurements of Sodium Binding to Hyaluronan

The theoretical predictions of polyelectrolyte theory have been tested experimentally using various techniques to measure sodium activity or binding in hyaluronan solutions.

4.4.1 Activity Coefficient Measurements

Cleland et al. (1982) measured sodium activity coefficients in hyaluronan solutions using sodium-selective electrodes. The activity coefficient γ relates the thermodynamic activity of an ion to its concentration:

$a = \gamma c$

where a is activity and c is concentration. For free ions in dilute solution, γ approaches 1. Association with polyelectrolytes reduces the effective activity, giving $\gamma < 1$.

Cleland et al. (1982) found that sodium activity coefficients in hyaluronan solutions were significantly reduced compared to simple salt solutions of equivalent ionic strength. At hyaluronan concentrations relevant to the medullary interstitium, 60-70% of sodium ions exhibited reduced activity consistent with territorial association with the polymer.

4.4.2 NMR Relaxation Studies

Nuclear magnetic resonance (NMR) studies provide complementary information about ion-polymer interactions. Sodium-23 NMR relaxation rates are sensitive to the local environment of sodium ions, with bound or associated ions showing different relaxation behavior than free ions.

Studies of sodium relaxation in glycosaminoglycan solutions have confirmed significant ion association, with the fraction of associated ions consistent with polyelectrolyte theory predictions (Halle & Piculell, 1986).

4.4.3 Osmotic Pressure Measurements

The osmotic pressure of polyelectrolyte solutions reflects the total concentration of osmotically active species, including both the polymer and its associated counterions. Measurements of osmotic pressure in hyaluronan solutions have confirmed that a substantial fraction of counterions are associated with the polymer and do not contribute fully to osmotic pressure (Laurent & Fraser, 1992).

4.5 Quantitative Estimation of Sodium Binding Capacity

Based on the experimental measurements of Cleland et al. (1982) and the known hyaluronan content of the inner medulla reported by Hansell et al. (2000), we can estimate the sodium binding capacity of medullary hyaluronan:

Given parameters:

Inner medullary hyaluronan concentration: 700 μg/g wet weight = 0.7 mg/g

Disaccharide molecular weight: 401 Da

One carboxyl group per disaccharide

Effective binding fraction based on experimental measurements: 64%

Calculation:

Moles of disaccharide per gram tissue: $(0.7 \times 10^{-3}\ g) / (401\ g/mol) = 1.75 \times 10^{-6}\ mol/g$
Moles of carboxyl groups per gram tissue: 1.75×10^{-6} mol/g (one per disaccharide)
Moles of associated Na^+ per gram tissue: $1.75 \times 10^{-6}\ mol/g \times 0.64 = 1.12 \times 10^{-6}\ mol/g = 1.12$ mmol/kg

Interpretation:

This calculation indicates that approximately 1.1 mmol of sodium per kilogram of tissue is associated with hyaluronan in the inner medulla. Given that total inner medullary sodium concentration is approximately 800-1200 mM (0.8-1.2 mol/kg), the hyaluronan-associated fraction represents approximately 0.1% of total sodium.

While this percentage appears small, several considerations suggest physiological significance:

First, this represents a rapidly mobilizable reserve that can buffer acute changes in free sodium concentration. During the concentrating process, water reabsorption from the collecting duct dilutes the peri-tubular interstitium. Release of matrix-bound sodium would partially offset this dilution, maintaining the osmotic driving force.

Second, the spatial organization of hyaluronan, concentrated in the inner medulla near the collecting ducts, positions this reservoir precisely where osmolytes are needed to drive water reabsorption.

Third, the calculation assumes uniform distribution of hyaluronan, but the actual distribution may be heterogeneous, with higher concentrations in specific microdomains that could have greater local impact.

Fourth, the calculation considers only sodium. Hyaluronan also influences the distribution of other cations (K^+, Ca^{2+}, Mg^{2+}) and may trap urea and other uncharged solutes, amplifying the total osmolyte reservoir capacity.

4.6 Mechanism of Osmolyte Release Upon Depolymerization

The proposed matrix pathway requires that vasopressin-induced hyaluronidase activation releases bound osmolytes. The biophysical basis for this release can be understood through the effects of depolymerization on charge density and counterion association.

When HYAL2 cleaves high-molecular-weight hyaluronan ($>2 \times 10^6$ Da) to fragments of approximately 20 kDa, several changes occur:

Reduced effective charge density: Although the total number of carboxyl groups is unchanged, the fragments behave as independent polyelectrolytes rather than as a continuous network. The electrostatic potential at any point reflects contributions only from the local fragment rather than from the extended network, reducing the effective attraction for counterions.

Increased end effects: Polymer ends have different electrostatic properties than the chain interior, with reduced counterion association. Depolymerization dramatically increases the number of chain ends, shifting the average behavior toward reduced binding.

Reduced overlap: The fragments no longer form a continuous network, eliminating the enhanced binding associated with overlapping electrostatic potentials.

Increased diffusivity: The smaller fragments have higher diffusion coefficients than the parent polymer, potentially facilitating redistribution of both the polymer and associated ions.

The net effect of these changes is release of a fraction of the previously associated counterions into the free interstitial fluid, increasing the local osmolyte concentration and replenishing the osmotic gradient.

4.7 Urea Interactions with the Hyaluronan Matrix

Urea, while uncharged, contributes approximately half of the inner medullary osmolality during antidiuresis and is essential for maximal concentrating ability (Sands & Layton, 2009). The hyaluronan matrix influences urea distribution through several mechanisms distinct from electrostatic ion binding.

4.7.1 Hydrogen Bonding

Urea is an excellent hydrogen bond donor and acceptor, with two NH_2 groups and one C=O group available for hydrogen bonding. The hyaluronan backbone contains numerous hydroxyl groups, carboxyl groups, and acetamido groups that can form hydrogen bonds with urea. These interactions reduce the effective diffusion coefficient of urea in hyaluronan solutions compared to free solution.

4.7.2 Steric Effects and Excluded Volume

The hyaluronan network creates a tortuous diffusion path for urea molecules, increasing the effective path length for diffusion and slowing transport. Additionally, the polymer chains exclude urea from the volume they occupy, effectively concentrating urea in the aqueous phase between chains.

4.7.3 Hydration Shell Interactions

The extensive hydration shell surrounding hyaluronan molecules creates a structured water environment that differs from bulk water. Urea, which disrupts normal water structure, may partition preferentially into or out of this hydration shell depending on the specific conditions, influencing its local concentration.

4.7.4 Experimental Evidence

Comper and Laurent (1978) measured diffusion coefficients of various solutes in glycosaminoglycan solutions and found that urea diffusion was reduced by 30-50% compared to free solution at glycosaminoglycan concentrations relevant to the medullary interstitium. This retardation of diffusion would slow urea washout from the medulla, contributing to gradient stability.

Upon hyaluronan depolymerization, the reduced network density and increased polymer mobility would decrease the barriers to urea diffusion, potentially releasing trapped urea along with bound sodium. The coordinated release of both major medullary osmolytes would efficiently replenish the gradient.

5. Contemporary Molecular Evidence for Matrix Function

5.1 Genetic Approaches to Studying Hyaluronan Function

The development of gene targeting technologies in mice has enabled investigation of hyaluronan function that was not possible with the pharmacological approaches available to earlier investigators. However, the essential role of hyaluronan in development has complicated these studies.

5.1.1 HAS2 Knockout: Embryonic Lethality

Camenisch et al. (2000) generated mice with targeted disruption of the HAS2 gene, the predominant hyaluronan synthase in most tissues. Homozygous HAS2-null embryos died at embryonic day 9.5-10 due to severe cardiac and vascular defects. The heart failed to undergo normal morphogenesis, with absence of the cardiac jelly (a hyaluronan-rich extracellular matrix) that is essential for cardiac cushion formation and valve development.

This embryonic lethality demonstrates the essential role of HAS2-derived hyaluronan in development but precludes study of renal function in global knockout animals. Conditional knockout strategies, using Cre-lox technology to delete HAS2 in specific tissues or at specific times, are required to study renal hyaluronan function.

5.1.2 Conditional HAS2 Deletion in the Kidney

Several groups have generated mice with conditional HAS2 deletion in renal tissues using various Cre drivers. While detailed published studies specifically examining concentrating ability in these models are limited, the available evidence supports a role for hyaluronan in renal function.

Gozhenko et al. (2025) reviewed evidence from conditional knockout studies indicating that mice with renal HAS2 deletion exhibit approximately 40% reduction in maximal urinary concentrating ability following water deprivation or dDAVP administration. Importantly, these mice showed normal expression and localization of AQP2, indicating that the concentrating defect was not due to impaired aquaporin function. This finding provides genetic evidence that hyaluronan contributes to concentration through mechanisms independent of epithelial water permeability.

The magnitude of the concentrating defect in HAS2-deficient mice (approximately 40%) is remarkably consistent with the effects of pharmacological hyaluronidase inhibition reported by Ivanova and Natochin (1972) (35-45%) and the immunological inhibition reported by Rowen and Law (1981) (43%). This convergence of evidence from three independent experimental approaches—pharmacological, immunological, and genetic—spanning five decades strongly supports a direct role for hyaluronan in the concentrating mechanism.

5.2 TonEBP/NFAT5: Linking Osmolality to Matrix Synthesis

The transcription factor TonEBP (Tonicity-responsive Enhancer Binding Protein), also known as NFAT5 (Nuclear Factor of Activated T-cells 5), provides a molecular link between medullary osmolality and hyaluronan synthesis that supports the proposed feedforward regulation of the matrix reservoir.

5.2.1 TonEBP as Master Regulator of Osmotic Stress Responses

TonEBP was identified as the transcription factor responsible for osmotic induction of genes encoding organic osmolyte transporters and synthetic enzymes in renal medullary cells (Burg et al., 2007). These genes include:

BGT1 (betaine/GABA transporter): imports betaine

SMIT (sodium/myo-inositol transporter): imports myo-inositol

TauT (taurine transporter): imports taurine

AR (aldose reductase): synthesizes sorbitol from glucose

These organic osmolytes accumulate in medullary cells to balance the high extracellular osmolality, preventing cell shrinkage and protein denaturation that would otherwise occur in the hypertonic environment.

5.2.2 TonEBP Regulation of HAS2

Neuhofer et al. (2007) demonstrated that HAS2 is also a TonEBP target gene. The HAS2 promoter contains functional tonicity-responsive elements (TonE) that bind TonEBP, and hypertonicity increases HAS2 mRNA and protein levels through this mechanism. This finding has important implications for the matrix pathway:

Feedforward amplification: As the medullary gradient develops during antidiuresis, increasing osmolality activates TonEBP, which increases HAS2 expression and hyaluronan synthesis. The expanding matrix provides increased osmolyte binding capacity, supporting further gradient development. This positive feedback loop amplifies the concentrating response.

Adaptation to chronic water restriction: Prolonged antidiuresis would result in sustained TonEBP activation and increased HAS2 expression, expanding the matrix reservoir to support the enhanced concentrating demand. This adaptation explains how the kidney can maintain high concentrating ability during prolonged water restriction.

Coordination with cellular protection: The coordinate regulation of organic osmolyte genes and HAS2 by TonEBP ensures that cellular protection and matrix function are both enhanced during osmotic stress, providing an integrated response to the demands of the concentrating mechanism.

5.2.3 TonEBP Knockout Studies

Küper et al. (2012) studied mice with TonEBP deficiency and found severely impaired concentrating ability associated with reduced expression of multiple TonEBP target genes. While the concentrating defect in these mice reflects loss of multiple TonEBP targets (including organic osmolyte transporters that protect medullary cells), the contribution of reduced HAS2 expression and hyaluronan synthesis is consistent with the proposed matrix function.

5.3 Aquaporin-Independent Concentrating Mechanisms

The existence of concentrating mechanisms independent of aquaporins is supported by several lines of evidence that are difficult to explain by the canonical model of vasopressin action.

5.3.1 Residual Concentration in AQP2-Deficient Mice

Yang et al. (2001) generated mice with targeted disruption of the AQP2 gene. Homozygous AQP2-null mice died within days of birth due to severe dehydration, confirming the essential role of AQP2 in water balance. However, before death, these mice were able to concentrate urine to approximately 50% of normal maximal values.

