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**POTASSIUM INFUSION IN CHRONIC POTASSIUM DEPLETED
RATS RAPIDLY REVERSES DEFECTIVE THICK ASCENDING LIMB
CHLORIDE REABSORPTION BY AN ALDOSTERONE
INDEPENDENT MECHANISM.**

by

Andrea J. McKay

*** thesis submitted to the School of Graduate Studies of the
University of Ottawa in partial fulfillment of the requirements for
the degree of Doctor of Philosophy.**

Department of Physiology

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Ottawa, Canada.

June 1993



Andrea J. McKay, Ottawa, Canada, 1993



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ABSTRACT

Previous studies from our laboratory, using a modified loop microperfusion technique (microstop-flow conductivity) in vivo, have shown that NaCl transport by the thick ascending limb (TAL) is impaired in potassium depleted (K-DEP) rats. The degree of impairment in TAL NaCl transport is highly correlated with plasma potassium (K^+) concentration and is completely reversed when extracellular fluid potassium concentration ($ECF[K^+]$) is increased by acute potassium infusion. These experiments assessed NaCl transport by the TAL in the absence of axial flow by measuring the conductivity of tubular fluid emerging from an early distal tubule site after different intervals of stop-flow (10-60 seconds) in perfused nephrons. Since these measurements of TAL transport were made in the absence of axial flow, the significance of the defect in NaCl transport in K-DEP rats could not be determined under in vivo conditions of physiological flow and chloride (Cl^-) delivery.

To determine the quantitative importance of this impairment in NaCl transport by the TAL, Cl^- reabsorption was measured in functionally isolated perfused single loops of Henle in vivo, using the technique of continuous microperfusion. Cl^- reabsorption was measured in loop segments microperfused at 22 nL/minute using a modified perfusate which minimized proximal nephron transport. This modification of the loop perfusate allowed the measurement of furosemide-sensitive Cl^- reabsorption in the perfused loop segments such that

net Cl⁻ uptake in this study can be attributed primarily to carrier-mediated Cl⁻ transport by the TAL.

Others have provided evidence that TAL NaCl reabsorption is aldosterone dependent in adrenalectomized animals. In our rats given a K-free diet, both plasma [K⁺] and plasma [aldosterone] were significantly reduced. Since aldosterone release is regulated by ECF[K⁺], the purpose of the present study was to determine whether an aldosterone deficiency and/or reduced ECF[K⁺] mediates inhibition of TAL Cl⁻ transport in potassium depletion. Using the described microperfusion conditions, it was possible to show that the defect in TAL Cl⁻ reabsorption in K-DEP rats was quantitatively significant and can be rapidly reversed by the acute systemic infusion of potassium. Acute administration of aldosterone, in the presence of sustained hypokalemia, failed to reverse the impairment in TAL Cl⁻ reabsorption in K-DEP rats. However, the acute infusion of potassium, in the presence of an aldosterone antagonist, in K-DEP rats rapidly reversed the defect in TAL Cl⁻ reabsorption to control levels.

Additional studies showed that despite normalization of ECF[K⁺] by acute potassium infusion in K-DEP rats, aldosterone levels failed to increase to control levels within this time period. This is the first study to demonstrate that the restoration of plasma [K⁺] in K-DEP rats is not immediately associated with a parallel rise in plasma [aldosterone]. As well, new knowledge was obtained from the present studies which showed that although a minimal [aldosterone] is required for normal TAL Cl⁻ transport to occur, this steroid hormone does not

regulate Cl⁻ reabsorption by this nephron segment. Therefore, these results provide conclusive evidence that in K-DEP rats the rapid reversal of defective TAL Cl⁻ reabsorption seen with acute potassium infusion occurs via an aldosterone independent mechanism.

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CHAPTER 1

The mammalian kidney is composed of numerous, virtually identical subunits called nephrons of which there are approximately 1 million located in the human kidney and approximately 35 000 found in the rat kidney (reviewed by Kriz and Kaissling, 1992). Each nephron is composed of a number of heterogeneous segments, each having specific characteristics and transport properties that are unique to that segment. Within the kidney, several populations of nephrons have been identified on the basis of the length of the loop of Henle as illustrated in Figure 1. As a first approximation, one can identify two distinct populations of nephrons which are referred to as (1) long loop or juxtamedullary nephrons and (2) short loop or cortical (superficial) nephrons. In cortical nephrons, the loop of Henle turns at the junction of the outer and inner medulla whereas in juxtamedullary nephrons, the loop of Henle extends deeply into the inner medulla as illustrated in Figure 1. However, the thick ascending limb (TAL) of both types of nephron populations begins at the junction of the outer and inner medulla. The ratio of short loops to long loops varies among different species and has been associated with urine concentrating ability (reviewed by Kriz and Kaissling, 1992).

In some species, such as the mountain beaver and muskrat, only short loop nephrons are found within the kidney such that these species have a low

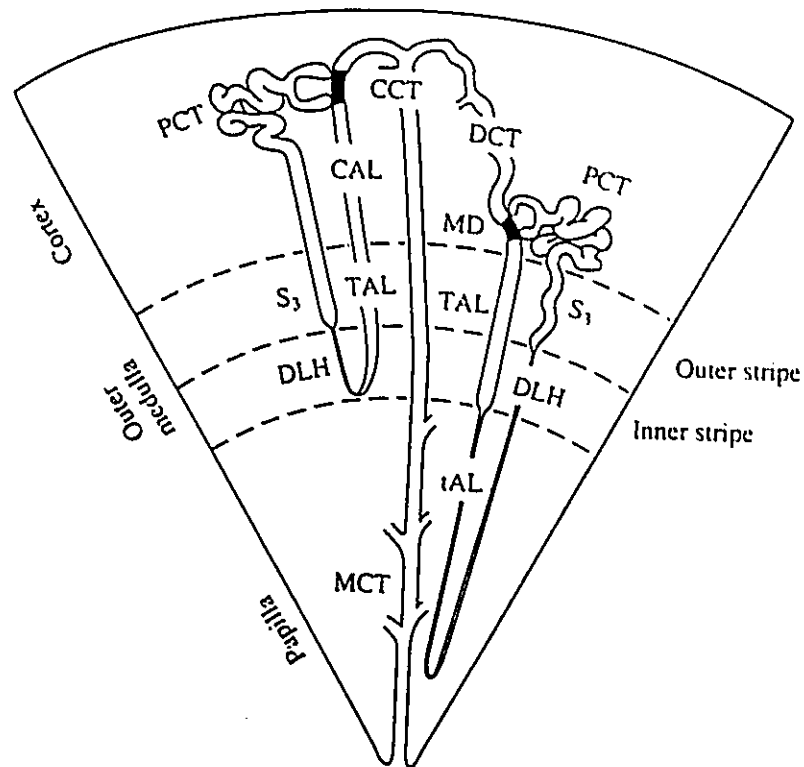


Figure 1. Schematic illustration of the various segments composing short loop (cortical) and long loop (juxtamedullary) nephrons found within the mammalian kidney. PCT = proximal convoluted tubule. S_3 = last portion of the proximal tubule. DLH = descending limb of Henle. TAL = thick ascending limb. CAL = cortical ascending limb of Henle. CCT = cortical collecting tubule. MCT = medullary collecting tubule. MD = macula densa. tAL = thin ascending limb.

concentrating ability due to the lack of an inner medulla which is responsible for the development of maximal urine osmolality (reviewed by Kriz and Kaissling, 1992). Among the rodent species, which includes the rat, mouse and golden hamster, there is a greater number of short loop to long loop nephrons and as a result, these animal species have a high urine concentrating ability (reviewed by Kriz and Kaissling, 1992).

The TAL of the loop of Henle is a highly specialized nephron segment which has transport characteristics that are vastly different from the other tubular segments that compose a typical mammalian nephron. The highly specialized transport properties of the TAL are ultimately responsible for the production of a concentrated urine during an anti-diuresis and a dilute urine during a water diuresis. The ability of the TAL to absorb salt, at a rate similar to the proximal tubule (Culpepper, 1989), in the absence of water absorption results in the generation of a hypertonic interstitium and a reduction in the osmolality of the luminal fluid, processes that are essential for the modulation of the urine concentrating mechanisms (Greger, 1985; Hebert et al, 1987; Culpepper, 1989; Molony et al, 1989).

The significant functional importance of the TAL in urine concentrating ability, in terms of the mechanisms underlying NaCl absorption, were initially difficult to analyze due to its subsurface location within the kidney. Early micropuncture studies examining the composition of the loop of Henle fluid in desert rats demonstrated that the tubular fluid in the early distal tubule was

hypoosmotic to plasma while fluid sampled from the tip of the loop was hyperosmolar (Gottschalk and Mylle, 1959; Gottschalk et al, 1963). In fact, classic micropuncture experiments by Gottschalk and Mylle (1959), demonstrated in rats that the collected fluid from the tip of the loop of Henle showed a comparable hyperosmolality to the collected fluid sampled from a collecting duct at the same level in the kidney. Such findings suggested the presence of an osmotic gradient, generated from the cortex to the inner medulla, with significant increases in the interstitial NaCl concentration and urea concentration as one approached the papillary tip (Gottschalk and Mylle, 1959; Gottschalk et al, 1963; Rocha and Kokko, 1973). Direct analysis of transport by the TAL was not possible until the development of an in vitro perfusion technique using isolated rabbit medullary and cortical TALs of Henle's loop (Burg and Green, 1973). The development of in vitro perfusion allowed direct analysis of transepithelial transport of salt and water within the TAL for the first time.

Using in vitro perfusion of the TAL, Burg and Green (1973) and Rocha and Kokko (1973) were the first to demonstrate four important features concerning transport in this nephron segment. First of all, the most striking feature was the discovery that the transtubular potential difference was oriented lumen positive in the TAL. This was in contrast to the proximal convoluted tubule and cortical collecting duct where the transtubular potential difference was negative and to the descending limb of Henle where the

potential was zero (Rocha and Kokko, 1973). Using isolated perfused rabbit TALs, the mean transtubular potential difference was measured at $+6.7 \pm 0.3$ mV and could be reversed in magnitude by cooling of the bath to 23 °C from 37 °C, and by the addition of ouabain (10^{-5} M) (Burg and Green, 1973; Rocha and Kokko, 1973). Secondly, chloride was shown to be the principle ion transported against both an electrical and chemical gradient (Rocha and Kokko, 1973; Burg and Green, 1973). Sodium transport, although not clearly understood, was postulated to involve a passive mechanism, diffusing down the electrical gradient generated by the movement of chloride (Rocha and Kokko, 1973). It was more recently shown that salt reabsorption by the TAL could be virtually abolished by the addition of the loop diuretic furosemide (10^{-4} M or 10^{-5} M) to the lumen (Hebert et al, 1981a). Thirdly, it has been shown that the measured positive transtubular potential and net chloride (and sodium) transport was dependent on the activity of the Na^+/K^+ -ATPase pump (Burg and Green, 1973; Rocha and Kokko, 1973), which has also been confirmed to be present in large quantities along the basolateral membranes of TAL cells (Katz et al, 1979). Finally, passive tracer permeability measurements have suggested that the TAL is 'electrically leaky' and cation selective, with the TAL being three times more permeable to sodium than to chloride (Rocha and Kokko, 1973). Hebert et al (1981a) later confirmed these findings, demonstrating both electrically and isotopically, that the TAL is essentially water impermeable and permselective for sodium with respect to chloride.

The low permeability to the transport of water is an absolute requirement by the TAL in the concentrating ability of the mammalian nephron (Hebert et al, 1981a; Hebert et al, 1981b; Hebert et al, 1986) and in the amphibian (salamander) early distal tubule (Guggino et al, 1985); both segments are essential for the dilution of the luminal fluid. The amphibian early distal tubule appears to function in a manner similar to the mammalian TAL, and these segments are referred to as the diluting segment (Greger, 1985). Using isolated mouse medullary TALs, Hebert et al (1981a) have demonstrated that the luminal membrane is the rate-limiting site for transepithelial water flow and that the transepithelial water permeability is negligible in this nephron segment. To determine that the luminal membrane was the site of low transepithelial water permeability, isolated mouse medullary TALs were perfused in vitro. Cell volume was determined after 30 to 60 second intervals following dilution of either the perfusate or the perfusate and bath osmolality from 290 mOsm/Kg H₂O to 240 mOsm/Kg H₂O (Hebert et al, 1986). This reduction in bath and/or perfusate osmolality is similar to that previously observed by Hebert et al (1981a) from medullary TAL isolated segments perfused (<5 nl/min) with isotonic NaCl containing solutions. Changes in perfusion osmolality did not alter cell volume. However, changes in bath osmolality were associated with a significant (30%) elevation in cell volume, suggesting that the water permeability of the basolateral cell membrane is significantly higher than the luminal cell membrane of this nephron segment. These findings of Hebert et

al (1986) clearly demonstrate the diluting ability of the TAL nephron segment, indicating the negligible transepithelial water permeability of the TAL luminal membrane. Therefore, the luminal cell membrane of mammalian TAL cells provides the major barrier to transcellular water flux within the TAL. It has also been observed (Hebert et al, 1986) that a functional nephron heterogeneity exists between cortical and medullary mouse TALs in terms of a negligible transepithelial water movement.

It has been confirmed using the amphibian diluting segment, the early distal tubule, that the apical cell membrane also functions as a major barrier to the movement of water. Guggino et al (1985) determined using analysis of cell volume, that the water permeability of the apical cell membrane of the early distal tubule of Amphiuma is negligible compared to the basolateral cell membrane, an important characteristic when coupled with ion transport which allows this segment to modulate urine concentrating ability.