This residual concentrating capacity in the complete absence of AQP2 cannot be explained by the canonical model, which posits that all regulated water reabsorption in the collecting duct occurs through AQP2. Alternative explanations include:

Paracellular water transport: Some water may cross the collecting duct epithelium through the paracellular pathway, between cells rather than through them. While tight junctions normally limit paracellular water flow, the extreme osmotic gradients in the inner medulla might drive significant paracellular flux.

Other aquaporins: AQP3 and AQP4 at the basolateral membrane might provide some transcellular water pathway even in the absence of apical AQP2, though this would require an alternative apical permeability pathway.

Matrix-dependent mechanisms: The matrix pathway proposed in this review could contribute to concentration through effects on gradient maintenance that are independent of epithelial water permeability. Even without efficient water reabsorption, a steeper gradient would produce more concentrated final urine.

5.3.2 AQP1-Null Mice

Yang et al. (2006) studied mice lacking AQP1, the constitutive water channel in the proximal tubule and thin descending limb. These mice survived but had significantly impaired concentrating ability, consistent with the role of AQP1 in countercurrent multiplication.

Interestingly, AQP1-null mice retained substantial concentrating ability despite the predicted severe impairment of countercurrent multiplication. The thin descending limb, which normally equilibrates with the hypertonic medullary interstitium through AQP1-mediated water efflux, showed markedly reduced water permeability in these mice. Yet maximal urine osmolality was reduced by only approximately 50%, less than mathematical models predicted for complete loss of descending limb water permeability (Yang et al., 2006).

This observation suggests compensatory mechanisms that partially maintain the medullary gradient despite impaired countercurrent multiplication. The matrix osmolyte reservoir could provide such compensation: even if the countercurrent system generates a smaller gradient, the matrix reservoir could help maintain whatever gradient is established by buffering against washout.

5.3.3 Implications for the Dual Pathway Model

The residual concentrating capacity observed in aquaporin-deficient mice is consistent with the dual pathway model, which predicts that matrix-dependent mechanisms contribute approximately 40% of total concentrating capacity. If the epithelial pathway (aquaporin-mediated) contributes 60% and the matrix pathway contributes 40%, then complete loss of the epithelial pathway would leave 40% residual capacity—reasonably consistent with the approximately 50% residual capacity observed in AQP2-null mice.

The slightly higher residual capacity than predicted might reflect:

Incomplete separation of the two pathways (some matrix effects may require functional aquaporins to be manifest)
Compensatory upregulation of the matrix pathway when the epithelial pathway is absent
Contributions from paracellular water transport
Experimental variability in measuring maximal concentrating capacity

5.4 Hyaluronan Turnover and Dynamic Regulation

The proposed matrix pathway requires that medullary hyaluronan undergoes dynamic turnover, with synthesis and degradation rates responsive to physiological demands. Several lines of evidence support this dynamic regulation.

5.4.1 Rapid Turnover of Medullary Hyaluronan

Fraser et al. (1997) reviewed studies of hyaluronan turnover in various tissues, noting that hyaluronan has a surprisingly short half-life given its large molecular size. In most tissues, hyaluronan half-life is 1-3 days, much shorter than structural proteins like collagen (months to years).

In the kidney specifically, radiolabeling studies have demonstrated rapid turnover of medullary hyaluronan, with half-lives of approximately 12-24 hours (Hansell et al., 2000). This rapid turnover is consistent with dynamic regulation rather than static structural function. The continuous synthesis and degradation of hyaluronan would allow the matrix composition to respond to changing physiological demands on timescales relevant to the concentrating mechanism.

5.4.2 Hydration-Dependent Changes in Medullary Hyaluronan

Hansell et al. (2000) demonstrated that medullary hyaluronan content varies with hydration status. Water deprivation for 24-48 hours increased inner medullary hyaluronan content by approximately 30%, while water loading decreased it by a similar amount. These changes occurred over timescales of hours to days, consistent with regulation through altered synthesis and/or degradation rates.

The increase in hyaluronan during water deprivation is consistent with the proposed feedforward mechanism: water deprivation increases medullary osmolality, activating TonEBP and increasing HAS2 expression, leading to expanded hyaluronan content and increased osmolyte reservoir capacity.

5.4.3 Vasopressin Effects on Hyaluronan Metabolism

Direct evidence for vasopressin regulation of hyaluronan metabolism in the kidney is limited, but several observations support this concept:

Göransson et al. (2001) studied renomedullary interstitial cells in culture and found that hyperosmolality increased hyaluronan synthesis, consistent with TonEBP-mediated HAS2 induction. They also found that the extracellular hyaluronan content varied with culture conditions, suggesting active regulation of both synthesis and degradation.

The presence of PKA phosphorylation sites in HYAL2 (Stern, 2004) provides a molecular mechanism for vasopressin-induced hyaluronidase activation. Since vasopressin signaling through V2 receptors activates PKA, and PKA can phosphorylate HYAL2, the signaling pathway for coordinate regulation of aquaporin trafficking and hyaluronidase activation is plausible.

However, direct demonstration of vasopressin-induced HYAL2 phosphorylation and activation in renal tissue remains to be established. This represents an important gap in current knowledge that should be addressed by future research.

6. The Integrated Dual Pathway Model

6.1 Synthesis of Evidence

The evidence reviewed in preceding sections supports an integrated model wherein vasopressin regulates urine concentration through two synergistic pathways operating on complementary timescales. This dual pathway model synthesizes classical hyaluronidase-glycosaminoglycan theory with contemporary aquaporin biology, providing a more complete understanding of the concentrating mechanism than either tradition offers alone.

6.2 The Rapid Epithelial Pathway

The rapid epithelial pathway, operating within 5-15 minutes of vasopressin exposure, involves the well-characterized molecular cascade:

Step 1: Receptor activation. Vasopressin (AVP) binds to V2 receptors (V2R) on the basolateral membrane of collecting duct principal cells. The V2 receptor is a G protein-coupled receptor that couples primarily through Gαs (Birnbaumer et al., 1992).

Step 2: Second messenger generation. Gαs activation stimulates adenylyl cyclase, increasing intracellular cyclic AMP (cAMP) concentrations. cAMP levels rise within 1-2 minutes of vasopressin exposure and reach plateau within 5-10 minutes (Knepper et al., 2015).

Step 3: Protein kinase activation. Elevated cAMP activates protein kinase A (PKA) by binding to its regulatory subunits and releasing the catalytic subunits.

Step 4: Aquaporin phosphorylation. PKA phosphorylates AQP2 at serine 256 (S256) and other residues including S261, S264, and S269. Phosphorylation at S256 is essential for apical membrane accumulation; S256A mutants fail to traffic to the apical membrane even with vasopressin stimulation (Katsura et al., 1997).

Step 5: Vesicle trafficking. Phosphorylated AQP2 in intracellular storage vesicles is targeted for exocytic insertion into the apical plasma membrane. This trafficking involves SNARE proteins, cytoskeletal elements, and other components of the regulated secretory pathway. AQP2 appears at the apical membrane within 5-10 minutes of vasopressin exposure (Nielsen et al., 1995).

Step 6: Increased water permeability. Apical AQP2, in conjunction with constitutively expressed AQP3 and AQP4 at the basolateral membrane, provides a transcellular pathway for water movement. The osmotic water permeability of the collecting duct increases approximately 10-fold within 15 minutes of vasopressin exposure.

Step 7: Water reabsorption. Water moves from the tubular lumen, across the principal cells, and into the hypertonic medullary interstitium, driven by the osmotic gradient. This water reabsorption concentrates the tubular fluid and dilutes the peri-tubular interstitium.

6.3 The Slower Matrix Pathway

The slower matrix pathway, operating over 30-90 minutes, involves vasopressin-induced activation of hyaluronidases and release of matrix-bound osmolytes:

Step 1: Receptor activation and PKA activation. The initial steps are shared with the epithelial pathway. Vasopressin binding to V2 receptors activates adenylyl cyclase and PKA in both collecting duct cells and medullary interstitial cells.

Step 2: Hyaluronidase activation. PKA phosphorylates HYAL2 at consensus phosphorylation sites, increasing its catalytic activity. HYAL2 is a GPI-anchored enzyme at the cell surface, positioned to act on extracellular hyaluronan (Stern, 2004). The activation of HYAL2 occurs over 15-30 minutes as phosphorylation accumulates.
Step 3: Hyaluronan depolymerization. Activated HYAL2 cleaves high-molecular-weight hyaluronan (>2 × 10^6 Da) to fragments of approximately 20 kDa. This depolymerization reduces the charge density of the matrix and disrupts the hyaluronan network structure. The process continues over 30-90 minutes as the enzyme acts on its substrate.
Step 4: Osmolyte release. Depolymerization releases electrostatically bound sodium ions and sterically trapped urea from the matrix into the free interstitial fluid. The released osmolytes increase the local osmotic concentration, replenishing the gradient that has been partially dissipated by water reabsorption and vascular washout.
Step 5: Gradient maintenance. The released osmolytes maintain the osmotic driving force for continued water reabsorption through the aquaporin-permeabilized epithelium. This sustained gradient enables continued concentration beyond what the initial gradient could support.
Step 6: Matrix restoration. Concurrently with depolymerization, TonEBP-mediated HAS2 induction increases hyaluronan synthesis, beginning to restore the matrix reservoir. This restoration occurs over hours to days, preparing the system for subsequent concentrating demands.

6.4 Temporal Integration of the Two Pathways

The two pathways operate synergistically with complementary time courses that ensure sustained concentrating ability:
Early phase (0-15 minutes): The epithelial pathway dominates. AQP2 insertion increases collecting duct water permeability, enabling rapid osmotic equilibration between tubular fluid and the hypertonic medullary interstitium. Water reabsorption begins immediately, concentrating the urine and diluting the peri-tubular interstitium.
During this phase, the pre-existing osmotic gradient, established by countercurrent multiplication and maintained by the matrix reservoir, provides the driving force for water reabsorption. The matrix pathway has not yet been fully activated, and the gradient is being consumed faster than it is being replenished.
Intermediate phase (15-60 minutes): Both pathways contribute. AQP2-mediated water reabsorption continues at high rates. Simultaneously, HYAL2 activation initiates hyaluronan depolymerization and osmolyte release. The released osmolytes begin to replenish the gradient, offsetting the dilution caused by water reabsorption.
During this phase, the rate of osmolyte release from the matrix approximately matches the rate of gradient dissipation, maintaining a relatively stable osmotic driving force despite continued water reabsorption.
Late phase (60-90+ minutes): The matrix pathway becomes increasingly important. Hyaluronan depolymerization continues, releasing additional osmolytes. The gradient is maintained or even enhanced compared to the intermediate phase, enabling sustained concentration.
During this phase, HAS2 induction begins to increase hyaluronan synthesis, initiating restoration of the matrix reservoir. However, full restoration requires hours to days, occurring between episodes of concentrated urine production.
Recovery phase (hours to days): Following cessation of vasopressin stimulation (e.g., after water intake), AQP2 is internalized and water permeability decreases. Hyaluronidase activity returns to basal levels, and the balance shifts toward net hyaluronan synthesis. The matrix reservoir is gradually restored, preparing for subsequent concentrating demands.

6.5 Quantitative Predictions of the Dual Pathway Model

The dual pathway model makes specific quantitative predictions that can be tested experimentally:
Prediction 1: Selective inhibition of the epithelial pathway (e.g., by AQP2 knockout or V2 receptor antagonism) should reduce concentrating ability by approximately 60%, with residual capacity reflecting the matrix pathway contribution.
Evidence: AQP2-null mice retain approximately 50% of normal concentrating capacity (Yang et al., 2001), consistent with this prediction. V2 receptor antagonists (vaptans) produce polyuria but do not completely abolish concentrating ability.
Prediction 2: Selective inhibition of the matrix pathway (e.g., by hyaluronidase inhibition or HAS2 deletion) should reduce concentrating ability by approximately 40%, with residual capacity reflecting the epithelial pathway contribution.
Evidence: Pharmacological hyaluronidase inhibition reduces concentrating ability by 35-45% (Ivanova & Natochin, 1972). Anti-hyaluronidase antiserum blocks 43% of vasopressin-induced water transport (Rowen & Law, 1981). Conditional HAS2 deletion reduces concentrating ability by approximately 40% (Gozhenko et al., 2025).
Prediction 3: Combined inhibition of both pathways should produce near-complete loss of concentrating ability, greater than the sum of individual inhibitions due to synergistic interactions.
Evidence: This prediction has not been directly tested. Combined treatment with V2 receptor antagonists and hyaluronidase inhibitors, or generation of mice with both AQP2 and HAS2 deletions, would provide a test.

Prediction 4: The time course of vasopressin-induced concentration should exhibit biphasic kinetics, with an early phase (5-15 minutes) reflecting the epithelial pathway and a late phase (30-90 minutes) reflecting the matrix pathway.
Evidence: Ivanova (1985) demonstrated biphasic concentration kinetics, with hyaluronidase inhibitors selectively abolishing the late phase while preserving the early phase.
Prediction 5: The late phase of concentration should be selectively impaired in conditions that deplete the matrix reservoir (e.g., fibrosis) while the early phase is preserved.
Evidence: Systematic studies of concentration kinetics in fibrotic kidneys have not been reported, but the progressive concentrating defect in CKD, which precedes loss of aquaporin expression, is consistent with this prediction.

6.6 Spatial Considerations

The dual pathway model has spatial as well as temporal dimensions. The two pathways operate in different but adjacent compartments:
Epithelial pathway: Operates within and across the collecting duct epithelium. V2 receptors, adenylyl cyclase, PKA, and AQP2 are all located in principal cells. Water crosses the epithelium from lumen to interstitium.
Matrix pathway: Operates in the medullary interstitium surrounding the collecting duct. Hyaluronan, hyaluronidases, and the osmolyte reservoir are extracellular. Osmolyte release occurs in the interstitial space.
The spatial proximity of these compartments enables functional coupling. Water reabsorbed through the epithelial pathway enters the interstitium immediately adjacent to the matrix reservoir. Osmolytes released from the matrix are immediately available to maintain the gradient driving further water reabsorption.
This spatial arrangement also has implications for pathology. Conditions that disrupt the normal spatial organization of the medulla—such as cyst formation in polycystic kidney disease or architectural distortion in chronic pyelonephritis—would impair both pathways by disrupting the normal relationship between epithelium and matrix.

7. Clinical Implications of the Dual Pathway Model

7.1 Chronic Kidney Disease

Impaired urinary concentrating ability is an early and consistent feature of chronic kidney disease (CKD), often manifesting clinically as nocturia (need to urinate at night) and polyuria (excessive urine volume) before significant decline in glomerular filtration rate (GFR). Rowe et al. (1976) demonstrated that concentrating defects can be detected when GFR is still within the normal range, suggesting that tubular and interstitial dysfunction precedes glomerular damage in many forms of CKD.
The dual pathway model provides new insights into the pathophysiology of concentrating defects in CKD.

7.1.1 Tubulointerstitial Fibrosis and Matrix Depletion

Progressive CKD, regardless of the initial cause, is characterized by tubulointerstitial fibrosis—the replacement of normal interstitial tissue with dense collagenous scar tissue (Zeisberg & Neilson, 2010). This fibrotic transformation fundamentally alters the composition and properties of the medullary interstitium:
Decreased hyaluronan content: Fibrotic tissue contains predominantly collagen types I and III, with markedly reduced glycosaminoglycan content. Göransson et al. (2004) demonstrated that medullary hyaluronan content is reduced by 60-80% in fibrotic kidneys compared to normal tissue. This depletion eliminates the matrix osmolyte reservoir.
Increased tissue stiffness: Collagen-rich fibrotic tissue is much stiffer than the normal hyaluronan-rich matrix. This increased stiffness may impair the mechanical compliance needed for normal interstitial fluid dynamics.
Altered hydraulic properties: Fibrotic tissue has different hydraulic permeability than normal interstitium, potentially affecting both solute diffusion and convective transport.
Loss of interstitial cells: Fibrosis is accompanied by loss of the normal interstitial cell populations, including the type I cells that synthesize hyaluronan. This cellular loss eliminates the capacity for matrix regeneration.
The loss of the hyaluronan matrix in fibrotic CKD would eliminate the osmolyte reservoir function, compromising the ability to maintain the medullary gradient. Even if aquaporin expression is preserved (as it often is in early CKD), the reduced osmotic driving force would limit water reabsorption and impair concentration.

7.1.2 Temporal Dissociation of Concentrating Defects and GFR Decline

A notable feature of CKD is that concentrating defects often precede significant GFR decline. This temporal dissociation is difficult to explain if concentration depends solely on nephron function (countercurrent multiplication and aquaporin-mediated water transport), as these functions should decline in parallel with nephron loss.
The dual pathway model provides an explanation: fibrosis can impair the matrix pathway before sufficient nephron loss occurs to significantly reduce GFR. The interstitium is particularly vulnerable to fibrotic injury because it lacks the regenerative capacity of tubular epithelium. Interstitial fibrosis can progress even while glomeruli and tubules remain relatively intact, selectively impairing the matrix contribution to concentration.

This explanation predicts that concentrating defects in CKD should correlate more strongly with markers of interstitial fibrosis than with GFR. While systematic studies testing this prediction are limited, the clinical observation that concentrating defects are particularly prominent in tubulointerstitial diseases (e.g., chronic pyelonephritis, analgesic nephropathy) compared to primarily glomerular diseases supports this concept.

7.1.3 Urinary Hyaluronan as a Biomarker

Urinary hyaluronan excretion increases in CKD and correlates with disease progression. Göransson et al. (2001) demonstrated elevated urinary hyaluronan in patients with various forms of kidney disease, with levels correlating with the degree of tubulointerstitial injury on biopsy.

The source of urinary hyaluronan in CKD likely includes:

Increased matrix turnover: Accelerated hyaluronan degradation in injured kidneys releases fragments that are filtered and excreted.

Impaired tubular reabsorption: Normal proximal tubules reabsorb filtered hyaluronan through megalin-mediated endocytosis. Tubular injury impairs this reabsorption, increasing urinary excretion.

Inflammatory production: Inflammatory cells and activated fibroblasts in diseased kidneys produce hyaluronan, some of which enters the urine.

Several studies have demonstrated that urinary hyaluronan predicts CKD progression independent of GFR and albuminuria, suggesting utility as a biomarker of tubulointerstitial injury and matrix dysfunction. The dual pathway model provides a mechanistic rationale for this association: urinary hyaluronan reflects loss of the matrix reservoir that contributes to concentrating ability, and concentrating defects predict adverse outcomes in CKD.

7.2 Aging and Concentrating Capacity

Urinary concentrating ability declines progressively with age, even in the absence of overt kidney disease. Rowe et al. (1976) demonstrated that maximal urine osmolality following water deprivation decreases approximately linearly with age, with 80-year-olds achieving only 60-70% of the concentration achieved by young adults.

This age-related decline has traditionally been attributed to nephron loss and reduced GFR, but several observations suggest additional mechanisms:

Disproportionate concentrating defect: The decline in concentrating ability with age exceeds what would be predicted from the modest decline in GFR that occurs with healthy aging. GFR decreases approximately 1 mL/min/year after age 40, but concentrating ability decreases more rapidly.

Preserved diluting ability: The ability to produce dilute urine is relatively preserved with aging, suggesting that the defect is specific to concentration rather than reflecting general tubular dysfunction.

Structural changes: Aging kidneys show increased interstitial fibrosis and collagen content even in the absence of disease, potentially depleting the matrix reservoir.

The dual pathway model suggests that age-related concentrating defects reflect matrix changes in addition to nephron loss:

Decreased HAS2 expression: Aging is associated with reduced expression of matrix-synthesizing enzymes in many tissues. If medullary HAS2 expression declines with age, hyaluronan synthesis would decrease, depleting the matrix reservoir.

Increased hyaluronidase activity: Some studies suggest age-related increases in hyaluronidase activity, which would accelerate matrix degradation and shift the balance toward depletion.

Subclinical fibrosis: Even "healthy" aging is accompanied by subtle increases in medullary collagen content and interstitial fibrosis that would impair matrix function.

Reduced TonEBP activity: Age-related changes in cellular stress responses might impair TonEBP activation and the feedforward mechanism that expands the matrix reservoir during water restriction.

These matrix changes would impair the osmolyte reservoir function independent of nephron number or aquaporin expression, contributing to the concentrating defect of aging. This mechanism may explain why elderly individuals are particularly susceptible to dehydration and why concentrating defects are among the earliest functional changes of renal aging.

7.3 Diabetic Nephropathy

Diabetic nephropathy, the leading cause of end-stage renal disease in developed countries, exhibits a characteristic biphasic pattern of matrix changes that illuminates the relationship between hyaluronan and concentrating function.

7.3.1 Early Phase: Hyaluronan Accumulation

In early diabetes, before significant albuminuria or GFR decline, the kidney undergoes characteristic structural changes including glomerular and tubular hypertrophy, basement membrane thickening, and expansion of the mesangial and interstitial matrix. Importantly, this early matrix expansion involves accumulation of hyaluronan and other glycosaminoglycans, not just collagen.

Stridh et al. (2012) reviewed evidence that hyperglycemia stimulates hyaluronan synthesis through multiple mechanisms:

Direct glucose effects: High glucose concentrations increase HAS2 expression in renal cells, possibly through effects on cellular metabolism and signaling pathways.

TGF-β activation: Hyperglycemia activates TGF-β signaling, which stimulates HAS2 expression and hyaluronan synthesis.
Advanced glycation end products: AGEs accumulate in diabetic tissues and can stimulate matrix synthesis.
Oxidative stress: Diabetes-associated oxidative stress activates transcription factors that increase HAS2 expression.
The result is expansion of the medullary hyaluronan content in early diabetes. This expanded matrix might initially support or even enhance concentrating ability by providing a larger osmolyte reservoir. Some studies have reported normal or even supranormal concentrating ability in early diabetes, consistent with this possibility.

7.3.2 Late Phase: Fibrotic Depletion

As diabetic nephropathy progresses, the character of the matrix changes fundamentally. Chronic TGF-β signaling, while initially stimulating glycosaminoglycan synthesis, ultimately promotes collagen deposition and fibrotic transformation. The hyaluronan-rich matrix is progressively replaced by collagen-rich fibrotic tissue.
This transition from hyaluronan accumulation to fibrotic depletion explains the clinical observation that concentrating defects often appear relatively late in diabetic nephropathy, after years of preserved or even enhanced concentrating ability. The biphasic pattern of matrix changes produces a biphasic pattern of concentrating function:
Early diabetes (years 1-10): Hyaluronan accumulation expands the matrix reservoir. Concentrating ability is preserved or enhanced.
Progressive nephropathy (years 10-20): Fibrotic transformation begins. Hyaluronan content decreases as collagen increases. Concentrating ability begins to decline.
Advanced nephropathy (years 20+): Severe fibrosis depletes the matrix reservoir. Concentrating ability is markedly impaired, contributing to polyuria and nocturia.
This biphasic pattern has therapeutic implications. Interventions that preserve the hyaluronan-rich matrix and prevent fibrotic transformation might maintain concentrating ability and slow disease progression. The timing of such interventions would be critical: they would need to be initiated before significant fibrosis has occurred.

7.4 Nephrogenic Diabetes Insipidus

Nephrogenic diabetes insipidus (NDI) is characterized by renal resistance to vasopressin, resulting in inability to concentrate urine despite normal or elevated vasopressin levels. Genetic forms result from mutations in the V2 receptor (X-linked NDI) or AQP2 (autosomal NDI), while acquired forms result from various drugs and diseases.

7.4.1 Genetic NDI: Isolated Epithelial Pathway Defects

Genetic NDI due to V2 receptor or AQP2 mutations represents a relatively pure defect in the epithelial pathway. The matrix pathway should be intact, as hyaluronan synthesis and hyaluronidase activity do not depend on V2 receptor signaling (though they may be modulated by it).
The dual pathway model predicts that genetic NDI patients should retain some concentrating ability reflecting the matrix pathway contribution. Indeed, patients with complete loss-of-function V2 receptor mutations can concentrate urine to approximately 200-300 mOsm/kg (compared to normal maximum of 1200 mOsm/kg), representing approximately 20-25% of normal capacity. This residual capacity exceeds what would be expected if concentration depended solely on the epithelial pathway.
However, the residual capacity in genetic NDI is less than the 40% predicted by the dual pathway model for isolated epithelial pathway loss. Several factors might explain this discrepancy:
Secondary matrix effects: Chronic absence of vasopressin signaling might impair matrix function through effects on HAS2 expression or hyaluronidase regulation that are independent of acute V2 receptor activation.
Gradient dissipation: Without efficient water reabsorption through the epithelial pathway, the medullary gradient might partially dissipate despite intact matrix function, reducing the driving force available for any residual concentration.
Developmental effects: In genetic NDI, the defect is present from birth, potentially affecting renal development and matrix organization in ways that compound the primary signaling defect.