1.1 Proposed Model for NaCl Transport by TAL of the Loop of Henle

It is now clear that the ability of the mammalian TAL to dilute urine and provide energy for the development of an osmotic gradient within the renal interstitium is a direct consequence of its ability to transport salt in the absence of water transport (Hebert et al, 1984; Greger and Velasquez, 1987; Hebert et al, 1987; Molony et al, 1989). During the past decade, our understanding and knowledge concerning this complex nephron segment has been significantly advanced through electrophysiological and biochemical studies using (a) intact perfused TAL segments and (b) luminal and basolateral membranes obtained from TALs. The currently proposed model for NaCl transport by the mammalian TAL is schematically illustrated in Figure 2, and is remarkably similar to that observed in non-mammalian TALs, including amphibian, avian and elasmobranch (dogfish shark) diluting segments (Miwa and Nishimura, 1986; Guggino et al, 1988; Friedman and Hebert, 1990). The proposed model indicates that transport of luminal NaCl occurs via a passive $1\text{Na}^+:1\text{K}^+:2\text{Cl}^-$ cotransporter found along the luminal membrane, with the energy provided by a Na^+/K^+ -ATPase pump located along the basolateral membrane. Reabsorbed Na^+ and Cl^- exits the TAL cells by the basolateral Na^+/K^+ -ATPase pump and a basolateral Cl^- conductance pathway. The reabsorbed K^+ is recycled across the luminal membrane into the lumen via a K^+ conductance pathway, creating a lumen positive potential and hence, allowing 50% of the luminal Na^+ to be

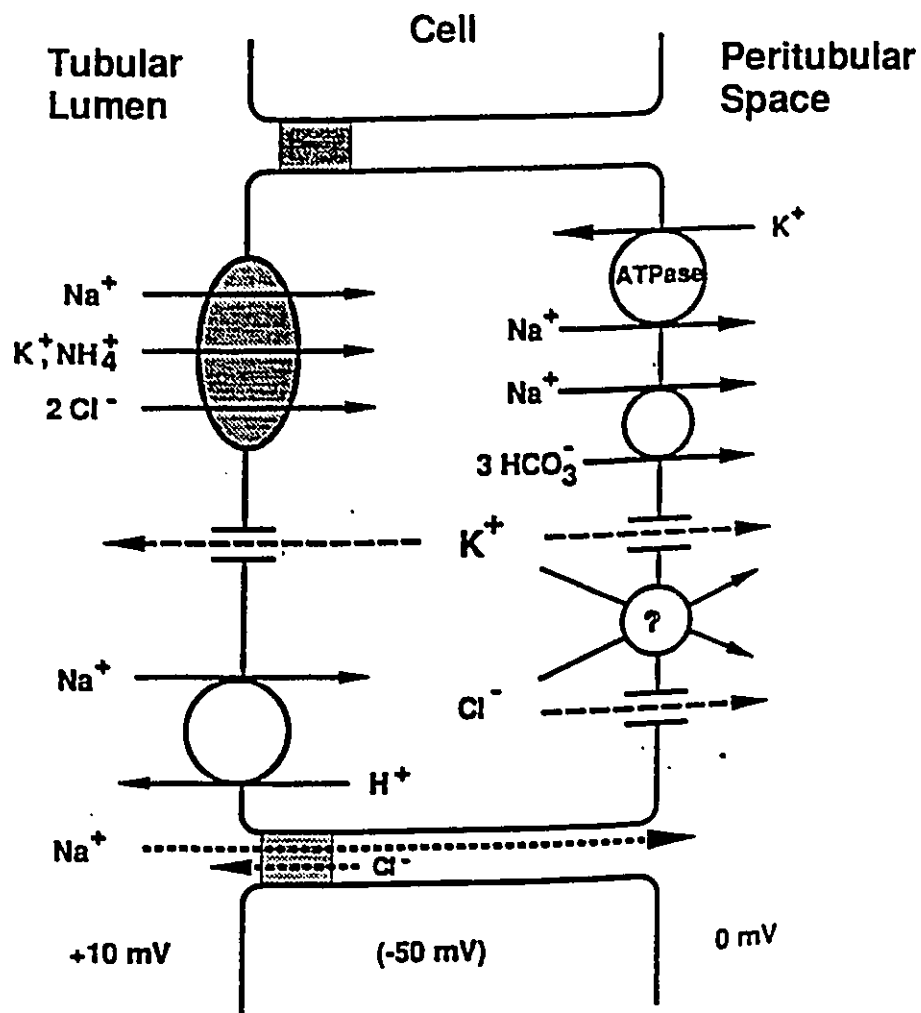


Figure 2. Schematic illustration of the currently proposed model for NaCl transport in the mammalian TAL. (Reeves and Andreoli, 1992).

passively reabsorbed through a paracellular pathway. A number of different factors are known to either stimulate or inhibit medullary TAL NaCl reabsorption. Factors which stimulate NaCl reabsorption by the medullary TAL include vasopressin, glucagon, β -adrenergic agents, mineralocorticoids and high protein diet while peritubular hypertonicity, PGE₂, calcium and acidosis have been demonstrated to inhibit NaCl transport (reviewed by Reeves and Andreoli, 1992). A detailed description of the proposed transport model is given in the following sections.

1.1.1 Apical 1Na⁺:1K⁺:2Cl⁻ Cotransporter and Basolateral Na⁺/K⁺-ATPase Pump.

As shown in Figure 2, net Cl⁻ transepithelial transport in the TAL involves a secondary active transport process such that luminal Cl⁻ is permitted to enter the cells via an electroneutral apical cotransporter (1Na⁺:1K⁺:2Cl⁻) mechanism (Hebert and Andreoli, 1984; Hebert et al, 1987; Molony et al, 1989). Luminal Cl⁻ entry into TAL cells is also dependent upon the presence of a favourable electrochemical gradient for apical Na⁺ entry across the basolateral membrane; created by the basolateral cell membrane Na⁺/K⁺-ATPase pump (Molony et al, 1989). Using apical membrane vesicles prepared from rabbit and dog medullary TALs, tracer Na⁺ and Cl⁻ uptake studies (Eveloff and Kinne, 1983; Forbush and Palfrey, 1983) have demonstrated the dependence of active Cl⁻ uptake on the

presence of both Na^+ and K^+ within the TAL nephron segment. These findings indicate that the net driving force for the transepithelial transport of Cl^- into TAL cells is ultimately dependent on the electrochemical gradients available for Na^+ , K^+ and Cl^- .

Currently, there have been no measurements obtained for all three ions to describe the electrochemical gradients present for electroneutral transport of these three ions across the mammalian TAL apical membrane. The inability to measure these electrochemical gradients within the mammalian model is due primarily to the extreme difficulty in assessing intracellular ion activity within the small TAL cells (Hebert and Andreoli, 1984). However, electrochemical gradients have been measured in Amphiuma diluting segments, whose cells are much larger in size, and which are summarized in Table 1.

Using the diluting segment of Amphiuma as a model for transport in mammalian renal TAL cells, it has been shown that Cl^- entry across the luminal membrane is dependent upon the presence of Na^+ , which is dependent on the presence of K^+ in the luminal fluid (Oberleithner et al, 1982a; Oberleithner et al, 1983). Further evidence to support this model is the primary active Na^+/K^+ -ATPase pump located along the basolateral cell membrane of this nephron segment which establishes and maintains the electrochemical gradient for Na^+ , and ultimately, provides the energy for the movement of the three ions from the tubule lumen into the cells (Oberleithner et al, 1983). Therefore, the movement of Cl^- from the tubule lumen into the cell is dependent upon the activity of the

Table 1. The intracellular activities of Na^+ , K^+ and Cl^- in the diluting segment of the Amphiuma. Negative numbers (mV) represent the movement of the ions out of the cells. (Modified from Reeves and Andreoli, 1992).

Ion	Electrochemical Potential (mV)	Cell Activity (mM)
Na^+	129	12
K^+	-16	76
Cl^-	-40	64

primary active pump, the Na^+/K^+ -ATPase pump, located extensively along the basolateral membrane. Entry of Cl^- into the diluting segment cells of the Amphiuma can be abolished (a) in the presence of furosemide (10^{-5} M), a diuretic known to inhibit cotransporters localized in the apical membrane of the diluting segment in Amphiuma, avian erythrocytes, mammalian TAL and Ehrlich ascites tumour cells (Hebert et al, 1981; Oberleithner et al, 1982a; Oberleithner et al, 1983; Hebert and Andreoli, 1984) and (b) by the removal of Na^+ from the luminal fluid being presented to this nephron segment (Oberleithner et al, 1982b). Chloride transport by this nephron segment can also be inhibited by factors which inhibit the basolateral membrane primary active pump, the Na^+/K^+ -ATPase pump. Removal of K^+ from the bath, cooling of the bath to 23°C and the presence of bath ouabain are all factors which alter basolateral Na^+/K^+ -ATPase pump activity, leading to reduction of the electrochemical gradient for sodium and thus, inhibition of luminal Cl^- entry into the cells (Rocha and Kokko, 1973; Hebert et al, 1981; Hebert and Andreoli, 1984).

Recent evidence obtained from the mouse medullary TAL has demonstrated that the mode of NaCl transport in this nephron segment can be modulated by osmolality and vasopressin (AVP) (Eveloff and Warnock, 1987; Sun et al, 1991). Alvo et al (1985) observed both a $1\text{Na}^+:1\text{Cl}^-$, K^+ -independent and a $1\text{Na}^+:1\text{K}^+:2\text{Cl}^-$ cotransport system in cells obtained from the rabbit medullary TAL. In the presence of hypotonicity, NaCl transport across the apical membrane occurs via a $1\text{Na}^+:1\text{Cl}^-$ cotransporter as shown in Figure 3

(Eveloff and Warnock, 1987). As well, Sun et al (1991) have demonstrated in mouse medullary TAL that NaCl transport occurs in this mode ($1\text{Na}^+:1\text{Cl}^-$) in the absence of AVP. As indicated in Figure 3, in the presence of hypotonicity or the absence of AVP, the apical cotransporter is in the K^+ independent- $1\text{Na}^+:1\text{Cl}^-$ mode. This form of NaCl transport in the TAL is also blocked by furosemide and its more potent analogues (ie. bumetanide). This indicates that all Na^+ reabsorbed by the TAL is being transported via a transcellular pathway. The reabsorbed Na^+ exits the cells via basolateral Na^+/K^+ -ATPase pumps which are consuming ATP and therefore, oxygen (Sun et al, 1991).

However, as shown in Figure 3, when rabbit medullary TAL cells are exposed to hypertonicity or when mouse medullary TALs are stimulated with AVP, NaCl transport proceeds via the K^+ -dependent $1\text{Na}^+:1\text{K}^+:2\text{Cl}^-$ cotransporter mode (Eveloff and Warnock, 1987; Sun et al, 1991). In this transporting mode, the recycling of K^+ back into the lumen results in the generation of a lumen positive voltage which is responsible for the remaining 50% of luminal Na^+ being reabsorbed via paracellular pathways (Hebert and Andreoli, 1984; Hebert et al, 1987; Molony et al, 1989; Sun et al, 1991) as illustrated in Figure 3. The enhancement of NaCl transport by the K^+ -dependent $1\text{Na}^+:2\text{Cl}^-$ cotransport mode has important metabolic implications. In this mode, the rate of Na^+ entry into the cell is similar to the $1\text{Na}^+:1\text{Cl}^-$ mode, however, the total rate of NaCl reabsorption is increased two-fold due to the K^+ recycling which allows an equal quantity of Na^+ to be transported

through the paracellular pathways (Sun et al, 1991). This implies that for similar rates of Na^+ transport and thus oxygen consumption by these two modes of apical cotransporters, the K^+ -dependent transport mode allows a doubling in the rate of Na^+ transport such that 50% is reabsorbed by a transcellular pathway and 50% by a paracellular pathway (Figure 3).

Currently, it is unknown whether the $1\text{Na}^+:1\text{Cl}^-$ cotransporter and $1\text{Na}^+:1\text{K}^+:2\text{Cl}^-$ cotransporter are products obtained from the same gene product or are products from different genes (Sun et al, 1991). It has been observed by Sun et al (1991) that the AVP induced modulation of NaCl transport in the mouse medullary TAL to the high efficiency form can be imitated by forskolin and cAMP (via AVP). From this observation, they speculate that the conversion of the cotransporter to the high efficiency mode may involve phosphorylation of the $1\text{Na}^+:1\text{Cl}^-$ form which results in the K^+ -dependent site becoming exposed (Sun et al, 1991).

The medullary TAL, *in vivo*, exists in an environment containing a reduced oxygen tension (Hebert et al, 1987; Sun et al, 1991). It would be favourable to the cells in this environment to have a more efficient form of NaCl transport in this nephron segment for enhanced metabolic survival (Hebert et al, 1987). The ability to modulate the form of transport to the more efficient form, without increasing its oxygen consumption, is important for the TAL, particularly during an antidiuresis when AVP levels are elevated (Sun et al, 1991). It is not known whether net reabsorption of NaCl by the TAL is

regulated by varying the mode of NaCl cotransport.

1.1.2 Basolateral Chloride Conductance Pathway.

Although the transport of Cl⁻ from the tubule lumen into the cell is an electroneutral process via an apical cotransporter (1Na⁺:1K⁺:2Cl⁻), the extrusion of Cl⁻ from the cell across the basolateral membrane occurs via a conductive pathway (Hebert and Andreoli, 1984; Hebert and Andreoli, 1984a; Hebert et al, 1984; Schlatter and Greger, 1985). There is also evidence of Cl⁻ exit across the basolateral membrane occurring via an electroneutral KCl symport (Schlatter and Greger, 1985); although this has been primarily documented within the Amphiuma early distal tubule segments which have low basolateral conductance cells (Guggino et al, 1985).