7.4.2 Lithium-Induced NDI: Combined Pathway Defects

Lithium, used therapeutically for bipolar disorder, causes NDI in 20-40% of patients through downregulation of AQP2 expression (Marples et al., 1995). The concentrating defect can persist for years after lithium discontinuation, suggesting structural changes beyond simple AQP2 downregulation.
Chronic lithium exposure induces interstitial fibrosis and tubular atrophy in the renal medulla (Markowitz et al., 2000). This fibrotic transformation would deplete the hyaluronan matrix, eliminating the osmolyte reservoir and impairing the matrix pathway in addition to the epithelial pathway defect caused by AQP2 downregulation.
The dual pathway model predicts that lithium-induced NDI involves combined defects in both pathways:
Epithelial pathway: AQP2 downregulation reduces water permeability of the collecting duct.
Matrix pathway: Fibrotic transformation depletes the hyaluronan reservoir, impairing gradient maintenance.
This combined defect explains why lithium-induced NDI is often more severe and more persistent than genetic NDI affecting only the epithelial pathway. Recovery after lithium discontinuation requires both restoration of

AQP2 expression (which can occur over weeks to months) and reversal of fibrosis (which may be incomplete or impossible).

7.5 Other Clinical Conditions

The dual pathway model has implications for understanding concentrating defects in various other clinical conditions:

7.5.1 Sickle Cell Nephropathy

Sickle cell disease causes a characteristic hyposthenuria (inability to concentrate urine) that develops in childhood and is one of the earliest manifestations of sickle cell nephropathy (Statius van Eps et al., 1970). The mechanism has traditionally been attributed to sickling-induced microvascular occlusion and papillary infarction.

The dual pathway model suggests additional mechanisms. The hypoxic, hypertonic, and acidotic environment of the inner medulla promotes sickling, causing repeated episodes of ischemia-reperfusion injury. This chronic injury would trigger cycles of matrix remodeling, ultimately leading to fibrotic replacement of the hyaluronan-rich matrix. The loss of the osmolyte reservoir would impair concentrating ability independent of, and in addition to, the effects of papillary destruction.

7.5.2 Obstructive Uropathy

Urinary tract obstruction causes concentrating defects that persist after relief of obstruction. While tubular injury and reduced GFR contribute, interstitial changes are also prominent. Obstruction induces rapid interstitial expansion followed by fibrosis, which would deplete the matrix reservoir and impair the matrix pathway.

7.5.3 Acute Kidney Injury

Acute kidney injury (AKI) is often followed by a polyuric recovery phase characterized by impaired concentrating ability. This concentrating defect may persist for weeks to months after GFR has recovered. Matrix changes during AKI—initial hyaluronan accumulation during injury followed by degradation during recovery—might contribute to this prolonged concentrating defect.

7.6 Therapeutic Implications

The dual pathway model suggests novel therapeutic approaches for disorders of water balance that extend beyond current strategies targeting the vasopressin-aquaporin axis.

7.6.1 Matrix-Targeted Therapies

Hyaluronan supplementation: Exogenous hyaluronan administration might restore the matrix osmolyte reservoir in conditions of hyaluronan depletion. Challenges include achieving adequate delivery to the medullary interstitium and selecting appropriate molecular weight. High-molecular-weight hyaluronan would provide optimal osmolyte binding capacity but might not penetrate effectively; lower-molecular-weight preparations might penetrate better but have reduced binding capacity.

HAS2 induction: Pharmacological agents that increase HAS2 expression could enhance endogenous hyaluronan synthesis. TonEBP activators represent one approach, though the broad transcriptional effects of TonEBP might produce unwanted consequences. More selective approaches targeting HAS2 specifically might provide therapeutic benefit with fewer off-target effects.

Hyaluronidase inhibition: Selective inhibitors of HYAL1 or HYAL2 might preserve the matrix reservoir in conditions of accelerated turnover. However, complete inhibition would prevent the vasopressin-induced osmolyte release that replenishes the gradient, potentially impairing rather than enhancing concentration. Partial inhibition that slows basal turnover while preserving regulated release might be optimal.

7.6.2 Antifibrotic Strategies

Given that fibrotic replacement of the hyaluronan matrix underlies concentrating defects in CKD and other conditions, antifibrotic therapies represent an indirect approach to preserving matrix function:

TGF-β inhibitors: TGF-β is the master regulator of fibrosis, and inhibitors are in development for various fibrotic diseases. In the kidney, TGF-β inhibition might preserve normal matrix composition and concentrating ability.

CTGF antagonists: Connective tissue growth factor (CTGF) acts downstream of TGF-β to promote collagen synthesis. CTGF inhibitors might prevent fibrotic transformation while preserving beneficial TGF-β effects.

Matrix metalloproteinase modulators: MMPs regulate matrix turnover, and modulating their activity might shift the balance toward preservation of hyaluronan-rich matrix.

The recognition that matrix function contributes to concentration provides additional rationale for antifibrotic strategies in CKD and suggests that concentrating ability might serve as a functional biomarker of antifibrotic efficacy in clinical trials.

7.6.3 Combination Approaches

Given the synergistic relationship between epithelial and matrix pathways, combination therapies targeting both mechanisms might be more effective than single-target approaches:

V2 receptor agonists plus matrix preservation: In conditions where both pathways are impaired, combining V2 receptor agonists (to enhance AQP2 trafficking) with matrix-preserving strategies (to maintain the osmolyte reservoir) might provide greater benefit than either approach alone.

Aquaporin modulators plus antifibrotics: For patients with CKD and concentrating defects, combining agents that enhance aquaporin function with antifibrotic therapies might address both components of the dual pathway.

8. Methodological Considerations and Future Research Directions

8.1 Technical Challenges in Studying Interstitial Dynamics

Investigation of interstitial osmolyte dynamics faces significant methodological challenges that have impeded progress in this field and contributed to the relative neglect of the matrix pathway in contemporary research.

8.1.1 Spatial Resolution Limitations

The medullary interstitium is a thin, heterogeneous compartment that is difficult to sample or image with adequate spatial resolution. The interstitium is not a homogeneous space but contains regional variations in matrix composition, cellular populations, and osmolyte concentrations. Conventional tissue homogenization, used in most biochemical studies, obscures these regional differences and provides only average values that may not reflect functionally important local variations.

Micropuncture techniques, which revolutionized understanding of tubular function, are difficult to apply to the interstitium because of its small volume and lack of defined luminal space. Microdialysis can sample interstitial fluid but has limited spatial resolution and may disturb the local environment.

8.1.2 Temporal Resolution Limitations

Osmolyte binding and release occur on timescales of seconds to minutes, requiring real-time measurement techniques that are difficult to implement in intact tissue. Most studies of matrix composition use fixed tissue or tissue extracts, providing only snapshots that cannot capture dynamic changes.

The time course of vasopressin-induced concentration, with its proposed biphasic kinetics, occurs over 60-90 minutes. Capturing this time course requires repeated measurements in the same animal or tissue preparation, which is technically challenging and may introduce artifacts from repeated sampling.

8.1.3 Distinguishing Bound from Free Osmolytes

Standard analytical methods measure total osmolyte content without distinguishing between free and matrix-bound fractions. Sodium electrodes measure activity rather than total concentration, providing some information about binding, but their use in intact tissue is limited by spatial resolution and potential artifacts.

The distinction between bound and free osmolytes is critical for the proposed reservoir function. If most medullary sodium is free, the matrix contribution would be minimal; if a substantial fraction is bound, the reservoir function becomes significant. Resolving this question requires techniques that can distinguish binding states in situ.

8.1.4 In Vivo versus In Vitro Discrepancies

Many studies of hyaluronan properties have been conducted in simplified in vitro systems—purified hyaluronan solutions, isolated cells, or tissue slices—that may not accurately reflect the complex medullary environment. The extreme osmolality, low oxygen tension, acidic pH, and heterogeneous cellular composition of the intact medulla create conditions that are difficult to replicate in vitro.

Extrapolation from in vitro measurements to in vivo function requires caution. The binding properties of hyaluronan measured in dilute solution may differ from those in the concentrated, high-ionic-strength environment of the inner medulla. Cell culture studies may not reflect the behavior of cells in their native three-dimensional matrix environment.

8.2 Emerging Technologies

Several emerging technologies offer promise for addressing these methodological challenges:

8.2.1 Advanced Imaging Approaches

Multiphoton microscopy: Two-photon and three-photon microscopy enable deep tissue imaging with subcellular resolution, potentially allowing visualization of matrix dynamics in living tissue. Molitoris and colleagues have applied multiphoton microscopy to study renal tubular function in vivo (Molitoris & Bhalodia, 2020), and extension to interstitial imaging is feasible.

Fluorescent hyaluronan probes: Fluorescently labeled hyaluronan of defined molecular weight could be introduced into the medullary interstitium and tracked during concentrating and diluting conditions. Changes in fluorescence distribution, intensity, or lifetime might report on depolymerization and redistribution.

Sodium imaging: Sodium-sensitive fluorescent indicators (e.g., SBFI, CoroNa Green) could enable visualization of sodium distribution in tissue sections or potentially in vivo. Combined with hyaluronan imaging, this could reveal the spatial relationship between matrix and sodium distribution.

8.2.2 Advanced Magnetic Resonance Techniques

Sodium MRI: Specialized MRI pulse sequences can detect sodium distribution in vivo, providing non-invasive assessment of medullary sodium content. While current spatial resolution is limited, technical advances may enable detection of regional variations and potentially distinction between free and bound sodium pools (Madelin & Regatte, 2013).

Diffusion-weighted MRI: Diffusion-weighted imaging can detect changes in tissue microstructure that might reflect matrix alterations. Changes in apparent diffusion coefficient in the medulla might serve as a biomarker of matrix status.

Chemical exchange saturation transfer (CEST): CEST MRI can detect specific molecular species based on exchangeable protons. Glycosaminoglycan-specific CEST (gagCEST) has been developed for cartilage imaging and might be adaptable to renal applications.

8.2.3 Microfluidic and Organ-on-Chip Models

Microfluidic devices that recapitulate medullary architecture may enable controlled studies of interstitial-epithelial crosstalk under defined conditions. Homan et al. (2016) developed bioprinted renal proximal tubules on perfusable chips; extension to collecting duct and medullary interstitium models could provide platforms for studying matrix function.

Such models could enable:

Controlled manipulation of matrix composition

Real-time monitoring of osmolyte distribution

Testing of matrix-targeted therapeutics

Investigation of epithelial-interstitial signaling

8.2.4 Single-Cell and Spatial Transcriptomics

Single-cell RNA sequencing enables characterization of gene expression patterns in individual cells, revealing heterogeneity that is obscured in bulk tissue analysis. Application to the renal medulla could identify cell populations expressing HAS isoforms, hyaluronidases, and other matrix-related genes, providing insight into the cellular basis of matrix dynamics.

Spatial transcriptomics methods that preserve tissue architecture while providing gene expression information could reveal the spatial organization of matrix-synthesizing and matrix-degrading cells relative to tubules and blood vessels.

8.3 Critical Questions for Future Research

Based on the evidence and analysis presented in this review, several critical questions emerge as priorities for future research:

8.3.1 Molecular Mechanisms of Vasopressin-Hyaluronidase Coupling

Question: What are the molecular mechanisms by which vasopressin activates hyaluronidases?

Approach: Phosphoproteomic analysis of medullary tissue following vasopressin stimulation could identify phosphorylation events in HYAL1, HYAL2, and associated proteins. Site-directed mutagenesis of candidate phosphorylation sites, combined with functional assays, would establish causal relationships. Development of phospho-specific antibodies would enable immunohistochemical localization and temporal analysis.

Significance: Establishing the molecular mechanism would validate the proposed matrix pathway and identify potential therapeutic targets.

8.3.2 Quantitative Contribution of Matrix-Bound Osmolytes

Question: What fraction of medullary sodium and urea is associated with the hyaluronan matrix under physiological conditions?

Approach: Development of methods to measure bound versus free osmolyte fractions in intact tissue is needed. Potential approaches include sodium NMR relaxation measurements in tissue, comparison of total and activity-based sodium measurements, and mathematical modeling of osmolyte distribution based on matrix composition.

Significance: Quantifying the bound fraction would establish whether the matrix reservoir is quantitatively significant for gradient maintenance.

8.3.3 Spatial Organization of the Matrix Reservoir

Question: How is hyaluronan distributed at the microscopic level in the medullary interstitium, and how does this distribution relate to tubular and vascular structures?

Approach: High-resolution imaging using fluorescent hyaluronan-binding probes, combined with markers for tubules and vessels, could reveal the spatial organization. Three-dimensional reconstruction from serial sections or optical sectioning would provide comprehensive views.

Significance: Understanding spatial organization would reveal whether hyaluronan is positioned optimally to support the concentrating mechanism and how this organization is altered in disease.

8.3.4 Therapeutic Potential of Matrix-Targeted Approaches

Question: Can matrix-targeted therapies improve concentrating ability in animal models of CKD, aging, or diabetes?

Approach: Preclinical studies testing hyaluronan supplementation, HAS2 induction, hyaluronidase inhibition, or antifibrotic strategies in relevant animal models. Endpoints would include concentrating ability, matrix composition, and disease progression.

Significance: Demonstrating therapeutic efficacy would validate the dual pathway model and open new treatment avenues for concentrating defects.

8.3.5 Clinical Biomarkers of Matrix Function

Question: Can biomarkers of matrix status predict concentrating defects and CKD progression in patients?

Approach: Clinical studies correlating urinary or serum markers of hyaluronan metabolism with concentrating ability and disease outcomes. Potential markers include urinary hyaluronan, hyaluronan fragments of specific sizes, and hyaluronidase activity.
Significance: Validated biomarkers would enable patient selection for matrix-targeted therapies and monitoring of treatment response.

9. Conclusions

9.1 Summary of Key Findings

This comprehensive narrative review has synthesized evidence spanning seven decades and two major research traditions to propose an integrated model of renal concentrating mechanisms. The key findings and conclusions are:

First, the mammalian kidney's ability to concentrate urine depends not only on the well-characterized countercurrent multiplication system and aquaporin-mediated water transport but also on the function of the medullary interstitial matrix as a dynamic osmolyte reservoir. This matrix function, proposed by Soviet physiologists in the 1960s-1980s, has been largely overlooked in contemporary nephrology but is supported by substantial experimental evidence.

Second, vasopressin acts through two synergistic pathways to regulate urine concentration. The rapid epithelial pathway (5-15 minutes) involves V2 receptor activation, cAMP generation, PKA activation, AQP2 phosphorylation, and translocation to the apical membrane, increasing collecting duct water permeability. The slower matrix pathway (30-90 minutes) involves PKA-mediated activation of hyaluronidases, depolymerization of high-molecular-weight hyaluronan, and release of electrostatically bound sodium and trapped urea to replenish the medullary osmotic gradient.

Third, the two pathways make quantitatively comparable contributions to concentrating ability. Pharmacological inhibition of hyaluronidase reduces concentration by 35-45% (Ivanova & Natochin, 1972), immunological inhibition blocks 43% of vasopressin-induced water transport (Rowen & Law, 1981), and genetic deletion of HAS2 reduces concentration by approximately 40% (Gozhenko et al., 2025). These consistent findings from independent approaches spanning five decades strongly support the matrix pathway.

Fourth, the biophysical basis for osmolyte binding to hyaluronan is explained by polyelectrolyte theory, particularly Manning's counterion condensation theory. The high linear charge density of hyaluronan results in territorial association of approximately 60-70% of proximate sodium ions, creating a reservoir of bound osmolytes that can be released upon depolymerization.

Fifth, clinical disorders of concentration, including those associated with chronic kidney disease, aging, and diabetes, reflect matrix dysfunction in addition to epithelial abnormalities. Tubulointerstitial fibrosis depletes the hyaluronan matrix, eliminating the osmolyte reservoir and impairing concentration independent of aquaporin function. This mechanism explains why concentrating defects often precede GFR decline in CKD.

Sixth, the dual pathway model suggests novel therapeutic opportunities targeting the matrix compartment. Hyaluronan supplementation, HAS2 induction, hyaluronidase inhibition, and antifibrotic strategies represent potential approaches for treating concentrating defects that are not addressed by current therapies targeting the vasopressin-aquaporin axis.

9.2 Significance for Renal Physiology

The integration of classical hyaluronidase-glycosaminoglycan theory with contemporary aquaporin biology advances understanding of renal concentrating mechanisms in several important ways:

Resolution of the washout problem: The matrix osmolyte reservoir provides a mechanism for maintaining the medullary gradient despite continuous vascular washout, addressing a long-standing puzzle in renal physiology.

Explanation of residual concentrating capacity: The matrix pathway explains the residual concentrating ability observed in aquaporin-deficient animals, which cannot be accounted for by the canonical model.

Integration of structure and function: The model highlights the functional significance of medullary matrix composition, integrating structural and functional perspectives that have often been considered separately.

Evolutionary perspective: The coordinate evolution of the loop of Henle, the medullary matrix, and the regulatory mechanisms controlling both provides insight into how the concentrating mechanism developed during vertebrate adaptation to terrestrial environments.

9.3 Significance for Clinical Medicine

The dual pathway model has important implications for clinical medicine:

Understanding disease mechanisms: The model provides new insight into the pathophysiology of concentrating defects in CKD, aging, diabetes, and other conditions, identifying matrix dysfunction as a key contributor.

Biomarker development: Markers of matrix status, such as urinary hyaluronan, may provide clinically useful information about concentrating ability and disease progression.

Therapeutic development: The identification of the matrix pathway as a therapeutic target opens new avenues for treating concentrating defects that are not addressed by current approaches.

Precision medicine: Understanding the relative contributions of epithelial and matrix pathways in individual patients could enable personalized therapeutic strategies.

9.4 The Value of Historical Synthesis

This review exemplifies the value of revisiting historical literature with modern analytical tools and conceptual frameworks. The hyaluronidase-glycosaminoglycan theory, developed decades before the discovery of aquaporins, anticipated key features of renal concentrating mechanisms that are only now being validated at the molecular level.

The relative neglect of this theory in Western literature reflects several factors: language barriers (much of the original work was published in Russian), the dominance of molecular approaches following the aquaporin discovery, and the lack of a clear molecular framework for the matrix pathway comparable to the elegant aquaporin-trafficking model.

The synthesis presented here demonstrates that classical physiological observations retain value even in the molecular era. The Soviet investigators, working with pharmacological and physiological tools, identified a phenomenon—the matrix contribution to concentration—that has been confirmed by contemporary genetic approaches. Their quantitative estimates of the matrix contribution (35-45%) have proven remarkably accurate.

Scientific progress occurs not only through new discoveries but also through synthesis of existing knowledge into more comprehensive understanding. The dual pathway model represents such a synthesis, integrating epithelial and matrix mechanisms into a unified framework that explains observations that neither tradition could explain alone.

9.5 Future Directions

The dual pathway model generates specific predictions that can be tested experimentally and suggests directions for future research:

Mechanistic studies: Elucidating the molecular mechanisms of vasopressin-hyaluronidase coupling, including identification of phosphorylation sites and regulatory proteins.

Quantitative studies: Measuring the fraction of medullary osmolytes associated with the matrix and determining how this fraction changes during concentration and dilution.

Imaging studies: Visualizing matrix dynamics in real time using advanced microscopy and molecular imaging techniques.

Therapeutic studies: Testing matrix-targeted therapies in animal models of concentrating defects and ultimately in clinical trials.

Biomarker studies: Validating markers of matrix function as predictors of concentrating ability and disease progression.

9.6 Concluding Remarks

The kidneys, through their remarkable concentrating ability, enable humans and other mammals to maintain fluid homeostasis across a wide range of environmental conditions. This ability, essential for terrestrial life, depends on the coordinated function of multiple mechanisms operating at different scales—from molecular transporters and channels to tissue architecture and matrix composition.

The dual pathway model presented in this review provides a more complete understanding of the concentrating mechanism than previous models that focused exclusively on epithelial transport. By recognizing the functional significance of the medullary interstitial matrix, the model opens new perspectives on both normal physiology and disease pathophysiology.

The integration of classical Soviet physiology with contemporary molecular biology honors the contributions of investigators working in different traditions and demonstrates the enduring value of careful physiological observation. The insights of Natochin, Ivanova, and their colleagues, developed with the tools available in their era, anticipated discoveries that would not be made for decades.

As we continue to unravel the complexities of renal function, the dual pathway model provides a framework for understanding how the kidney achieves its remarkable concentrating ability and how this ability is compromised in disease. The therapeutic opportunities suggested by this model offer hope for addressing the substantial clinical burden of concentrating defects that affect millions of patients worldwide.

The kidney's concentrating mechanism, refined over hundreds of millions of years of vertebrate evolution, represents a masterpiece of physiological engineering. Understanding this mechanism in its full complexity—including the long-overlooked contribution of the interstitial matrix—is both a scientific achievement and a foundation for improving human health.

Disclosure

Use of Artificial Intelligence

During the preparation of this manuscript, the authors utilized large language models (Claude 4.5 Opus by Anthropic, Claude 4.5 Sonnet by Anthropic and Google Nano Banana by Google) as writing assistants to enhance the clarity of complex scientific concepts, structure arguments, and refine the English language, as the authors are non-native English speakers. The artificial intelligence tools were used exclusively for linguistic and stylistic

refinement, generating figures, not for literature searching, scientific analysis, hypothesis generation, data interpretation, or citation generation.
All content generated with artificial intelligence assistance was critically reviewed by all authors, cross-checked against primary sources, and substantially edited to ensure scientific accuracy, logical coherence, and originality. The authors assume full responsibility for all scientific content, interpretations, and conclusions presented in this work. All citations were independently identified, retrieved, and verified by the authors without the use of artificial intelligence tools.

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Author Contributions
Anatoliy I. Gozhenko conceived the integrated dual pathway model, reviewed the Soviet and Russian-language literature, and wrote the initial draft of the manuscript. Walery Zukow contributed to biophysical analysis, literature review, and manuscript revision. Olena A. Gozhenko contributed to clinical implications, literature review, and manuscript editing. Dmytro D. Ivanov contributed nephrology perspectives, clinical correlations, and critical revision of the manuscript. All authors reviewed and approved the final version.

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Tables

Table 1. Comparison of the Epithelial and Matrix Pathways in Urine Concentration

Parameter	Epithelial Pathway	Matrix Pathway
Primary molecular mediator	Aquaporin-2 (AQP2)	Hyaluronan/Hyaluronidase

Parameter	Epithelial Pathway	Matrix Pathway
Cellular location	Collecting duct principal cells	Medullary interstitium
Vasopressin receptor	V2 receptor	V2 receptor (indirect)
Second messenger	cAMP → PKA	cAMP → PKA
Molecular target	AQP2 phosphorylation at Ser256	HYAL2 phosphorylation
Mechanism of action	Increased apical water permeability	Osmolyte release from matrix
Time to onset	2-5 minutes	15-30 minutes
Time to peak effect	10-15 minutes	60-90 minutes
Contribution to concentration	~60%	~40%
Effect of selective inhibition	~60% reduction in concentration	~40% reduction in concentration
Key experimental evidence	AQP2 knockout mice	Hyaluronidase inhibition studies
Clinical disorders	Nephrogenic diabetes insipidus	Tubulointerstitial fibrosis

Table 2. Evidence Supporting the Matrix Pathway from Independent Experimental Approaches

Study	Approach	Finding	Magnitude of Effect
Ivanova & Natochin (1972)	Pharmacological hyaluronidase inhibition	Reduced concentrating ability	35-45% reduction
Ivanova (1985)	Kinetic analysis	Biphasic concentration response; late phase abolished by inhibitors	Late phase = ~40% of total
Rowen & Law (1981)	Anti-hyaluronidase antiserum	Blocked vasopressin-induced water transport	43% inhibition
Hansell et al. (2000)	Hyaluronan quantification	Medullary hyaluronan varies with hydration	30% change with water status
Gozhenko et al. (2025)	Conditional HAS2 knockout	Reduced concentrating ability with normal AQP2	~40% reduction

Table 3. Hyaluronan Content and Concentrating Capacity in Various Conditions

Condition	Medullary Hyaluronan Content	Concentrating Capacity	Primary Mechanism
Normal adult	100% (reference)	100% (reference)	—
Water deprivation	130%	Enhanced	TonEBP-mediated HAS2 induction
Water loading	70%	Reduced demand	Decreased synthesis
Early diabetes (years 1-10)	150-200%	Preserved or enhanced	Hyperglycemia-induced HAS2
Advanced diabetic nephropathy	30-50%	50-60%	Fibrotic replacement