Greger et al (1983) have successfully documented cellular Cl⁻ activity within the rabbit cortical TAL segment, a technical advance considering the small cell size which had to be impaled compared to the much larger cells of the Amphiuma which were easily impaled using conventional microelectrodes by Guggino et al (1982). Using perfused rabbit cortical TALs, in vitro, intracellular Cl⁻ activity was measured using Cl⁻ selective electrodes to be approximately 22 mmol/l under control conditions (Greger et al, 1983). This measured cellular Cl⁻ activity in cortical TAL cells is two to three times higher than that which had been predicted for passive Cl⁻ distribution (5 mM),

indicating the presence of a favourable electrochemical gradient for Cl^- efflux across the basolateral cell membrane (Oberleithner et al, 1982b; Greger et al, 1983). Similarly, a favourable electrochemical gradient, as measured by intracellular Cl^- activity, has been measured in the early distal segment of the Amphiuma kidney (Oberleithner et al, 1982b). This elevated Cl^- distribution above passive distribution measurements has also been recorded for the mammalian proximal tubule and the amphibian proximal tubule (Shindo and Spring, 1981; Oberleithner et al, 1982b; Guggino et al, 1982). Predictably, the high intracellular Cl^- activity measured in the cells of the TAL is maintaining a favourable electrochemical gradient via the continued entry of luminal Cl^- across the apical membrane by the $1\text{Na}^+:1\text{K}^+:2\text{Cl}^-$ cotransporter. In both the mammalian and amphibian models, the addition of furosemide (10^{-5} M, 10^{-6} M) in the tubule lumen blocked Cl^- entry across the luminal membrane (via the $1\text{Na}^+:1\text{K}^+:2\text{Cl}^-$ cotransporter), causing the intracellular Cl^- activity to fall dramatically to 7 mmol/l which closely resembled the measured passive distribution of Cl^- (Oberleithner et al, 1982b; Greger et al, 1983). Substitution of luminal Cl^- with gluconate also abolished Cl^- entry across the luminal membrane in both the mammalian and amphibian diluting segments, with intracellular Cl^- activity achieving a passive distribution (Oberleithner et al, 1982b; Greger et al, 1983).

Further evidence for the passive transport of Cl^- through chloride conductance channels localized on the basolateral membrane, is the

measurement of a large negative intracellular voltage, ranging from -40 mV to -70 mV, in the mouse medullary TAL and the rabbit cortical TAL (Greger et al, 1983; Greger and Schlatter, 1983; Hebert and Andreoli, 1984a; Molony et al, 1989). The presence of a large negative intracellular voltage provides a potent driving force for the movement of Cl^- across the basolateral membrane.

1.1.3 Apical Potassium Conductance Pathway.

The apical membrane of the diluting segments found in the mammalian and amphibian TAL also contains a large barium sensitive K^+ conductance pathway, in addition to the $1\text{Na}^+ : 1\text{K}^+ : 2\text{Cl}^-$ cotransporter (Greger and Schlatter, 1981; Greger and Schlatter, 1983; Hebert and Andreoli, 1984; Hebert et al, 1987; Molony et al, 1989). In fact, these conductive pathways for potassium along the apical membrane are the only conductive channels that have been currently identified within the diluting segment. In the mouse medullary TAL, it has been shown that the diluting segment shares many of its voltage and concentration dependence characteristics, as well as a barium/potassium concentration effect on conductance, as has been previously demonstrated in excitable tissues (Hebert and Andreoli, 1984; Hebert et al, 1984).

The contribution of K^+ to apical membrane conductance pathways has been tested directly (Hebert and Andreoli, 1984) by evaluating the apical membrane to basolateral membrane resistance ratio in mouse medullary TALs

of Henle microperfused *in vitro*. Luminal potassium concentration (5 mM) was exchanged for 5 mM barium (blocker of the apical membrane potassium conductance pathway); this produced a dramatic ten-fold increase in the apical membrane to basolateral membrane resistance ratio. This finding is similar to that observed in the early distal tubule of the *Amphiuma* and in the cortical TAL of the rabbit (Guggino et al, 1982; Greger and Schlatter, 1983; Oberleithner et al, 1983), providing strong evidence that K^+ is the major conductive ion moving across the luminal membrane.

Further evidence to support the finding that K^+ is the major species moving across the luminal membrane has been shown by examining the effect of changes in the luminal K^+ concentration on apical membrane voltage. Hebert and Andreoli (1984) found that increasing luminal potassium ten-fold, from 5 mM to 50 mM luminal K^+ , in the presence of luminal furosemide (10^{-4} M) produced a spontaneous lumen positive change of 53.1 mV. This change in apical membrane potential was calculated to represent approximately 87% of the 61.4 mV change that has been predicted for ideal Nernstian behaviour (Hebert and Andreoli, 1984), strong evidence indicating that K^+ is the major ion passing across the TAL apical membrane via a conductive pathway (Guggino et al, 1982; Greger and Schlatter, 1983; Oberleithner et al, 1983; Hebert and Andreoli, 1984; Hebert et al, 1984).

Therefore, according to the model illustrated in Figure 3, a large proportion of K^+ that enters the TAL cells from the lumen via the

$1\text{Na}^+:1\text{K}^+:2\text{Cl}^-$ cotransporter is recycled across the apical membrane by a parallel conductive pathway. Hebert et al (1981) have shown using mouse medullary TAL that the net K^+ secretion was less than 10% of the net Cl^- transport; indicating that a large proportion (approximately 90%) of the K^+ secreted into the lumen is recycled across the apical membranes back into the TAL cells. This allows for minimal K^+ transport but allows for continual electroneutral entry of one Na^+ with 2 Cl^- to be transported across the TAL luminal membrane (Hebert and Andreoli, 1984; Hebert et al, 1984; Molony et al, 1987; Hebert et al, 1987). As mentioned earlier, blockade of the K^+ conductance pathway by luminal barium virtually abolishes the positive apical potential and transepithelial currents such that net transport of Cl^- across the luminal membrane is prevented (Hebert et al 1984; Hebert and Andreoli, 1984; Molony et al, 1987). Recycling of K^+ across the luminal membrane in TAL cells also maintains NaCl transport by keeping a continual supply of K^+ for luminal $1\text{Na}^+:1\text{K}^+:2\text{Cl}^-$ cotransport. If this recycling of K^+ did not occur, then luminal K^+ concentration would be reduced and as a result, net NaCl transport would fall.

Another important aspect of apical K^+ recycling is that its current polarizes the lumen from cell to lumen. In the rabbit cortical TAL, potassium diffusion from cell to lumen causes an equal current to flow paracellularly (Greger and Schlatter, 1983; Molony et al, 1989). As indicated in Figure 3, dilution of the tubular fluid presented to the TAL occurs through the net

transport of equivalent quantities of Na^+ and Cl^- (Hebert and Andreoli, 1984). The stoichiometry of the transcellular pathway, involving the apical K^+ -dependent cotransporter results in one Na^+ for every 2 Cl^- reabsorbed from the luminal fluid. This accounts for approximately 50% of the net Na^+ reabsorbed by the TAL. The remaining 50% of Na^+ that is reabsorbed by this nephron segment occurs through a paracellular pathway (Hebert and Andreoli, 1984; Hebert et al, 1987; Molony et al, 1989).

Hebert et al (1984) have demonstrated that this paracellular pathway is four times more permeable to Na^+ than to Cl^- indicating that in the mouse medullary TAL, there is a strong Na^+ permselectivity. These studies have found that the passive Na^+ reabsorption by the paracellular pathway is approximately half of the net rate of transcellular Cl^- transport (Hebert et al, 1984). The magnitude of the lumen positive voltage produced by K^+ recycling and the permselectivity of the paracellular pathway for Na^+ provides the driving force required to transport Na^+ through this pathway at a rate which is approximately half of the net Cl^- transport (Hebert et al, 1984; Hebert and Andreoli, 1984). The paracellular pathway also increases the efficiency of Na^+ transport by the TAL since the oxygen required to absorb the Na^+ is reduced in half (Hebert et al, 1984; Hebert and Andreoli, 1984).

1.2 Potassium

It has been documented for over four decades that a consequence of potassium depletion is an impairment in renal concentrating ability (Relman and Schwartz, 1958; Bank and Aynedjian, 1964; Gottschalk et al, 1965; Berl, 1980). Early studies in rabbits, hamsters, dogs and rats postulated that the defect in urine concentrating ability in potassium depleted animals was the result of either (a) an impairment in the osmotic equilibrium developed in the collecting ducts, (b) an impairment in Na^+ transport by the medullary TAL of the loop of Henle or (c) reduced delivery of Na^+ from the proximal tubule to the loop of Henle (Relman and Schwartz, 1958; Bank and Aynedjian, 1964; Gottschalk et al, 1965).

Gottschalk et al (1965) measured directly, microcryoscopically, the osmolality of tubular fluid sampled from the loop of Henle and collecting ducts in rats and hamsters. The location of the collected sample was determined by microdissection. The results of these studies demonstrated that an osmotic equilibrium comparable to control animals was present across the collecting duct in potassium depleted (K-DEP) rodents (Gottschalk et al, 1965). The measured osmolality of the tubular fluid sampled (by micropuncture) leaving the loop of Henle at an early distal tubule site in K-DEP rats exhibited a similar osmolality to control rats (Gottschalk et al, 1965). These findings suggested that solute reabsorption by the TAL was not altered in K-DEP rats, and

suggested that the impairment in renal concentrating ability was due to a reduced sodium delivery to the TAL in these animals (Bank and Aynedjian, 1964; Gottschalk et al, 1965).

Micropuncture studies by Bank and Aynedjian (1964) have also shown that the osmolality measured in samples obtained from the early distal tubule from K-DEP rats was similar to the osmolality measured in control animals. This was further evidence to support the hypothesis that Na^+ transport by the TAL was not altered by potassium depletion. In addition, these micropuncture studies demonstrated a significantly elevated ratio of tubular fluid inulin concentration to plasma inulin concentration in K-DEP rats, which was suggestive that greater fractional reabsorption had occurred proximal to the collecting ducts (Aynedjian and Bank, 1964). Others have measured a reduction in medullary Na^+ concentration in renal tissue slice analysis in K-DEP rats (reviewed by Buckalew et al, 1967). These early findings described above suggest that the urine concentrating defect observed in K-DEP animals was mediated by a significant reduction in the delivery of Na^+ to the TAL (Bank and Aynedjian, 1964; Gottschalk et al, 1965).

Since micropuncture studies and renal tissue analysis concluded that the urine concentrating defect observed in K-DEP rats was likely mediated by a reduction in the delivery of Na^+ to the TAL; the effect of increasing Na^+ delivery to the TAL on the renal concentrating mechanism in K-DEP animals has been investigated. Buckalew et al (1967) increased Na^+ delivery to this

nephron segment using hypertonic saline or mannitol diuresis. No significant difference in glomerular filtration rate was observed between control and K-DEP rats, indicating that the delivery of Na^+ to the TAL in both groups of rats was similar (Buckalew et al, 1967). Sodium delivery to the distal nephron was dramatically increased by the presence of either a saline or mannitol diuresis, however, K-DEP rats demonstrated a significant reduction in free water reabsorption at any level of solute excretion (Buckalew et al, 1967). It is also highly likely that an impairment in Na^+ transport would not be observed in the distal nephron if there was reduced delivery of Na^+ to the nephron segment. If the critical level of sodium reabsorption is not achieved then hypertonicity of the renal interstitium would not occur and thus, a reduction in free water reabsorption would occur. These results challenge earlier findings (Bank and Aynedjian, 1964; Gottschalk et al, 1965) and demonstrate that the delivery of Na^+ to the TAL, alone, cannot explain the defect in the urine concentrating mechanism observed in K-DEP animals. Rather, the study by Buckalew et al (1967) provided strong support for the hypothesis that the defect in renal concentrating ability in these rats may be due to an impairment in Na^+ transport by the TAL.

Other mediators that have been postulated to be involved with the renal concentrating defect in potassium depletion include changes in (a) renal hemodynamics and (b) antidiuretic hormone (ADH) release (Whinnery and Kunau, 1979; Berl, 1980; reviewed by Gutsche et al, 1984; Peterson, 1984).

Whinnery and Kunau (1979) and Peterson (1984) have investigated whether changes in renal inner medullary blood flow may alter inner medullary solute concentration in K-DEP rats, resulting in a defect in the urine concentrating mechanism. This hypothesis was based on the assumption that an increase in blood flow through the medullary region would significantly increase the removal of solute from the interstitium. As a result, this would lead to a reduction in medullary solute concentration and may account for the reduction in renal concentrating ability measured in K-DEP rats (Whinnery and Kunau, 1979; Peterson, 1984).

Inner medullary plasma flow was measured using the ^{125}I albumin accumulation technique (described by Peterson, 1984). In initial studies, Whinnery and Kunau (1979) did not find an increase in blood flow, but rather a significant reduction in inner medullary blood flow in K-DEP rats fed a potassium deficient diet for three weeks. These findings suggested that a reduction in inner medullary blood flow in K-DEP rats may indicate a reduction in plasma delivery to the juxtamedullary nephrons which may have resulted in a decrease in solute delivery to the TAL (Whinnery and Kunau, 1979). However, a reduction in inner medullary blood flow in these rats would be expected to decrease solute removal and thus, significantly enhance the renal concentrating ability. Therefore, the results of Whinnery and Kunau (1979) demonstrate that a 'washing out' effect of medullary solute does not occur in K-DEP rats.