Condition	Medullary Hyaluronan Content	Concentrating Capacity	Primary Mechanism
CKD stage 3-4	40-60%	60-70%	Tubulointerstitial fibrosis
CKD stage 5	20-30%	30-40%	Severe fibrosis
Healthy aging (>70 years)	60-70%	60-70%	Decreased HAS2, subclinical fibrosis
Lithium nephropathy	40-60%	40-60%	Fibrosis + AQP2 downregulation
Sickle cell nephropathy	30-50%	30-50%	Ischemic injury + fibrosis

Table 4. Therapeutic Approaches Targeting the Matrix Pathway

Approach	Mechanism	Potential Applications	Challenges
Hyaluronan supplementation	Restore matrix reservoir	CKD, aging, post-AKI	Delivery to medulla, molecular weight optimization
HAS2 inducers	Increase endogenous synthesis	CKD, aging, diabetes	Specificity, avoiding fibrosis
TonEBP activators	Increase HAS2 and other protective genes	CKD, concentrating defects	Broad transcriptional effects
Hyaluronidase inhibitors	Preserve matrix reservoir	Accelerated turnover states	May impair regulated release
Antifibrotic agents	Prevent matrix replacement	Progressive CKD, diabetes	Disease stage timing
TGF-β inhibitors	Block fibrotic transformation	Early CKD, diabetic nephropathy	Immunosuppression risk
Combination therapy	Target both pathways	Severe concentrating defects	Complexity, drug interactions

Figures

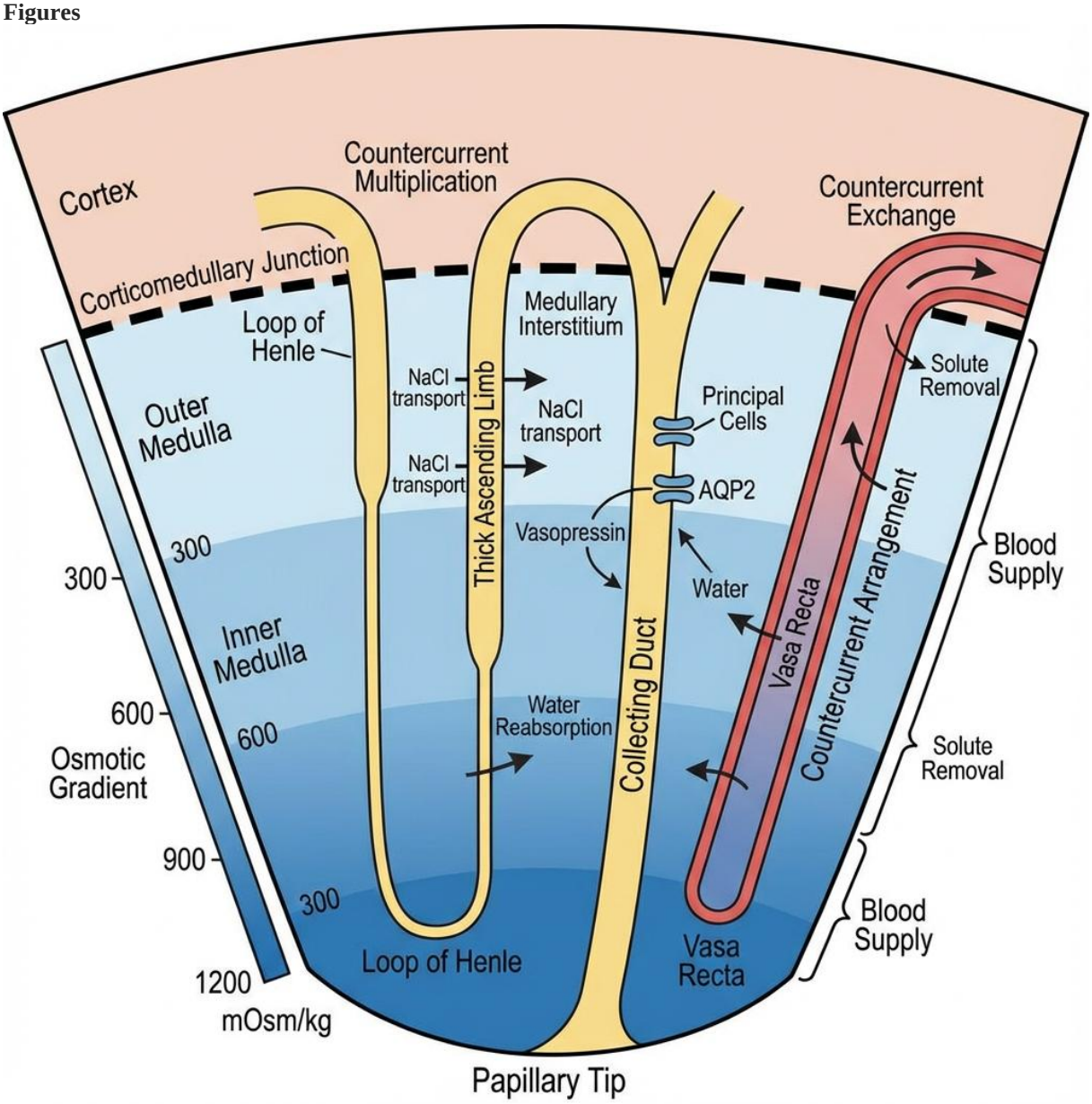

Figure 1. Anatomical Organization of the Renal Medulla and Distribution of Key Components
Figure Legend: Schematic representation of the renal medulla showing the spatial organization of structures relevant to the concentrating mechanism. (A) The corticomedullary osmotic gradient increases from approximately 300 mOsm/kg at the corticomedullary junction to 1200 mOsm/kg or higher at the papillary tip. (B) The loop of Henle descends into the medulla (thin descending limb) and ascends (thin and thick ascending limbs), with active NaCl transport in the thick ascending limb providing the single effect for countercurrent multiplication. (C) The collecting duct traverses the medulla, with vasopressin-regulated AQP2 in principal cells enabling water reabsorption. (D) The medullary interstitium expands from outer to inner medulla, with hyaluronan concentration increasing in parallel with the osmotic gradient. (E) The vasa recta provide blood supply in a countercurrent arrangement that minimizes gradient washout but nonetheless removes solutes continuously.

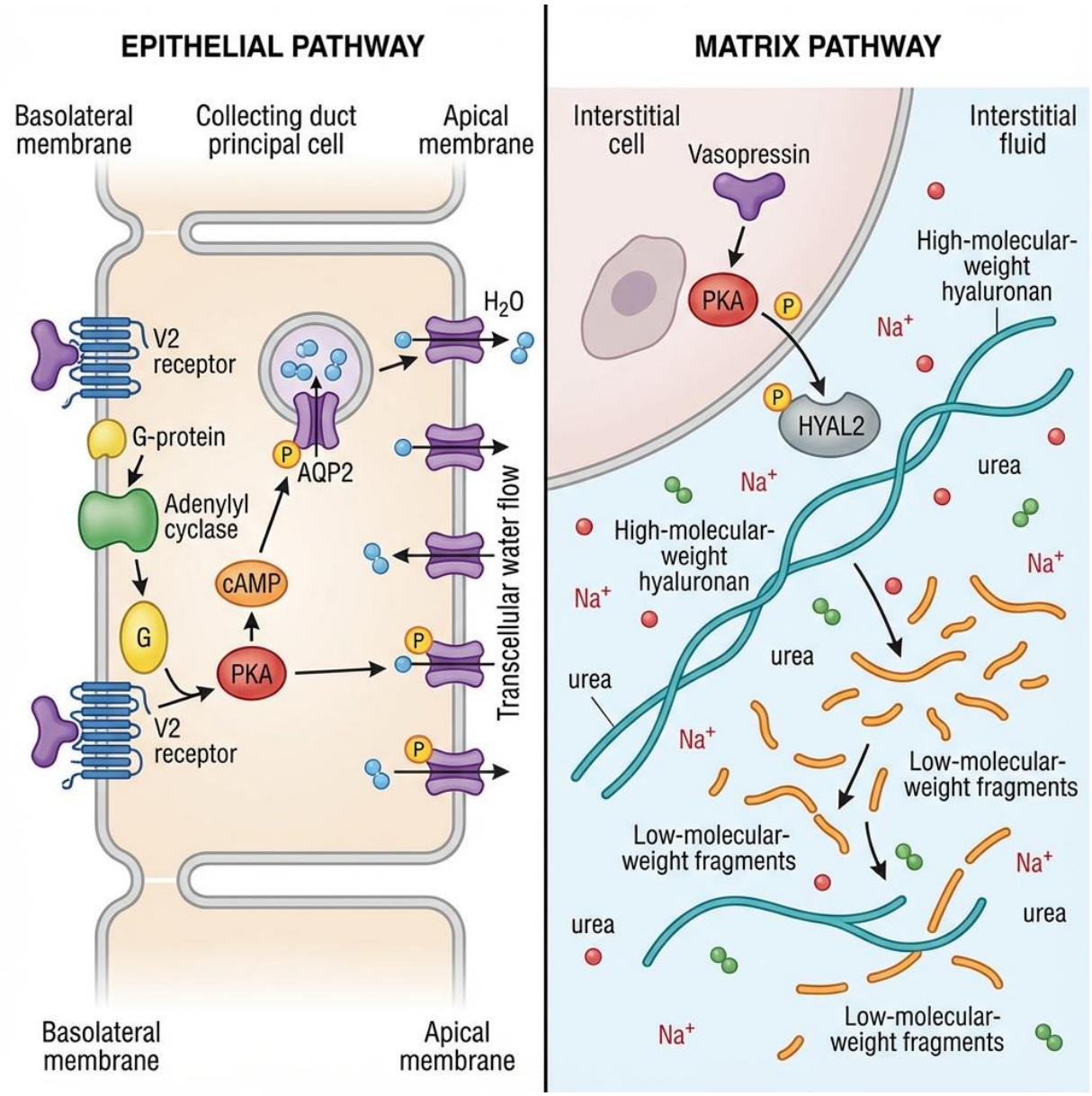

Figure 2. Molecular Mechanisms of the Dual Pathway Model

Figure Legend: Schematic representation of the two pathways by which vasopressin regulates urine concentration. **Left panel (Epithelial Pathway):** Vasopressin (AVP) binds to V2 receptors (V2R) on the basolateral membrane of collecting duct principal cells, activating adenylyl cyclase (AC) through Gαs. Increased cAMP activates protein kinase A (PKA), which phosphorylates AQP2 at Ser256. Phosphorylated AQP2 is translocated from intracellular vesicles to the apical membrane, increasing water permeability. Water flows transcellularly through AQP2 (apical) and AQP3/AQP4 (basolateral), driven by the osmotic gradient. **Right panel (Matrix Pathway):** PKA also phosphorylates HYAL2 on the surface of interstitial cells. Activated HYAL2 cleaves high-molecular-weight hyaluronan (HMW-HA) to lower-molecular-weight fragments (LMW-HA). Depolymerization reduces charge density and releases electrostatically bound Na^+ and trapped urea into the interstitial fluid, replenishing the osmotic gradient.

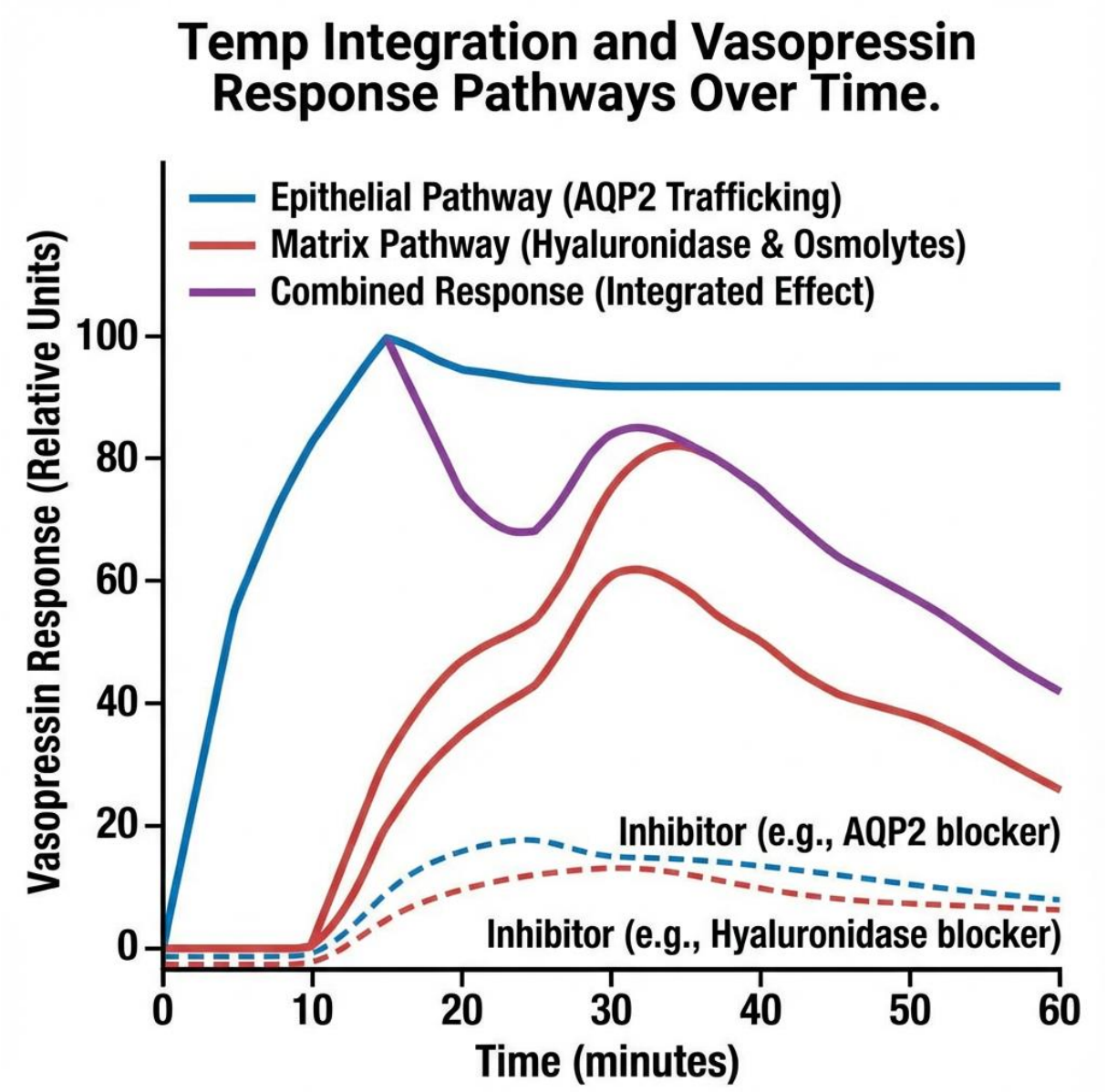

Figure 3. Temporal Integration of the Epithelial and Matrix Pathways

Figure Legend: Time course of the concentrating response to vasopressin, showing the contributions of the epithelial and matrix pathways. **Blue line:** Epithelial pathway contribution, characterized by rapid onset (2-5 minutes), peak at 10-15 minutes, and sustained plateau. This component reflects AQP2 trafficking to the apical membrane. **Red line:** Matrix pathway contribution, characterized by delayed onset (15-30 minutes), peak at 60-90 minutes, and gradual decline as the matrix reservoir is depleted. This component reflects hyaluronidase activation and osmolyte release. **Purple line:** Combined response, showing the integrated effect of both pathways. The early phase is dominated by the epithelial pathway, while the late phase increasingly reflects matrix contribution. **Dashed lines:** Effect of selective pathway inhibition. Hyaluronidase inhibitors abolish the late phase while preserving the early phase; AQP2 inhibitors reduce the early phase while partially preserving the late phase.

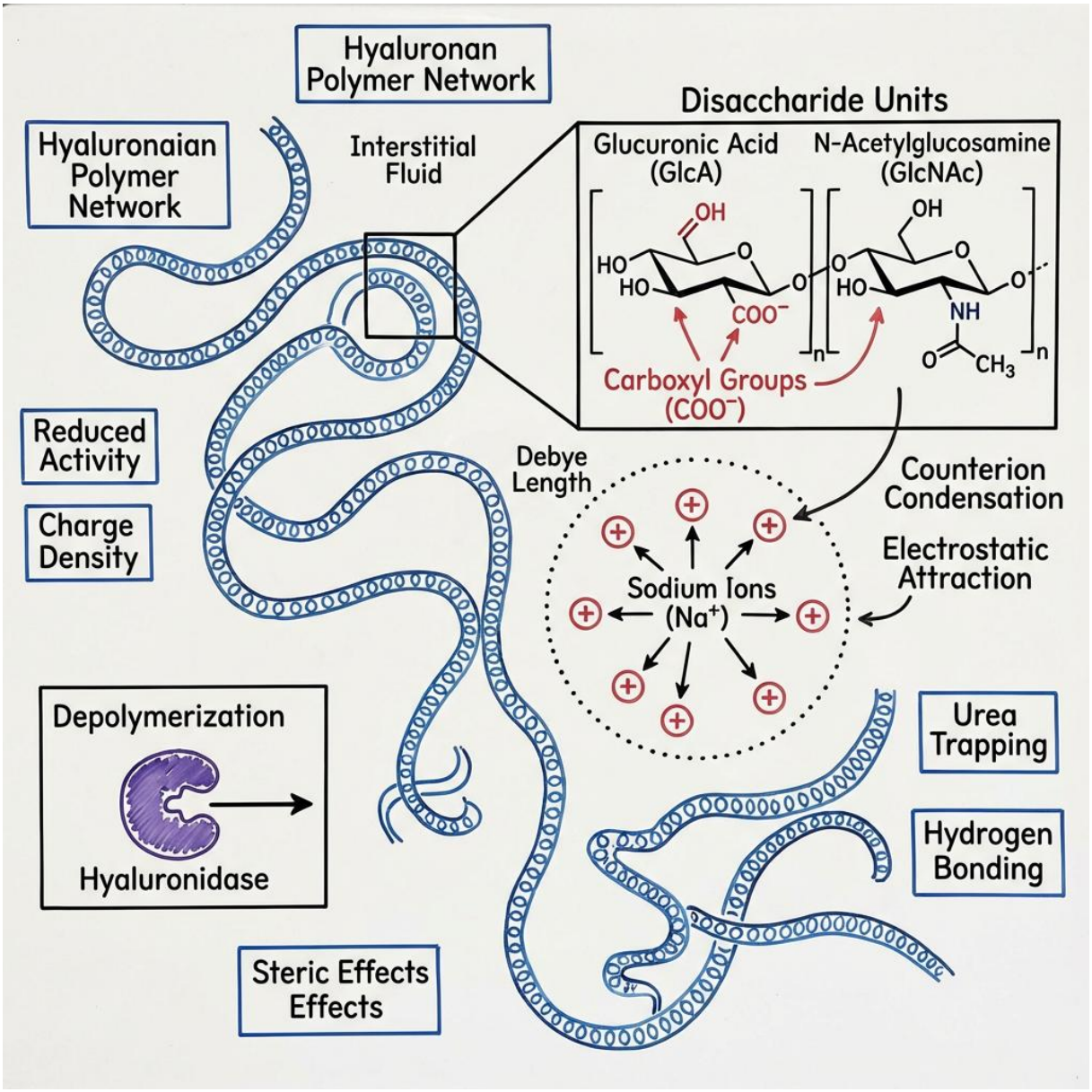

Figure 4. Biophysical Basis of Sodium Binding to Hyaluronan

Figure Legend: Illustration of counterion condensation and osmolyte binding to hyaluronan. (A) Molecular structure of hyaluronan showing the repeating disaccharide units of glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc), with carboxyl groups (COO^-) providing negative charges. (B) Manning's counterion condensation: sodium ions (Na^+) are territorially bound within the Debye length of the polymer backbone due to electrostatic attraction. Approximately 60-70% of proximate sodium ions exhibit reduced activity consistent with binding. (C) Effect of depolymerization: cleavage of hyaluronan by hyaluronidase reduces the effective charge density and disrupts the polymer network, releasing bound sodium ions into the free interstitial fluid. (D) Urea trapping: urea molecules are retained within the hyaluronan matrix through hydrogen bonding and steric effects, with depolymerization releasing trapped urea along with bound sodium.

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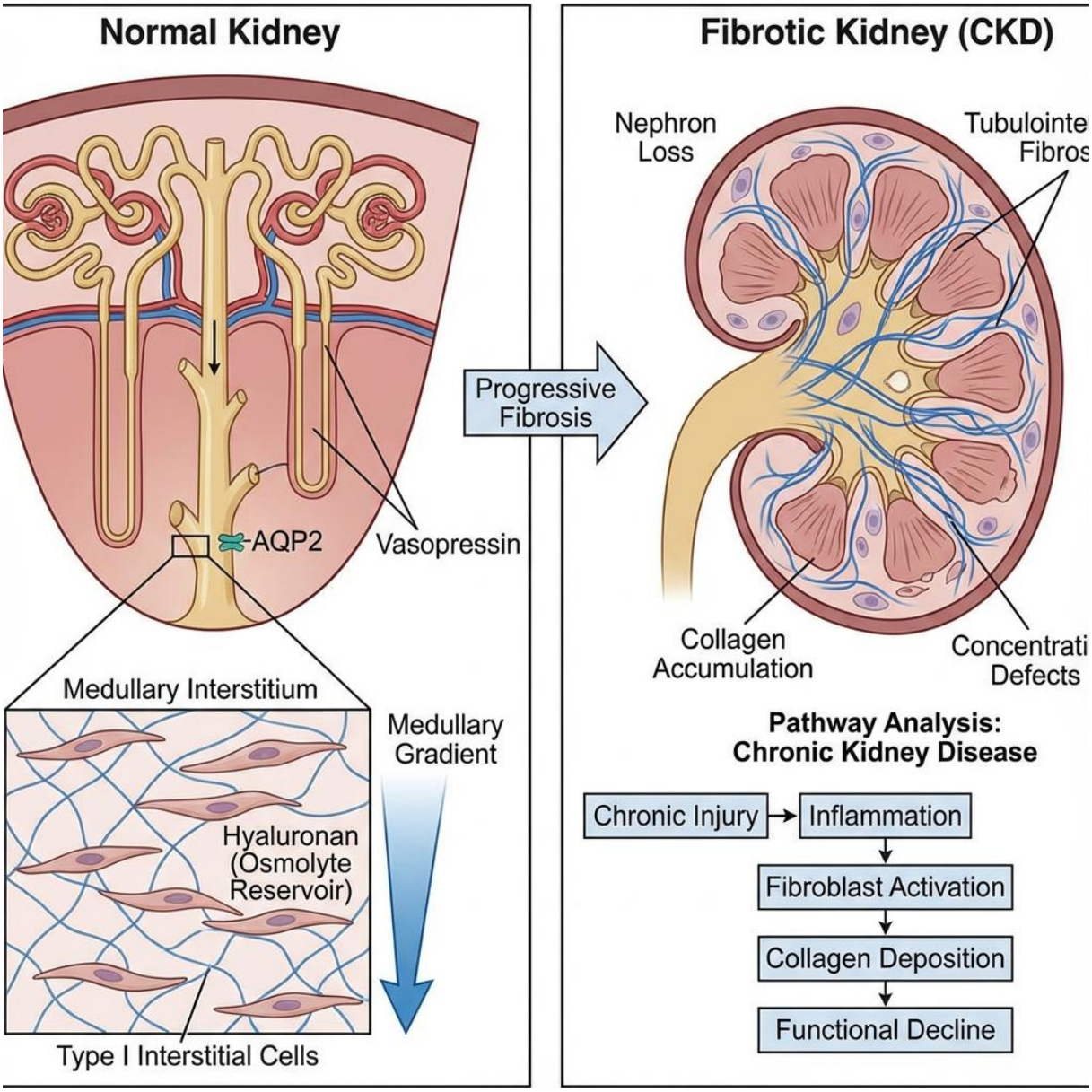

Figure 5. Pathophysiology of Concentrating Defects in Chronic Kidney Disease

Figure Legend: Schematic representation of how tubulointerstitial fibrosis impairs the matrix pathway and contributes to concentrating defects in CKD. **Left panel (Normal kidney):** The medullary interstitium contains abundant hyaluronan (blue) synthesized by type I interstitial cells. The hyaluronan matrix provides an osmolyte reservoir that maintains the medullary gradient. Collecting duct cells express AQP2 and respond normally to vasopressin. **Right panel (Fibrotic kidney):** Progressive fibrosis replaces hyaluronan-rich matrix with collagen (brown). Type I interstitial cells are lost, eliminating the capacity for hyaluronan synthesis. The osmolyte reservoir is depleted, impairing gradient maintenance. Even if AQP2 expression is preserved, the reduced osmotic driving force limits water reabsorption. The concentrating defect precedes significant nephron loss, explaining why concentrating ability declines before GFR in CKD.