Since a reduction in inner medullary blood flow was postulated to be involved with the renal concentrating defect associated with potassium depletion, Peterson (1984) proposed that the reduction in medullary blood flow should also be present when the defect in renal concentrating ability initially appears. Previously, it had been determined that the impairment in concentrating ability occurs between 7 and 14 days of dietary potassium depletion (Brokaw, 1953; Eknayan et al, 1970; Berl, 1980; Peterson, 1984). After two weeks of dietary potassium depletion, the inner medullary blood flow was not significantly different from that in control animals (K-DEP: 32.8 ± 1.52 ml/min/100g inner medulla versus Control: 35.1 ± 1.93 ml/min/100g inner medulla, $p > 0.05$) despite the presence of an impairment in urine concentrating ability in K-DEP rats (Peterson, 1984). However, a significant reduction in inner medullary blood flow was demonstrated following three weeks of dietary potassium depletion (Peterson, 1984), which confirmed the earlier findings of Whinnery and Kunau (1979). The results of Peterson (1984) clearly demonstrate that the renal concentrating defect can occur independently of alterations in inner medullary blood flow. Thus, the reduction in medullary solute concentration and the defect in concentrating ability measured in K-DEP animals is due to factors other than changes in inner medullary blood flow.

Alterations in ADH release have also been proposed to explain the defect in concentrating ability of K-DEP animals. It has been well established that dietary potassium depletion results in a significant increase in water intake or

polydipsia within three days of animals consuming a potassium deficient diet (Brokaw, 1953; Blyth et al, 1960; reviewed by Berl, 1980; Saikaley et al, 1986; McKay et al, 1990). In fact, the added stimulus of sodium chloride depletion superimposed on potassium depletion results in a greater increase in water intake than that measured in K-DEP rats alone (Blyth et al, 1960; Saikaley et al, 1986; McKay et al, 1990). The effect of water intake on impaired concentrating ability in K-DEP animals has been examined by Berl (1980), who found a significant increase in water intake in rats after six days of consuming a potassium deficient diet. Despite the presence of polydipsia at this time point, there was no difference in urine concentrating ability between control and K-DEP animals (Berl, 1980). Therefore, the polydipsia associated with dietary potassium depletion precedes the impairment in renal concentrating ability. It has been suggested that the impairment in concentrating ability may be due to an alteration in ADH release. However, following a 24 hour water deprivation period, Berl (1980) found that plasma levels of ADH were not significantly different between control and K-DEP rats. Administration of exogenous ADH to hypokalemic rats failed to correct the concentrating ability to control levels; indicating that this peptide hormone cannot account for the effects observed during potassium depletion (Berl, 1980).

Eknoyan et al (1970) have confirmed and extended the earlier work by Buckalew et al (1967), showing that decreased delivery of Na^+ to the TAL cannot explain the renal concentrating defect measured in K-DEP animals. In

these studies, the urine concentrating ability of K-DEP rats was studied by measuring free water clearance during a hypotonic mannitol diuresis. At similar rates of delivered Na^+ , free water clearance in these rats was significantly lowered compared to control animals (Eknoyan et al, 1970). Furthermore, Na^+ excretion, at any level of excretion or filtrate delivery to the distal nephron, was significantly higher in the K-DEP animals compared to control animals (Eknoyan et al, 1970). Since the reabsorption of sodium by the TAL is the major factor responsible for the generation of solute free water, these results (Eknoyan et al, 1970) provide strong evidence that the urinary concentrating defect observed in K-DEP animals involves an impairment in Na^+ transport by the TAL.

Dietary potassium depletion has also been associated with renal Cl^- wasting, which has been described as "continuing renal chloride excretion despite mounting cumulative chloride depletion" (Luke et al, 1978). Free flow micropuncture and microperfusion experiments of the superficial loop segment (latest proximal to earliest distal tubule) in rats have revealed a decrease in fractional Cl^- reabsorption within this loop segment of K-DEP rats (Luke et al, 1978; Luke et al, 1985). As well, K-DEP rats had a significantly higher distal tubule Cl^- concentration to perfusate Cl^- concentration ratio such that the luminal fluid sampled from the early distal tubule site had a significantly higher concentration in the collected sample (Luke et al, 1978). An infusion of indomethacin in K-DEP rats partially corrected the reduction in net Cl^- reabsorption by the loop segment (Luke et al, 1985). Based on these findings,

it appears that the impairment in net Cl⁻ transport occurs in the TAL segment of the loop of Henle in K-DEP rats, and may account for the defect in urine concentrating ability of these potassium deficient animals (Luke et al, 1978; Luke et al, 1985; reviewed by Mujais and Katz, 1992).

Gutsche et al (1984) were the first to provide direct in vivo evidence that the TAL was the site of sodium chloride (NaCl) inhibition in K-DEP rats. In these studies, the NaCl concentration in the TAL was measured as the tubular fluid was presented to an early distal tubule micropuncture site using the stop-flow conductivity probe technique developed by Gutsche et al (1980), as conventional micropuncture techniques do not allow direct access to the TAL. Using the stop-flow conductivity technique, Gutsche et al (1984) found that K-DEP rats have a significant impairment in NaCl reabsorption by the TAL, which could be rapidly reversed upon acute potassium repletion as shown in Figure 4. Analysis of change in the TAL fluid NaCl concentration over time showed a significantly larger NaCl concentration in the TAL tubular fluid of K-DEP rats (Gutsche et al, 1984). In addition, a striking correlation was observed between the degree of reduced extracellular fluid potassium concentration (ECF[K⁺]) and the severity of the impaired NaCl transport by the TAL (Gutsche et al, 1984). These results demonstrate a defect in NaCl transport by the TAL in K-DEP rats, but is this defect occurring at (a) the entrance step of NaCl into the cell across the apical membrane or (b) at the exit step across the basolateral membrane?

Transport of NaCl across the apical membrane has been well

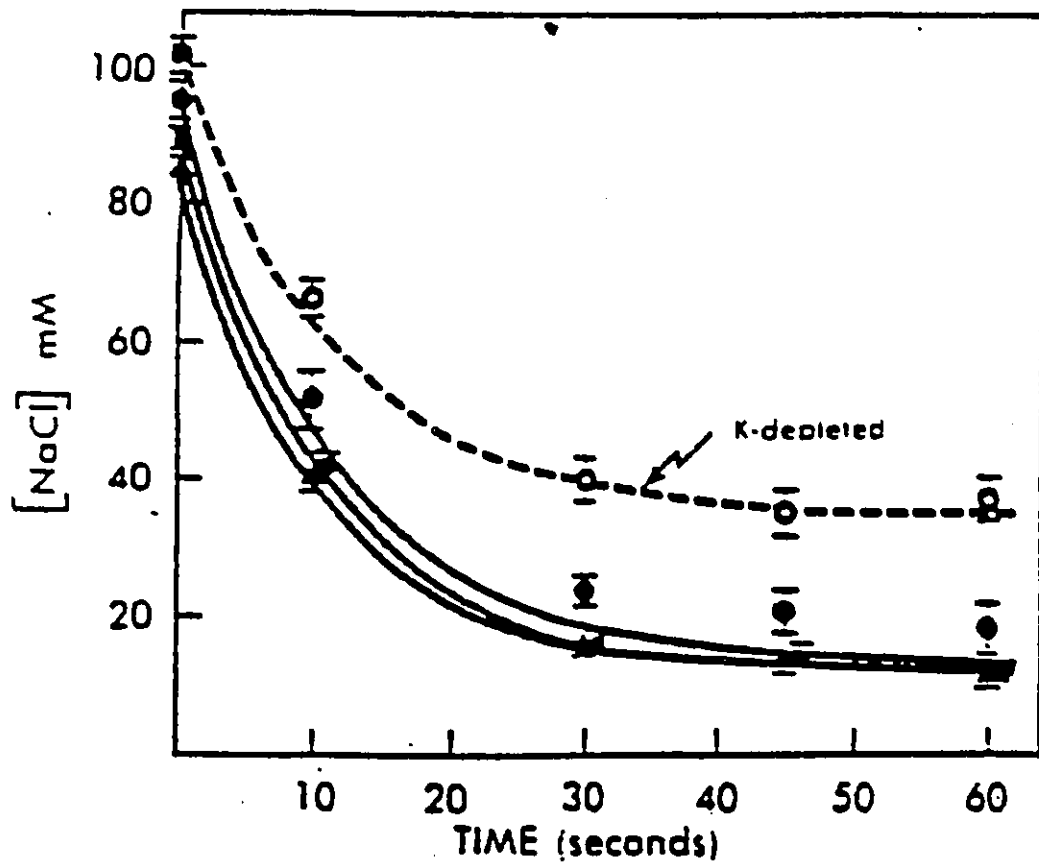


Figure 4. Changes in TAL tubular fluid [NaCl] at varying intervals of microstop-flow (10-60 seconds) in control, K-DEP and K-replaced rats (infusion or gavage). Filled circles represent control rats. Open circles represent K-DEP rats. K-replaced rats are represented by (a) filled squares (K^+ given by intravenous infusion) and (b) filled triangles (K^+ given by gavage).

demonstrated to be dependent upon the presence of K^+ (Burg and Bourdeau, 1978; Greger and Schlatter, 1981) and thus, it is possible that the impairment in NaCl transport by the TAL of K-DEP rats may be due to insufficient K^+ entering the perfused loops of Henle. This hypothesis was investigated by Gutsche et al (1984) who perfused the loop segments (90 nl/minute) with either a zero K^+ or a 5 mM K^+ containing luminal perfusate. Based on these studies, it was shown that K^+ delivery to the luminal membrane of K-DEP rats should not have been limiting for NaCl transport by the TAL (Gutsche et al, 1984). Furthermore, the defect in NaCl transport by the TAL was not restored to control values in K-DEP rats when luminal K^+ delivery was increased to that measured in control animals (Gutsche et al, 1984). Therefore, these studies demonstrated that reduced luminal K^+ delivery to the TAL cannot account for the impairment in TAL NaCl transport in K-DEP rats.

Sodium chloride transport across the luminal membrane into the cells of the TAL and its subsequent movement into the renal interstitium is driven by a Na^+/K^+ -ATPase pump as illustrated in Figure 3. Na^+/K^+ -ATPase activity has been examined in mouse medullary TAL in vitro with maximal activity of the pump measured at a bath K^+ concentration of 5 mM (Doucet et al, 1979). As the bath concentration of K^+ was reduced, there was a corresponding reduction in the pump activity (Doucet et al, 1979), as illustrated in Figure 5. The determination of the Na^+/K^+ -ATPase activity in the mouse medullary TAL coupled with the in vivo findings of Gutsche et al (1984), who showed a

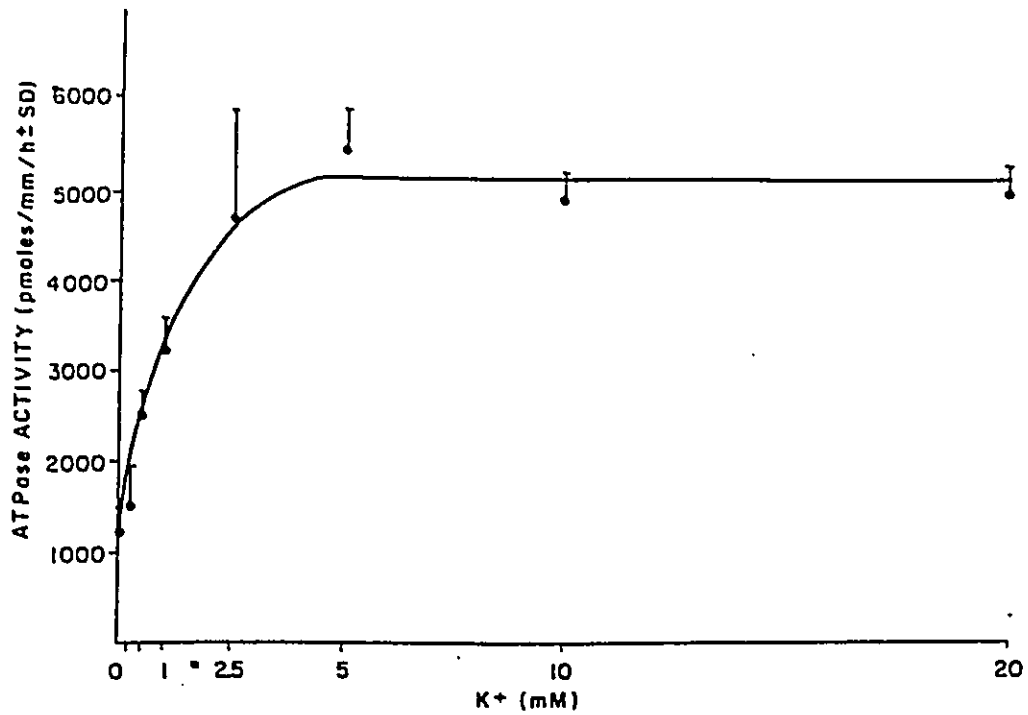


Figure 5. Effect of increasing bath [K⁺] on Na⁺/K⁺-ATPase activity in mouse medullary TAL. Bath [K⁺] was increased from 0 mM to 20 mM, with maximal activity of the Na⁺/K⁺-ATPase measured at a bath [K⁺] of 5 mM. (Doucet et al, 1979).

striking correlation between NaCl concentration in the TAL and $ECF[K^+]$, indicates that $ECF[K^+]$ has a strong regulatory effect on the pump activity along the basolateral membrane. Therefore, these findings suggest that reductions in $ECF[K^+]$ induced by dietary potassium depletion, limit the activity of the pump and hence, reduce the movement of NaCl by the cells of the TAL (Gutsche et al, 1984). It remains to be determined if the impairment in TAL NaCl transport in potassium depletion involves the luminal entry step and/or the basolateral exit step.