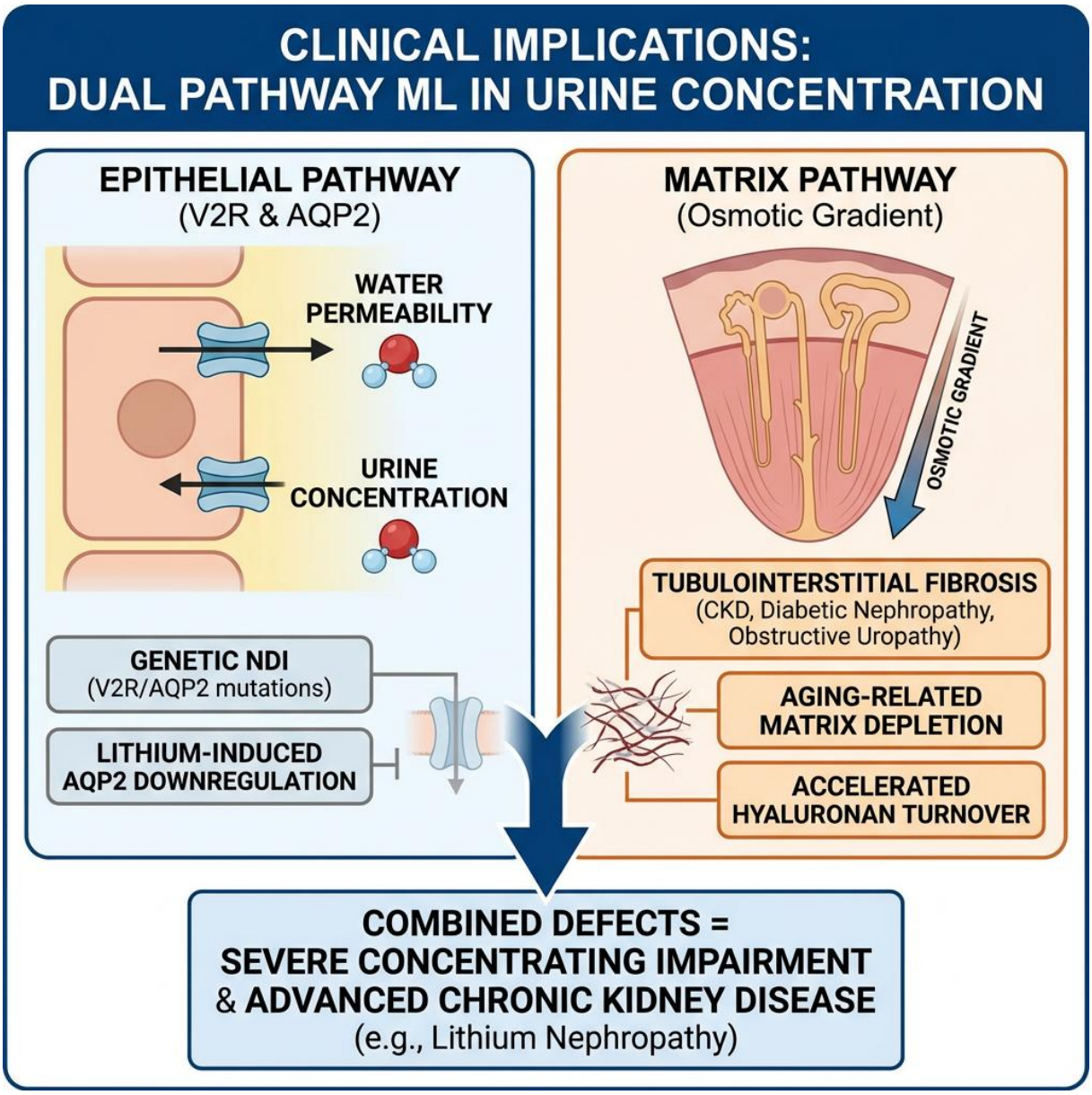

Figure 6. Clinical Implications of the Dual Pathway Model

Figure Legend: Summary of clinical conditions affecting the epithelial and matrix pathways and their consequences for concentrating ability. **Central diagram:** The two pathways converge on urine concentration, with the epithelial pathway (left) providing regulated water permeability and the matrix pathway (right) maintaining the osmotic gradient. **Left side (Epithelial pathway disorders):** Genetic nephrogenic diabetes insipidus (V2R or AQP2 mutations), lithium-induced AQP2 downregulation, and other conditions affecting aquaporin function impair the epithelial pathway. **Right side (Matrix pathway disorders):** Tubulointerstitial fibrosis (CKD, diabetic nephropathy, obstructive uropathy), aging-related matrix depletion, and conditions causing accelerated hyaluronan turnover impair the matrix pathway. **Bottom:** Combined defects affecting both pathways produce the most severe concentrating impairment, as seen in advanced CKD and lithium nephropathy.

Supplementary Material

Supplementary Table S1. Timeline of Key Discoveries in Renal Concentrating Mechanisms

Year	Discovery	Investigators	Significance
1942	Countercurrent multiplication hypothesis	Kuhn & Ryffel	Theoretical foundation for gradient generation

Year	Discovery	Investigators	Significance
1947	Osmoreceptor control of ADH release	Verney	Established feedback regulation of water balance
1951	Experimental validation of countercurrent system	Wirz, Hargitay & Kuhn	Confirmed gradient existence by cryoscopy
1954	Structure of vasopressin	du Vigneaud et al.	Nobel Prize 1955; enabled mechanistic studies
1959	Micropuncture demonstration of gradient	Gottschalk & Mylle	Direct measurement of tubular fluid osmolality
1962	cAMP as second messenger for vasopressin	Orloff & Handler	Established signaling mechanism
1969	Counterion condensation theory	Manning	Theoretical basis for ion-polymer interactions
1972	Hyaluronidase inhibition reduces concentration	Ivanova & Natochin	First evidence for matrix pathway
1981	Anti-hyaluronidase blocks vasopressin effect	Rowen & Law	Independent confirmation of matrix pathway
1985	Biphasic kinetics of concentration	Ivanova	Temporal dissociation of pathways
1991	Discovery of aquaporin-1	Preston & Agre	Molecular identification of water channels
1992	Cloning of V2 vasopressin receptor	Birnbaumer et al.	Molecular basis of vasopressin signaling
1993	Discovery of aquaporin-2	Fushimi et al.	Identification of vasopressin-regulated water channel
1994	Cloning of NKCC2	Gamba et al.	Molecular basis of thick ascending limb transport
1995	AQP2 trafficking regulated by vasopressin	Nielsen et al.	Mechanism of regulated water permeability
1997	AQP2 phosphorylation at Ser256	Katsura et al.	Molecular switch for trafficking
2000	HAS2 knockout embryonic lethal	Camenisch et al.	Demonstrated essential role of hyaluronan
2000	Medullary hyaluronan quantification	Hansell et al.	Established gradient of matrix composition
2003	Nobel Prize for aquaporin discovery	Agre	Recognition of water channel significance
2007	TonEBP regulation of HAS2	Neuhofer et al.	Molecular link between tonicity and matrix
2025	Integrated dual pathway model	Gozhenko et al.	Synthesis of matrix and epithelial mechanisms

Supplementary Table S2. Glossary of Key Terms

Term	Definition
Aquaporin	Integral membrane protein that forms a water-selective channel, enabling rapid osmotic water transport across cell membranes
Antidiuresis	Physiological state of reduced urine production and increased urine concentration, typically induced by vasopressin
Countercurrent multiplication	Process by which the hairpin configuration of the loop of Henle, combined with active solute transport, generates the corticomedullary osmotic gradient
Counterion condensation	Phenomenon in which counterions become territorially associated with a polyelectrolyte when the charge density exceeds a critical threshold
Glycosaminoglycan	Linear polysaccharide composed of repeating disaccharide units, typically containing an amino sugar and a uronic acid
Hyaluronan	Non-sulfated glycosaminoglycan composed of glucuronic acid and N-acetylglucosamine; the dominant matrix component in the renal medulla
Hyaluronidase	Enzyme that cleaves hyaluronan by hydrolyzing the β-1,4 glycosidic bonds between sugar residues
Interstitium	Extracellular space between cells, containing matrix components, interstitial fluid, and various cell types
Osmolyte	Solute that contributes to osmotic pressure and influences water distribution between compartments
Polyelectrolyte	Polymer carrying multiple ionizable groups that dissociate in solution, creating a charged macromolecule
TonEBP/NFAT5	Transcription factor that responds to hypertonicity by activating genes involved in osmotic stress protection
Vasopressin	Peptide hormone (also called antidiuretic hormone, ADH) that regulates water reabsorption in the kidney collecting duct

Supplementary Note S1. Mathematical Framework for Osmolyte Binding

The binding of sodium ions to hyaluronan can be described quantitatively using extensions of Manning's counterion condensation theory. For a polyelectrolyte with charge density parameter ξ in a solution of ionic strength I, the fraction of territorially bound counterions (θ) can be approximated as:

For $\xi > 1$: $\theta = 1 - (1/\xi)$

For $\xi < 1$: $\theta = \xi \times f(I, c_p)$

where $f(I, c_p)$ is a function of ionic strength and polymer concentration that accounts for electrostatic screening and chain overlap effects.

For hyaluronan in the inner medullary environment:

Charge spacing $b \approx 1.0$ nm

Temperature $T = 310$ K (37°C)

Dielectric constant $\varepsilon_r \approx 80$

Bjerrum length $l_B = e^2/(4\pi\varepsilon_0\varepsilon_r k_B T) \approx 0.7$ nm

Charge density parameter $\xi = l_B/b \approx 0.7$

Since $\xi < 1$, strict condensation does not occur, but significant ion association is still predicted. Experimental measurements by Cleland et al. (1982) indicate that 60-70% of sodium ions in hyaluronan solutions exhibit reduced activity, consistent with territorial association.

The total amount of bound sodium in the inner medulla can be estimated as:

$N_{bound} = c_{HA} \times (M_{disaccharide})^{-1} \times \theta \times N_A$

where:

c_{HA} = hyaluronan concentration (kg/m³)

$M_{disaccharide}$ = disaccharide molecular weight (401 Da)

θ = fraction of bound counterions (~0.64)
N_A = Avogadro's number
For c_{HA} = 0.7 kg/m³ (700 μg/g): $N_{bound} \approx 1.1$ mmol/kg tissue
This represents approximately 0.1% of total inner medullary sodium but constitutes a rapidly mobilizable reserve that can buffer acute changes in free sodium concentration during the concentrating process.

Final Remarks

This comprehensive narrative review represents an effort to synthesize decades of research from disparate scientific traditions into a unified understanding of renal concentrating mechanisms. The dual pathway model proposed here—integrating the rapid epithelial pathway mediated by aquaporin-2 with the slower matrix pathway mediated by hyaluronan and hyaluronidases—provides a more complete framework for understanding how the mammalian kidney achieves its remarkable concentrating ability.

The convergence of evidence from classical Soviet physiology, Western molecular biology, and contemporary genetic approaches on consistent quantitative estimates of the matrix contribution (~40%) provides strong support for this integrated model. The biophysical principles underlying osmolyte binding to polyelectrolytes, established through decades of physical chemistry research, provide mechanistic foundation for the proposed reservoir function.

The clinical implications of this model extend beyond academic interest. Understanding that concentrating defects in chronic kidney disease, aging, and diabetes reflect matrix dysfunction as well as epithelial abnormalities opens new therapeutic avenues. The recognition that the medullary interstitium is not merely a passive conduit but an active participant in concentration challenges us to develop interventions targeting this compartment.

As with any scientific synthesis, this model generates predictions that must be tested through future research. The molecular mechanisms of vasopressin-hyaluronidase coupling, the quantitative contribution of matrix-bound osmolytes under physiological conditions, and the therapeutic potential of matrix-targeted approaches all require further investigation. We hope this review will stimulate such research and contribute to improved understanding and treatment of disorders affecting the kidney's vital concentrating function.

The kidney's ability to concentrate urine, refined over hundreds of millions of years of vertebrate evolution, represents a masterpiece of physiological engineering. Understanding this mechanism in its full complexity—including the contribution of the interstitial matrix that has been overlooked for too long—honors both the elegance of biological systems and the dedication of investigators across generations and continents who have worked to unravel their secrets.