The direct in vivo studies of Gutsche et al (1984) agree with earlier studies using rat isolated and perfused segments of TALs (Burg and Bourdeau, 1978; Greger and Schlatter, 1981). These in vitro studies have shown that removal of K^+ from the lumen and the bath results in the inhibition of NaCl transport across the TAL segment (Burg and Bourdeau, 1978; Greger and Schlatter, 1981).

In vitro studies by Greger and Velasquez (1987), using rat medullary and cortical TALs, are not consistent in terms of the level of $ECF[K^+]$ required to impair NaCl transport by the TAL compared to the in vivo findings of Gutsche et al (1984). These in vitro studies showed that NaCl reabsorption, measured by the equivalent short circuit current, was not altered when the K^+ concentration in the bath was reduced to as low as 1.5 mM to 2.0 mM (Greger and Velasquez, 1987). However, when the bath K^+ concentration was reduced to less than 1.5 mM, a very significant reduction in NaCl reabsorption was

measured, which led to the proposal that there may be an impairment of the Na^+/K^+ -ATPase pump on the basolateral membrane (Greger and Velasquez, 1987). These results taken with the in vivo findings of Gutsche et al (1984) suggest that the inhibition of TAL NaCl transport may not be mediated directly by reduced $\text{ECF}[\text{K}^+]$, but by some factor which is regulated by $\text{ECF}[\text{K}^+]$.

1.2.1 What is the Mediator Responsible for Impaired NaCl Transport in K-DEP Animals?

Consumption of a potassium free (K-Free) diet in animals and man results in a very rapid decline in urinary K^+ excretion which is virtually complete by 72 hours (Linas et al, 1979), indicating that renal K^+ conservation occurs rapidly after the initiation of a potassium deficient diet. The fall in urinary K^+ excretion appears to be a mechanism to prevent a large reduction in $\text{ECF}[\text{K}^+]$. As well, it has been demonstrated in rats that following 24 hours of potassium depletion, there is a significant reduction in plasma $[\text{K}^+]$ and plasma [aldosterone] compared to animals fed a control diet (Linas et al, 1979). Plasma $[\text{K}^+]$ and plasma [aldosterone] were further reduced after 72 hours of dietary potassium depletion (Linas et al, 1979). These findings indicate that during dietary potassium depletion, plasma [aldosterone] are significantly suppressed and parallel the decrease in plasma $[\text{K}^+]$ (Linas et al, 1979).

Potassium has been shown to influence aldosterone production in

numerous species, including man, dogs and rats (Boyd et al, 1971). The classic study by Boyd et al (1971) demonstrated that K^+ influences the synthesis of aldosterone at the level of the conversion of corticosterone to aldosterone. Potassium has also been shown to stimulate widening of the zona glomerulosa region of the adrenal cortex (Boyd et al, 1971). These investigators found that aldosterone release was suppressed in response to reduced $ECF[K^+]$ induced by dietary potassium depletion in rats (Boyd et al, 1971). In addition, oral loading of K^+ significantly increased aldosterone release; a significant increase in the conversion of corticosterone to aldosterone was measured as early as four hours following the oral load of potassium (Boyd et al, 1971). The peptide hormone, angiotensin II, has also been demonstrated to be a powerful stimulus for inducing aldosterone release from the adrenal cortex. However, studies by our laboratory have demonstrated that aldosterone release is suppressed in K-DEP rats in the presence of elevated angiotensin II (Saikaley et al, 1986; McKay et al, 1990). Furthermore, dietary potassium depletion results in a very significant suppression of plasma aldosterone levels within 24 hours (Linas et al, 1979) and occurs even in the presence of the powerful stimulus of added NaCl depletion (McKay et al, 1990).

1.3 Aldosterone

Recent in vivo microperfusion studies and in vitro perfusion studies have provided evidence that the mineralocorticoid hormone, aldosterone, may have a regulating effect on NaCl transport by the TAL.

Early studies using dogs proposed that aldosterone was necessary for Na⁺ transport by the TAL and that its absence would result in a defect in maximal urine concentrating ability (Sigler et al, 1965; Green et al, 1970; Rogers et al, 1975; Schwartz and Kokko, 1980). These studies measured free water clearance in adrenalectomized mongrel dogs, rabbits and rats. Rogers et al (1975) were unable to demonstrate any significant effect of an aldosterone deficiency on free water clearance. However, they speculated that their clearance techniques may not have been sensitive enough to be able to measure a small but significant effect of this mineralocorticoid hormone on Na⁺ transport by the TAL (Rogers et al, 1975). In earlier studies by Green et al (1970), a significant reduction in free water clearance was measured in adrenalectomized animals and could be increased to control values upon aldosterone repletion. In addition, it has been demonstrated in rats and rabbits, that the replacement of aldosterone in adrenalectomized animals increased their ability to maximally concentrate their urine (Sigler et al, 1965; Schwartz and Kokko, 1980).

The source of physiological endogenous mineralocorticoids and

glucocorticoids in the mammalian body is the adrenal gland. Mineralocorticoid receptors are generally referred to as Type 1 receptors (Marver, 1984; Funder et al, 1988). This classification refers to mineralocorticoid hormones " with a relative affinity that mimics their biological potency with respect to sodium reabsorption" (Marver, 1984). These receptor sites have been localized in a number of tissues, including the kidney, parotid gland, colon, hippocampus and heart (Funder et al, 1988). More specifically within the kidney, the major target sites for aldosterone, in rat and rabbit nephron, are the distal tubule and collecting ducts (Farman and Bonvalet, 1983; Marver, 1984). However, autoradiography and binding studies have also demonstrated a significant binding of [³H] aldosterone in both the medullary and cortical TAL in the rabbit nephron (Farman and Bonvalet, 1983; Marver, 1984). In vivo studies have shown that tissues with Type 1 receptor sites are selective for the mineralocorticoid hormone, aldosterone, with little corticosterone binding while in vitro work demonstrates that these receptor sites can bind aldosterone, corticosterone and cortisol with equal affinity (Funder et al, 1988; reviewed by Reeves and Andreoli, 1992).

The highly specific binding of aldosterone to mineralocorticoid receptors in target tissues (ie. kidney, parotid gland, colon) in vivo appears to be due to the presence of an enzyme known as 11 β -hydroxysteroid dehydrogenase (Funder et al, 1988; Bonvalet et al, 1990). This enzyme causes the conversion of corticosterone and cortisol to their respective 11-keto analogs; however, it

has no effect on aldosterone (Funder et al, 1988; Bonvalet et al, 1990). The aldosterone group is protected from the actions of this enzyme by forming either 11,18-hemiketal or 11,18,20-hemiacetal bonds (Funder et al, 1988). Conversion of corticosterone and cortisol to 11-keto analogues results in a structure which has a much lower affinity for the Type 1 mineralocorticoid receptor site (Funder et al, 1988; Bonvalet et al, 1990). Therefore, the Type 1 receptor site is left free for aldosterone binding and action (Bonvalet et al, 1990), even in the presence of physiological levels of circulating glucocorticoids which are much higher than the circulating level of aldosterone (Funder et al, 1988; Bonvalet et al, 1990).

Recently, Bonvalet et al (1990) have investigated the distribution of this enzyme, 11 β -hydroxysteroid dehydrogenase, along the rabbit nephron, as earlier work was controversial. Receptor binding studies by Funder et al (1988) showed that high activity of this enzyme was demonstrated in well-known target tissues such as the kidney and colon. However, enzyme activity was significantly lower in the heart, a tissue which has been shown to bind mineralocorticoids but the physiological significance of this action is unclear (Funder et al, 1988). Bonvalet et al (1990) predicted that the distribution of the enzyme in the rabbit nephron would be highly localized to the aldosterone sensitive epithelia (distal tubule, TAL, collecting duct) with little localization of this enzyme along the aldosterone insensitive epithelia, such as the proximal tubule. However, earlier studies were unable to show any distinct or significant

difference in terms of the localization of the 11β -hydroxysteroid dehydrogenase between the aldosterone sensitive and insensitive portions of the rat nephron (Edwards et al, 1988). Immunohistochemical studies localized the enzyme to the proximal nephron (Rundle et al, 1989), which does not fit with the currently known distribution of the Type 1 mineralocorticoid receptors along the nephron (Bonvalet et al, 1990). Using HPLC, Bonvalet et al (1990) have demonstrated in the rabbit nephron, a wide distribution of the enzyme. A high activity was reported in the cortical TAL and distal nephron segments. A much smaller percentage of activity was measured in the medullary TAL. These findings suggest that the 11β -hydroxysteroid dehydrogenase activity is highest in those segments where Type 1 mineralocorticoid receptors have been localized, which supports their hypothesis that this is a key enzyme in terms of mineralocorticoid tissue specificity (Bonvalet et al, 1990).

Some studies have demonstrated that aldosterone has a modulating influence on TAL basolateral Na^+/K^+ -ATPase pump activity (Hendler et al, 1972; Schmidt et al, 1975; Horster et al, 1980; Grossman and Hebert, 1988) such that activity of this pump is reduced following adrenalectomy in rabbits and rats. However, these effects are controversial as other investigators (Garg et al, 1981; El Mernissi and Doucet, 1984; Rane and Aperia, 1985) have been unable to demonstrate any effect of aldosterone on the activity of this enzyme in the TAL.

Earlier work has shown that six hours following adrenalectomy, there

was a significant 75% reduction in Na^+/K^+ -ATPase in the TAL (reviewed by Schmidt et al, 1975). Based on these findings, the effect of a single low dose of aldosterone on Na^+/K^+ -ATPase activity has been investigated. One hour following the injection of the mineralocorticoid, there was a rapid and complete reversal of the pump activity to control levels (Schmidt et al, 1975). The activity of the rabbit cortical TAL following adrenalectomy and incubation for one hour with aldosterone (5×10^{-7} M) has been investigated by Horster et al (1980). In these studies, the difference between total ATPase and ouabain-insensitive ATPase was used as an index of basolateral cortical TAL Na^+/K^+ -ATPase activity. Adrenalectomy reduced Na^+/K^+ -ATPase activity by 75% and activity of the pump was restored to control levels by the presence of the mineralocorticoid aldosterone (Horster et al, 1980). Incubation of rabbit cortical TAL with either (a) a glucocorticoid (dexamethasone) or (b) aldosterone antagonist (spironolactone) failed to restore pump activity. These findings indicate a modulating effect of aldosterone on Na^+/K^+ -ATPase activity in the cortical TAL (Horster et al, 1980).

More recently, Grossman and Hebert (1988) have demonstrated in mice with intact adrenal function that treatment with pharmacological doses of the mineralocorticoid deoxycorticosterone acetate (DOCA) increased by 28% the Na^+/K^+ -ATPase activity in the medullary TAL. This evidence suggests a possible direct action of DOCA on the medullary TAL.

However, other studies have been unable to measure any significant

effect of DOCA on Na^+/K^+ -ATPase activity in rabbit cortical and medullary TALs (Garg et al, 1981; El Mernissi and Doucet, 1984). The inability to measure a change in pump activity by these earlier studies may involve a species difference or differences in the dose of the mineralocorticoid administered (Grossman and Hebert, 1988).

Morphological studies by Stanton (1986) examining the ultrastructure of the loop of Henle, using qualitative and morphometric analysis, have shown that adrenalectomy (and thus, aldosterone depletion) had no significant alteration on this segment's ultrastructure. In fact, cell structure in the adrenalectomized rats was similar to control animals and to that previously reported in the literature (Stanton, 1986). Earlier studies (Stanton, 1985) have shown that aldosterone deficiency reduced the area of the basolateral membrane of principal cells in the late distal tubule and in the initial collecting tubule. These changes were correlated with an impairment in sodium reabsorption and potassium secretion by these nephron segments (Stanton, 1985). In addition, Garg et al (1985) have suggested that the significant reduction in basolateral membrane surface area is associated with a reduction in the Na^+/K^+ -ATPase activity in the late distal nephron. Based on these findings, Stanton (1986) proposed that the lack of change observed in the ultrastructure of the loop of Henle in adrenalectomized rats may reflect either (a) that aldosterone does not mediate a regulating influence on this cell structure or (b) that the effect of the adrenalectomy may not have been

detected by the morphometric analysis.

In addition to the evidence presented which suggests that aldosterone can modulate Na^+/K^+ -ATPase activity in the TAL, there is also evidence that aldosterone can influence another transport enzyme in the TAL. Citrate synthase is a putative marker enzyme for both mineralocorticoids and Na^+/K^+ -ATPase activity (reviewed by Marver, 1984). This enzyme is a component of the citric acid cycle and provides the energy (ATP) for enhanced Na^+ transport (Marver and Lombard, 1981; Marver, 1984). Adrenalectomy reduces citrate synthase activity in rabbits. Acute administration of aldosterone but not dexamethasone in adrenalectomized rabbits restores the enzyme activity to control values (Marver and Lombard, 1981). This data taken with the Na^+/K^+ -ATPase data provides strong evidence that aldosterone has an important influence in regulating NaCl reabsorption by the mammalian TAL.

Recent in vivo microperfusion and in vitro perfusion studies have shown that NaCl reabsorption by the loop of Henle is dependent upon normal plasma aldosterone levels (Stanton, 1986; Work and Jamison, 1987). In vivo microperfusion of superficial loop segments in adrenalectomized rats demonstrated a 33% reduction in loop Na^+ transport (Stanton, 1986). Physiological aldosterone replacement ($0.5 \mu\text{g}/100\text{g}$ body weight per day) in adrenalectomized rats (Field et al, 1984; Stanton et al, 1985) returned Na^+ reabsorption in the loop segments to normal levels (Stanton, 1986). Physiological glucocorticoid (dexamethasone) replacement had no effect in

reversing the reduction in loop Na^+ reabsorption in the adrenalectomized rats (Stanton, 1986), further evidence to support a role for this hormone in regulating NaCl transport in the TAL. In vitro perfusion studies (Work and Jamison, 1987) using rat medullary TAL have recently confirmed the in vivo findings. In fact, net Na^+ and net Cl^- fluxes in the medullary TAL of adrenalectomized rats were significantly lower than in control animals and adrenalectomized rats replaced with physiological levels of aldosterone (Work and Jamison, 1987). Associated with this impairment in NaCl transport by the TAL in adrenalectomized rats was a significant reduction in transepithelial voltage compared to adrenalectomized rats infused with aldosterone (Work and Jamison, 1987). Physiological aldosterone replacement in adrenalectomized rats returned both NaCl transport and the transepithelial voltage back to control levels (Work and Jamison, 1987). These findings described above suggest an important role for the mineralocorticoid hormone, aldosterone, in TAL NaCl transport and ultimately, in the urine concentrating mechanism in the kidney.

1.4 General Aims:

NaCl transport across the luminal membrane into the TAL cells and its subsequent movement into the renal interstitium is driven by a Na^+/K^+ -ATPase enzyme. In vitro studies have shown using mouse medullary TAL that maximal activity of the Na^+/K^+ -ATPase enzyme occurs at a bath K^+ concentration of 5

mM, with no increase in enzyme activity when bath K^+ concentration is increased to 20 mM (Doucet et al, 1979). However, as bath K^+ concentration was reduced, there was a corresponding reduction in enzyme activity, with marked reduction of Na^+/K^+ -ATPase activity when bath K^+ concentration fell below 2.5 mM (Doucet et al, 1979).

Earlier studies from our laboratory were the first to provide direct in vivo evidence that the TAL was the site of NaCl inhibition in potassium depletion (Gutsche et al, 1984). Using the microstop-flow conductivity technique developed by Gutsche et al (1980), it was shown that K-DEP rats have a significant impairment in TAL NaCl reabsorption which could be rapidly reversed (30 minutes) upon acute, partial replacement of the potassium deficit (Gutsche et al, 1984). Furthermore, a striking correlation was observed between the degree of reduced $ECF[K^+]$ and the severity of the impairment in TAL NaCl transport (Gutsche et al, 1984). Based on these results, Gutsche et al (1984) postulated that the impairment in NaCl reabsorption by the TAL was due to reductions in $ECF[K^+]$ induced by potassium depletion which could limit the activity of the Na^+/K^+ -ATPase enzyme, and hence, reduce the movement of the NaCl by the TAL. These in vivo findings correspond with earlier in vitro studies that have shown that removal of K^+ from the lumen and bath results in the inhibition of NaCl transport across the TAL (Burg and Bourdeau, 1978; Greger and Schlatter, 1981). However, more recent in vitro perfusion studies have shown, in rat cortical and medullary TAL, that bath K^+ concentration must be

reduced to less than 1.5 mM before a significant impairment in NaCl reabsorption was measured (Greger and Velasquez, 1987). Therefore, these findings suggest that inhibition of TAL NaCl reabsorption may not be mediated directly by reduced ECF[K⁺] but by some factor which is regulated by ECF[K⁺].

It has been well-established that aldosterone release is regulated by ECF[K⁺] (Boyd et al, 1971) such that aldosterone release is significantly suppressed in response to reduced ECF[K⁺] in potassium depletion (Boyd et al, 1971; Linas et al, 1979). In addition, our laboratory has previously shown that aldosterone release is suppressed in K-DEP rats when NaCl depletion is superimposed on potassium depletion (McKay et al, 1990). Recent *in vivo* microperfusion and *in vitro* perfusion studies in adrenalectomized rats have shown that NaCl reabsorption by the loop of Henle is dependent upon normal plasma aldosterone levels (Stanton, 1986; Work and Jamison, 1987). The results of these studies suggest that the impairment in TAL Cl⁻ reabsorption in potassium depletion may be due directly to an aldosterone deficiency and secondarily to reduced ECF[K⁺]. The time course of potassium repletion described by Gutsche et al (1984) was of sufficient duration (1-3 hours) to allow the steroid hormone effects of aldosterone to occur. Therefore, the hypothesis of the present study was that an aldosterone deficiency accounts for impaired TAL NaCl transport in K-DEP rats. The overall aim of this study was to investigate, *in vivo*, Cl⁻ transport in the TAL to determine whether aldosterone deficiency and/or reduced ECF[K⁺] mediates inhibition of TAL NaCl

transport in potassium depletion.

Initial studies examined the effect of dietary-induced potassium depletion on urine concentrating ability in rats. Water intake, food intake, growth, kidney weight and systemic effects of potassium depletion were examined over a 14 day period of study. The second series of experiments examined the contribution of the TAL to net Cl^- reabsorption using the loop diuretic, furosemide, added to the luminal perfusate in standard Purina chow fed rats. These studies examined Cl^- transport in vivo using the technique of continuous microperfusion of single loops. Furosemide-sensitive J_{Cl} was used as an index of TAL Cl^- reabsorption. The abolishment of Cl^- reabsorption in loop segments perfused with furosemide was attributed to furosemide-sensitive inhibition of carrier-mediated Cl^- transport. To determine whether an aldosterone deficiency and/or reduced extracellular fluid potassium concentration ($\text{ECF}[\text{K}^+]$) mediates inhibition of TAL NaCl transport in K-DEP rats, solute transport in the TAL of control and K-DEP rats was examined at the single nephron level using the technique of continuous microperfusion of functionally isolated single loops of Henle. Initial microperfusion studies examined the quantitative significance of the impairment in TAL NaCl transport in K-DEP rats in the presence of continuous axial flow. As well, these studies assessed whether an acute potassium infusion (to partially replace the potassium deficit induced by the potassium-deficient diet) could rapidly reverse the defect in TAL NaCl transport in K-DEP rats. The next series of studies examined the effect of a physiological

aldosterone infusion in repairing defective TAL Cl⁻ reabsorption in K-DEP rats, in the presence of reduced ECF[K⁺]. As well, the effect of an acute potassium infusion in repairing defective TAL Cl⁻ reabsorption, in the absence of increased aldosterone action, in K-DEP rats was investigated. Spironolactone, a potassium-sparing diuretic and aldosterone antagonist, was used to competitively block increased aldosterone action. Biological activity of aldosterone and spironolactone was confirmed in a separate series of experiments using adrenalectomized rats. Additional studies examined whether plasma [aldosterone] in K-DEP rats achieved control values in response to an acute potassium infusion. Lastly, studies were carried out to determine whether replacement of the total calculated potassium deficit would result in normalization of plasma [aldosterone] in K-DEP rats.

CHAPTER 2

2.1 Method: Description of the Microperfusion Protocol Used to Examine Defective TAL Cl⁻ Transport in K-DEP Rats.

2.1.1 Animal Preparation and Diets

Studies were carried out using 40 male Sprague-Dawley rats (Charles River, Quebec, Canada) weighing between 200 and 250 grams. Animals were individually housed with free access to food and distilled water for ten consecutive days. Potassium depletion was induced in 27 rats by feeding a basal electrolyte free diet (TD78093, Teklad Test Diets, Madison, WI.) deficient in potassium and supplemented with sodium, chloride, and neutral phosphate salts. This potassium deficient diet is referred to as the K-free diet. Control measurements were made in 13 rats fed the identical diet with the addition of potassium and was called the control diet. All electrolytes were added to the basal electrolyte free diet in our laboratory. The complete chemical composition (g/Kg) is given in Table 2. The basal electrolyte free diet consisted of 26% protein, 60% carbohydrate in the form of corn starch, 10% fat, 1.7% fiber and a complete vitamin and mineral mixture.

Table 2. Chemical Composition (g/Kg) of electrolytes added to the basal electrolyte free diet (TD78093, Teklad Test Diets, Madison, WI.).

CHEMICAL	CONTROL DIET (g/Kg)	K-FREE DIET (g/Kg)
NaCl	9.50	4.07
NaH ₂ PO ₄ ·H ₂ O	----	12.90
K ₂ HPO ₄	15.94	----
K ₃ Citrate	9.92	----
4MgCO ₃ ·Mg(OH) ₂ ·2H ₂ O	8.36	4.31
MgCl ₂ ·6H ₂ O	----	9.60
CaCO ₃	14.24	14.24
Distilled H ₂ O	600 mL	600 mL
Basal Diet	942.0	955.0
TOTAL	999.96	1000.12

In microperfusion studies to determine the contribution of the TAL to net Cl⁻ reabsorption, furosemide (10^{-3} M, Abbott, Montreal, Quebec, Canada) was added to the luminal perfusate. These studies were carried out using 8 rats from our colony at the University of Ottawa, weighing between 296 and 393 grams. In these studies, animals were fed standard rat chow (Purina, Canada) and drank tap water ad libitum.

2.1.2 Surgical Preparation for Microperfusion Studies

Rats were anaesthetized intraperitoneally with Inactin (100 mg/Kg, BYK Gulden Kontanz, FRG.) and prepared for microperfusion. The left flank region of the animal was shaved approximately 8 cm. Once the animal had reached a state of unconsciousness, it was placed dorsally on a thermoregulated table warmed to a normal body temperature of 37 °C. A small temperature probe was inserted into the rat's rectum to allow regulation of core body temperature. If the body temperature of the animal fell below 37 °C, then the thermoregulated table would turn on until the animal's core body temperature returned to 37 °C. Conversely, if the animal's body temperature became elevated, then the thermoregulated table would turn off, until the animal's body temperature had returned to normal. This negative feedback pathway initiated by the thermoregulated table allowed the animal's core body temperature to be maintained. Paraffin oil was then placed on the neck region to facilitate the

surgery and to minimize hair from circulating in the air.

2.1.2A Neck Surgery

An incision was made in the ventral surface of the midline, from the bottom of the mandible to the clavicle. The remainder of the surgery used blunt dissection techniques. The left jugular vein was located and freed from the surrounding mesentery for approximately one centimeter. A doubled silk ligature (size 4-0) was passed under the vessel and cut into two strands, with the ligature distal to the heart tied off firmly to stop blood flow. A small v-shaped incision was cut into the surface of the left jugular vein, with the aid of a dissecting microscope (Carl Zeiss Jena). The vein was then cannulated with both a PE-50 line and a PE-10 line. The PE-50 line allowed infusion of solutions. The PE-10 line was an anaesthetic line, allowing maintenance of anaesthesia throughout the experiment (1:10 dilution - Inactin: 0.9% NaCl) and euthanasia at the termination of the experiment. The remaining proximal ligature was used to tie the two cannulae firmly in the vein. Both the distal and proximal ligatures were then tied together for added strength.

The right jugular vein was isolated and cannulated in the identical method as described for the left jugular vein. The right jugular vein was also cannulated with two lines, a PE-50 and PE-10. The PE-50 line allowed continuous infusion of solutions while the PE-10 line allowed the bolus

administration (0.1 ml) of lissamine green dye for the assessment of transit time in the kidney.

Next, the left carotid artery was located by its pulsating nature and carefully separated from the vagus and sympathetic nerves until a one centimeter portion was cleared. A doubled silk ligature (size 4-0) was passed under the artery and cut into two strands. The ligature distal to the heart was firmly tied off to stop blood flow. A bulldog clamp was placed on the ends of the proximal ligature to maintain tension on the vessel and to maximize distance available to cannulate. A second clamp, an alligator clamp, was clamped onto the artery below the untied proximal ligature. Between the two ligatures, a small v-shaped cut was made on the surface of the artery, with the aid of a dissecting microscope. A PE-50 cannula, filled with heparin-saline (1:10), was advanced towards the alligator clamp (8 mm) and tied firmly into the vessel using the proximal ligature. The bulldog clamp was removed from the distal ligature and the proximal and distal ligatures were tied together. At this point, the alligator clamp was removed from the artery. Cannulation of the left carotid artery allowed continuous monitoring of the animal's blood pressure throughout the experiment. Blood pressure was measured via a pressure transducer (Statham, Hato Rey, Puerto Rico) which transformed the mechanical signal into an electrical signal which was then displayed digitally on an electromanometer (Inly Systems International Ltd., Gloucester, Ontario, Canada). Blood samples were collected from the left carotid artery cannula by

removing the end of the cannula from the pressure transducer. Only those preparations with a mean arterial pressure greater than or equal to 95 mmHg were used in these studies.

The final part of the neck surgery involved a tracheostomy. The trachea was dissected free from the surrounding tissue and a PE-90 cannula was placed under it, to prevent aspiration of interstitial fluid. The trachea was cut below the larynx, between two cartilage rings, and approximately one centimeter of PE-240 tubing was inserted into the trachea towards the lungs. Completion of the neck surgery usually required eleven to twelve minutes. An illustration of the cannulations and of the tracheostomy performed during the neck surgery is shown in Figure 6.

2.1.2B Left Kidney Preparation for Microperfusion

Prior to the preparation of the left kidney for microperfusion, the animal was checked to ensure an adequate level of anaesthesia was present. The animal was then placed on its right side and the fur in the left flank region was soaked with paraffin oil. The left kidney was exposed by a subcostal skin incision, approximately six centimeters in length, along the left flank of the animal. The underlying muscle layer was cauterized open to prevent bleeding, using a thermocautery (Burton Medical Products Corp., Model 41104, California, USA.). Perirenal fat and the left adrenal gland were carefully



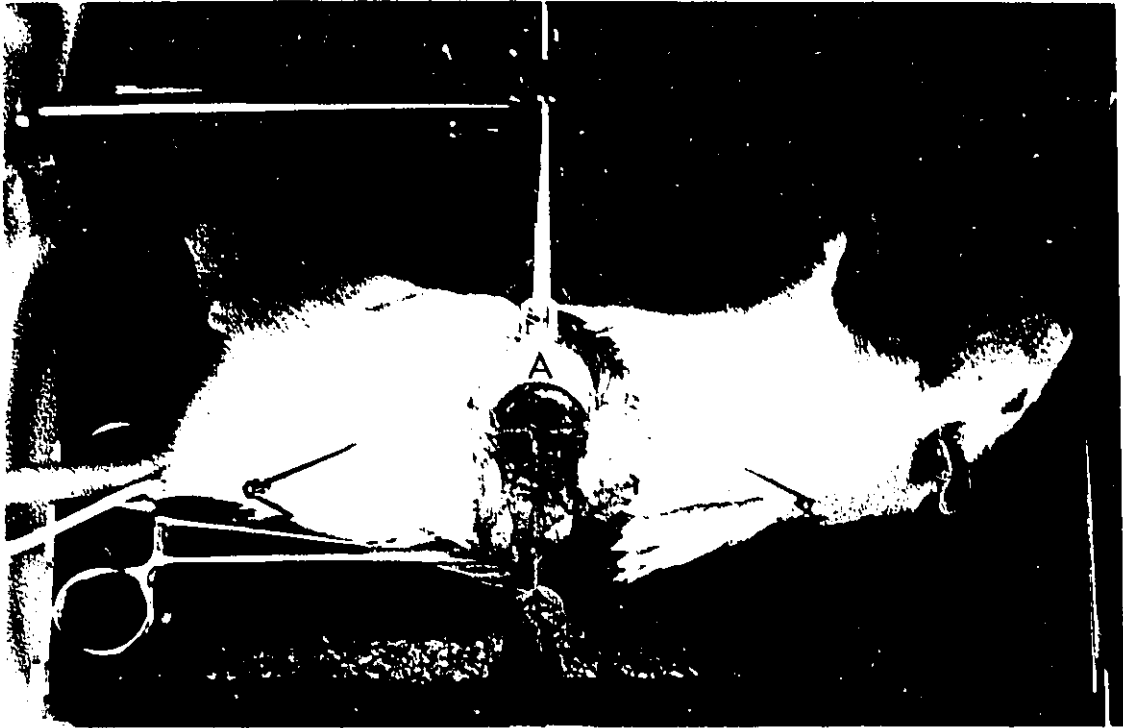
Figure 6. Diagram representing typical neck cannulations and tracheostomy in a rat being prepared for microperfusion studies. (A) left jugular vein cannulated with a PE-50 line and PE-10 line which allowed for a continuous infusion of 0.9% saline and maintenance of anaesthesia. (B) right jugular vein cannulated with PE-50 and PE-10 lines for the infusion of plasmanate and assessment of transit time in the kidney. (C) cannulation of the left carotid artery (PE-50 line) to allow continuous monitoring of the rat's blood pressure throughout the experiment. (D) tracheostomy (PE-240 cannula) to prevent aspiration of interstitial fluid.

dissected away from the curvature and poles of the kidney, using curved forceps. Special care was taken to prevent any damage to the renal artery, renal vein or to the ureter. Once the left kidney was cleaned of surrounding perirenal fat, it was placed in a translucent, lucite kidney cup that was attached to the back of the thermoregulated table. Warmed agar (2% agar in 0.9% NaCl) was placed around the kidney, forming a well in the kidney cup, and the kidney was then bathed in water-saturated paraffin oil.

Next, the left ureter was located and dissected free of surrounding fat. A doubled silk ligature (size 6-0) was passed under the ureter and cut into two strands. The ligature distal to the left kidney was firmly tied off. With the aid of a dissecting microscope, a small v-shaped cut was quickly made into the surface of the ureter. A PE-10 cannula was carefully inserted into the ureter, approximately 1.0-1.5 cm, and tied firmly into place by the proximal ligature. Tension was maintained on the ureter by an alligator clamp which was positioned onto the ends of the distal ligature. The distal end of the PE-10 cannula was then placed into a 1.5 ml Eppendorf tube, attached to the front of the thermoregulated table, and urine collections were made. The surface of the kidney was cleaned of any debris using the tips of microsponges (Sugi, Germany) which were slightly moistened with 0.9% NaCl. The placement of the left kidney and cannulation of the ureter is shown in Figure 7A and 7B. Completion of the surgery for microperfusion experiments generally required 30 minutes.

Figure 7A. A picture illustrating the placement of the left kidney and the cannulation of the left ureter in a rat being prepared for in vivo microperfusion studies. (A) left kidney in lucite kidney cup. (B) left ureter cannula (PE-10) for urine collection.

Figure 7B. Close-up view of the left kidney placement in the lucite kidney cup. (C) perfusion micropipet. (D) Hampel nanoliter perfusion pump. (E) collection micropipet.



Upon completion of the surgery, the transit time of the kidney was measured using lissamine green dye. A small bolus of lissamine green dye (approximately 0.1 ml) was injected in the right jugular vein and the appearance of the dye in the proximal and distal surface tubules was timed. The appearance of the dye in the late surface proximal tubules formed a proximal star image and occurred in less than 12 seconds. The time for the dye to reach the distal tubules was under 45 seconds after the first appearance of the dye was visualized in the kidney. Complete clearance of the dye from the kidney occurred in under two minutes from the first appearance of the dye in the kidney. Preparations were not used if: (a) proximal star formation time was greater than 12 seconds, (b) the time for the dye to reach the surface distal tubules was greater than 45 seconds or (c) clearance time of the dye from the kidney was longer than 2 minutes. The exclusion criteria used in all microperfusion experiments are summarized in Table 3. Transit times were also measured prior to the initiation of microperfusion and upon termination of the experiment.

2.1.3 Infusions Used During Microperfusion Studies

In the majority of microperfusion studies, rats were infused intravenously at 1% body weight per hour with 0.9% NaCl (Razel syringe pump, Model A-99). In some studies, rats were infused intravenously with 150 mM KCl at 1%

TABLE 3. A summary of the exclusion criteria used in all microperfusion studies.

(1) Blood pressure < 95 mmHg.

(2) Plasma [K] < 2.50 mM for all animals consuming the control diet. Plasma [K⁺] > 2.50 mM for all animals consuming the K-free diet.

(3) Transit time of lissamine green dye to proximal star formation > 12 seconds.

(4) Transit time of lissamine green dye to surface distal tubules > 45 seconds from bolus administration of the dye.

(5) Clearance time of lissamine green dye from kidney > 2 minutes.

(6) $0.95 < CF/P [Inulin] > 1.15$

(7) $19.8 < \text{Calculated Perfused Rate (nL/minute)} > 24.2$

body weight per hour. In other studies, rats were infused intravenously with aldosterone ($0.3 \mu\text{g}/100\text{g}$ body weight per hour) solubilized in 0.05% absolute alcohol-0.9% NaCl or its vehicle (0.05% absolute alcohol-0.9% NaCl) at 1% body weight per hour. In all studies, animals were infused intravenously with Plasmanate (5% human plasma protein, Cutter Laboratories, Berkeley, CA.) in buffered saline at a rate of 1 ml/hr (Sage Instruments syringe pump, Model 355). In studies involving the use of the aldosterone antagonist, spironolactone (Sigma), potassium depleted (K-DEP) rats were gavaged with spironolactone at $3 \text{ mg}/100\text{g}$ body weight as a 3 mg/ml solution in 2.5% Gum Tragacanth (Sigma), 2 hours prior to the acute administration of potassium.

2.1.4 Blood Sampling

An arterial blood sample was obtained from all animals at the completion of the neck surgery. Heparinized arterial blood was collected from the left carotid artery. Collected blood was analyzed for $[\text{K}^+]$, $[\text{Na}^+]$ and $[\text{Cl}^-]$. In studies where the animal was infused with 150 mM KCl, arterial blood samples were collected at the end of the neck surgery and at the termination of the microperfusion experiment. Potassium depleted (K-DEP) rats were excluded from the study if the measured plasma $[\text{K}^+]$ was greater than 2.50 mM as indicated in Table 3.

2.1.5 Microperfusion

Microperfusion studies began following a 120 minute equilibration period measured from the start of the neck surgery. As shown in Figure 8, microperfusion studies involved pump-driven perfusate from a late surface proximal tubule to an early surface distal tubule of the same nephron.

A filled perfusion micropipet (described in section 2.1.6) was mounted onto a calibrated Hampel nanoliter perfusion pump (Type III, Wolfgang Hampel, West Germany). Figure 9 illustrates the components of a Hampel nanoliter perfusion pump which was used in these studies. The lucite holder of the perfusion pump was filled with silicone oil (1:1 mixture of 20 cs and 50 cs silicone oil, Dow Corning). The filled perfusion micropipet, with its knurled screw and o-ring, was placed in the oil-filled lucite holder of the perfusion pump and the knurled screw was tightened. The cock of the lucite holder was tightened with an allen key and the lucite holder chamber checked carefully to make sure that there was no air trapped in the lucite holder. A shield was then carefully placed over the tip of the perfusion micropipet to maintain a constant flow rate in the presence of temperature fluctuations. The mounted perfusion micropipet was placed onto a nanoliter drive pump (Wolfgang Hampel, West Germany) which was connected to a Leitz micromanipulator. The tip of the perfusion micropipet was immersed into the water-saturated paraffin oil covering the surface of the left kidney. The nanoliter drive pump was turned

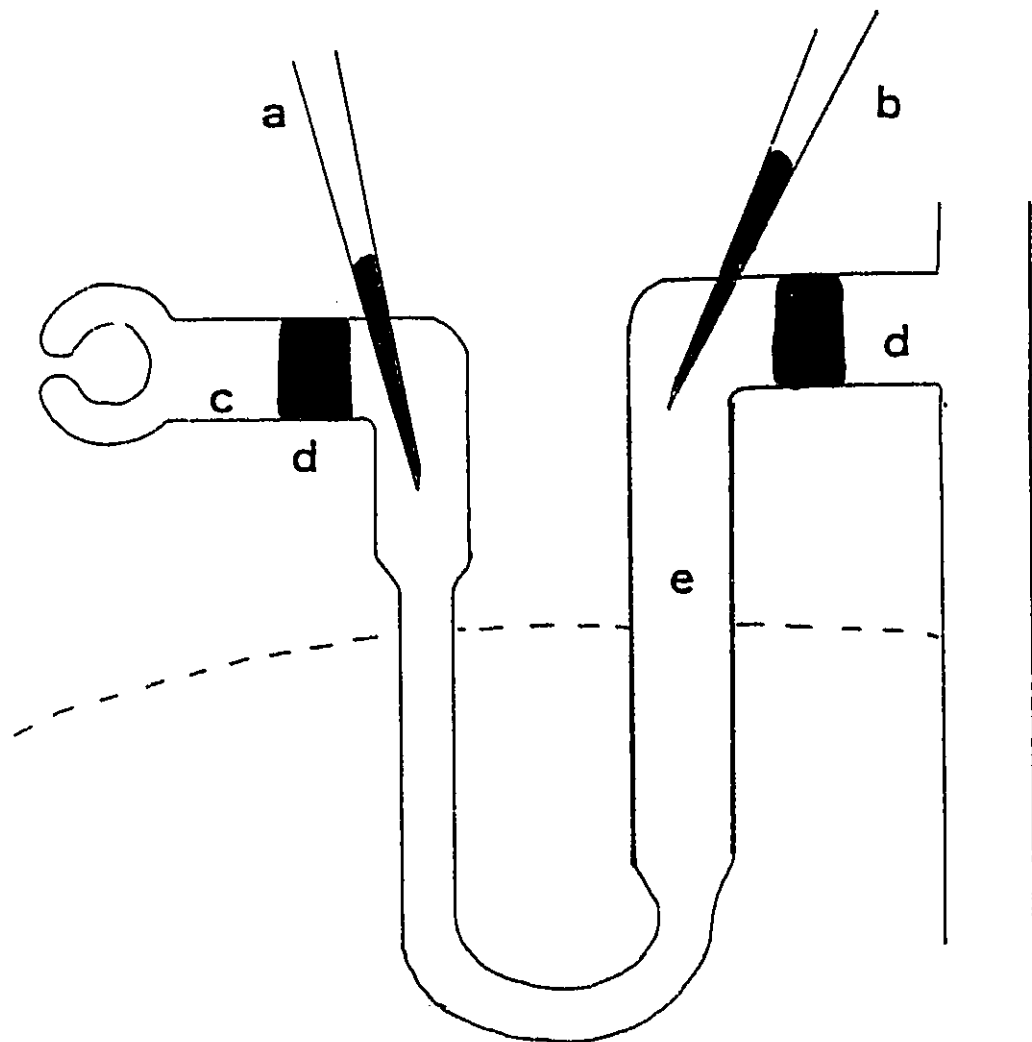


Figure 8. Schematic representation of the technique of continuous microperfusion *in vivo* in functionally isolated loop of Henle segments. (a) perfusion micropipet. (b) collection micropipet. (c) vent. (d) oil block. (e) TAL.

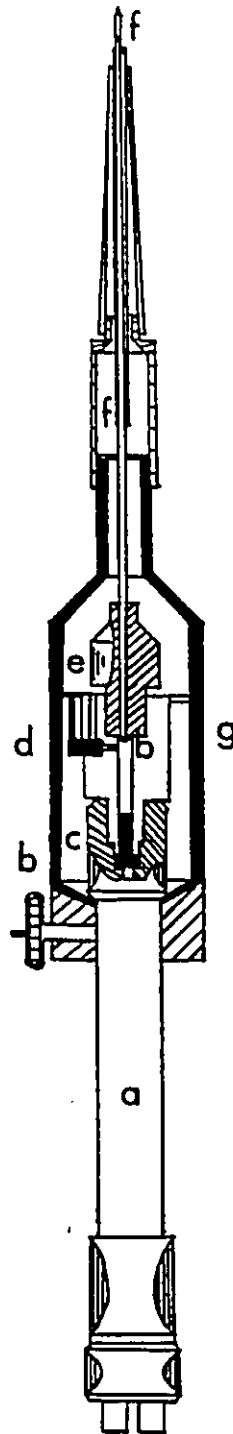


Figure 9. Components of a Hampel nanoliter perfusion pump. (a) microperfusion pump. (b) o-ring. (c) pump chamber. (d) cock. (e) knurled screw. (f) microperfusion pipet. (g) thermo-protector.

on and off to ensure satisfactory mounting of the perfusion micropipet. The nanoliter drive pump was then turned on at a flow rate of 5 nl/minute until the microperfusion experiment commenced.

A perfusion micropipet filled with perfusate was randomly inserted into surface proximal tubules. Perfusion was then started and stopped to find the latest surface proximal segment and to determine if the system had an early surface distal segment. Once a system was found which contained both a late surface proximal and an early surface distal segment, the perfusion micropipet was then placed in the latest surface proximal segment. The nephron was perfused at 22 nl/minute using a calibrated Hampel nanoliter perfusion pump (Type III, Wolfgang Hampel, West Germany) which is described in Figure 9. A collection micropipet was inserted upstream from the perfusion micropipet, and a sudan black stained paraffin oil block was injected. Upstream from the injected oil block, a vent was made in the proximal tubule using the tip of the collection micropipet, which allowed normal glomerular filtration to continue in the nephron. At this point, the partially closed system was perfused for a minimum of 2 minutes to ensure satisfactory flow in the nephron. A collection micropipet filled with sudan black stained paraffin oil was then inserted into the earliest surface distal tubule and a second oil block was injected distal to the tip of the collection micropipet. Timed collections of perfused fluid were collected for a minimum of 1.5 minutes and a maximum of 3 minutes. The average collection time in all experiments was 2 minutes.

All reported tubules were perfused at 22 nl/minute with a modified chloride perfusate stained with FD & C Green #3 (Keystone Aniline and Chemical Company, Chicago, Ill.) which allowed visualization of the tubules. The composition of the perfusate was physiologically similar to the composition of normal TAL tubular fluid. The loop perfusate was designed to minimize proximal nephron transport by approximating equilibrium concentration of NaCl in the proximal tubule. The non-reabsorbable solute, mannitol, was added to the perfusate to bring the total osmolality of the perfusate to 300 mOsm/Kg H₂O. This osmolality of the perfusate would theoretically impair water reabsorption in tubule segments proximal to the TAL. The complete composition of the modified loop perfusate used in these studies is given in Table 4.

In some studies, the loop diuretic, furosemide (10^{-3} M, Abbott, Montreal, Quebec, Canada.), was added to the perfusate. Carboxy-¹⁴C-inulin (New England Nuclear, Montreal, Quebec, Canada) was dialysed (Schafer et al, 1974) and added to the perfusate to give 80-100 dpm/nl. Microperfusion collections from the TAL were considered acceptable only when the ratio of collected fluid [inulin] to perfusate [inulin] was between 0.95 and 1.15. Table 3 summarizes the complete set of exclusion criteria used in all microperfusion studies.

Table 4. Composition of Modified Chloride Perfusate.

[Na ⁺]	110 mM
[Cl ⁻]	100 mM
[HCO ₃ ⁻]	10 mM
[K ⁺]	5 mM
[Ca ²⁺]	1 mM
[Mg ²⁺]	1 mM
[UREA]	4 mM
[MANNITOL]	61 mM

2.1.6 Perfusion Micropipets

Borosilicate glass, (KG-33, Friedrich-Dimmock, Millville, NJ., USA.) with an outer diameter of 0.98 mm and an inner diameter of 0.68 mm, was used to make perfusion micropipets. Perfusion micropipets were double-pulled using a horizontal micropipet puller (Model KHB 6, Rechnung, West Germany) set at 18.5 Amps. The perfusion micropipets were initially pulled at 18.5 Amps and allowed to cool. Once the pipets were cool, they were then repulled at 18.5 Amps. Double pulling the perfusion micropipet made a tip that was easier to insert into the late surface proximal tubule and also minimized the resistance to flow. The tips of the perfusion micropipets were then briefly dipped into a platinum resin (Blythe Matthey, Brampton, Ontario, Canada) and dried in a muffle furnace (Blue M Electric Company, Blue Island, Ill., USA.) at 360 °C for a minimum of one hour. This enhanced the visibility of the perfusion micropipet tips during the study. The tips of the perfusion micropipets were then re-dipped into the platinum resin and again dried in the muffle furnace for a minimum of one hour at 360 °C. The perfusion micropipets tips were then ground open to an outer diameter of 6-8 microns using a motor driven grinder (Groschopp & Co., West Germany).

The perfusion micropipets were then prepared for perfusion of functionally isolated nephrons in vivo. A perfusion micropipet was filled with mineral oil using a 25 gauge needle attached to a 3 ml syringe. A knurled

screw and o-ring was placed onto the end of the perfusion micropipet which was then connected to a pipet holder attached to a Brinkmann micromanipulator. The pipet holder was connected to a 60 ml syringe by tygon tubing. Fresh perfusate was filtered using a 0.45 micron Millipore filter and a 10 μ l sample was pipetted onto the bottom of a water-saturated paraffin oil filled quartz glass concave dish. The tip of the perfusion micropipet was carefully immersed into the sample of perfusate. Using negative pressure by pulling back on the syringe, the perfusion micropipet was filled with approximately 7 μ l of perfusate.

2.1.7 Collection Micropipets

Collection micropipets were made from borosilicate glass (KG-33, outer diameter: 0.98 mm, inner diameter: 0.68 mm) obtained from Friedrich-Dimmock (Millville, NJ., USA.). The collection micropipets were made in an identical manner to the perfusion micropipets with the exception being that these micropipets were not dipped into a platinum resin. The tips of the collection micropipets were ground open to 4-6 microns as described previously. On the day of the experiment, the tips of the collection micropipets were backfilled with sudan black stained paraffin oil. The end of the collection micropipet was inserted through a knurled screw and o-ring and placed onto a pipet holder which was connected to a Leitz micromanipulator. A 60 ml syringe, partially

filled with distilled water, was attached to the pipet holder by tygon tubing. The knurled screw was tightened. The tip of the collection micropipet was then immersed into the water-saturated paraffin oil covering the surface of the left kidney.

2.1.8 Description of Analytical Methods

2.1.8A Volume Determination of Collected Sample

The volume of the collected sample from perfused nephrons was measured on the same day using a calibrated constant bore quartz glass capillary (Vitro Dynamics, Inc., NJ. USA.). The constant bore quartz glass capillary (outer diameter: 0.08 mm; inner diameter: 0.05 mm) was filled with water-saturated paraffin oil. The volume of the sample was measured by transferring the contents of the collection micropipet into the oil-filled constant diameter bore quartz glass capillary whose length to volume ratio had been previously calculated using a known concentration of a radioactive solution. The collected sample was capped at both ends with water-saturated paraffin oil to minimize air contact and evaporation of sample. The length of the column of fluid was measured using a micrometer slide comparator (Gaertner Scientific Company, Chicago, USA.). The volume was determined by multiplying the measured length by the volume constant (nl/mm) of the constant quartz glass

capillary. The quartz glass capillary was siliconized with 1% Prosil-28 (SCM Chemicals, Gainesville, Florida, USA.) to prevent samples from adhering to the inside of the capillary. The measured volume of collected sample was generally between 40 and 60 nl. The measured sample was then aliquotted into two samples for liquid scintillation counting (approximately 10 nl) for ^{14}C -inulin and for nanoliter [Cl] analysis (approximately 30-50 nl). The constant bore quartz glass capillary was cleaned with distilled water and acetone between each sample volume measurement.

2.1.8B ^{14}C -Inulin

Measured volumes of perfusate and collected samples were dispensed into 7 ml plastic scintillation vials, containing 0.8 ml distilled water, for ^{14}C -inulin determination. Scintillation fluor (3.2 ml, Formula 963, New England Nuclear, Montreal, Quebec, Canada) was added to each vial. All vials were then shaken until the contents in the vial had formed a gel. Samples were counted for 10 minutes using a benchtop, liquid scintillation counter (Beckman Model LS 3801). Disintegrations per minute (DPM) were used in all calculations. DPMs were used in all microperfusion experiments because of quenching of the samples. This allowed comparisons of the samples to be made independent of the quench level and thus, provided a measure of the rate of absolute activity in the samples.

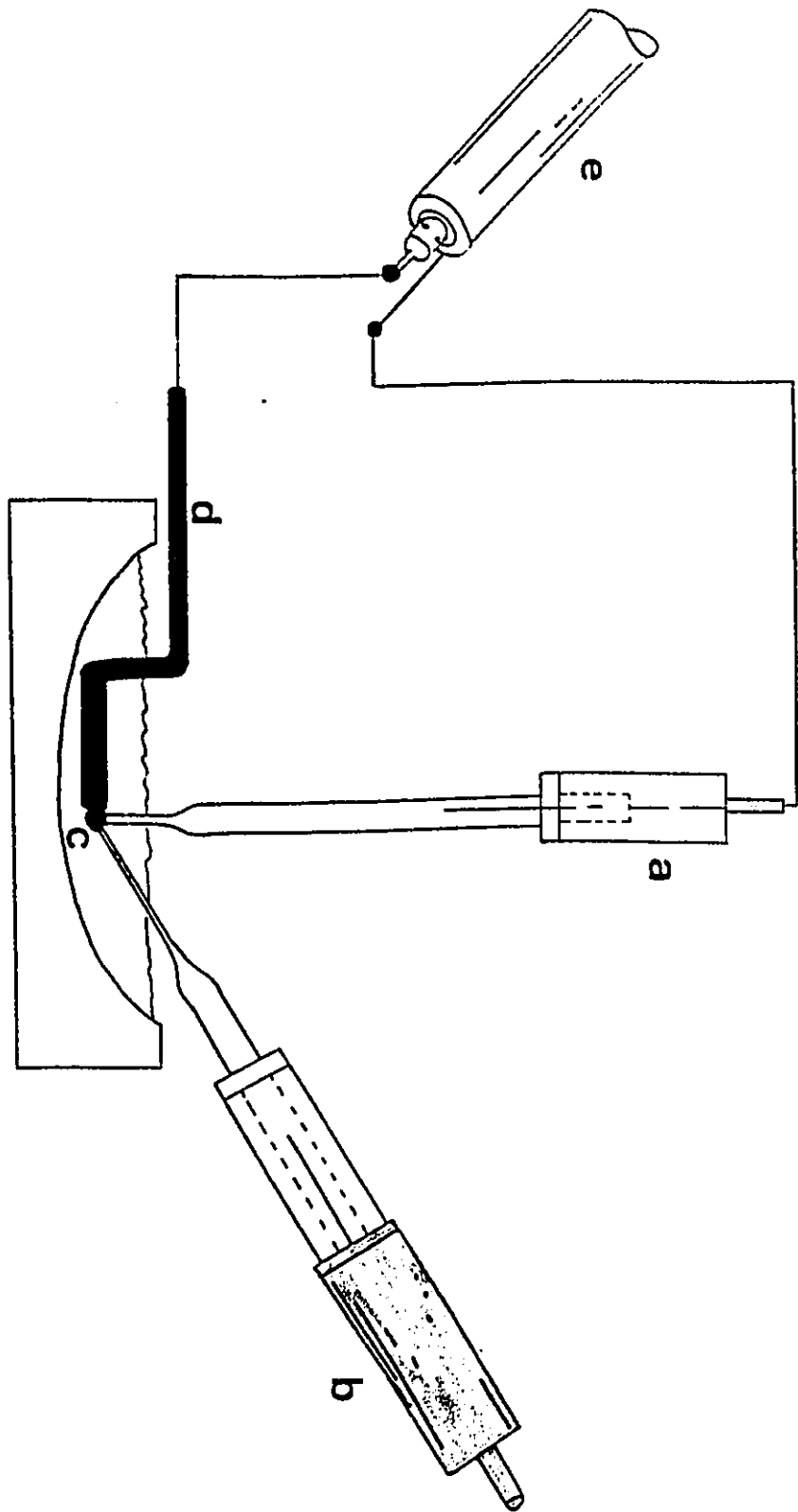
2.1.8C Coulometric Titration of Nanoliter Volumes of Chloride

Perfusate and collected sample $[Cl^-]$ were determined by coulometric titration (Microtitre ET-1, World Precision Instruments, New Haven, Conn., USA.) according to the method of Ramsey et al (1955). The Microtitre ET-1 used a current delivery system to determine $[Cl^-]$ in nanoliter samples by titrating silver and potentiometrically sensing the depletion of chloride. Figure 10 represents a diagrammatic illustration of the Microtitre ET-1 used in these experiments.

Borosilicate glass (KG-33, outer diameter: 0.98 mm, inner diameter: 0.68 mm, Friedrich-Dimmock, Millville, NJ., USA.) was used to make two glass micropipets. Micropipets were single-pulled at 18.5 Amps, using a horizontal micropipet puller (Model KHB 6, Rechnung, West Germany). The tips of the micropipets were ground open to an outer diameter of 1.2 microns. Each micropipet was filled with a warmed solution of 2% agar in 1 M $NaNO_3$. The shank of each micropipet was then backfilled with 1 M $AgNO_3$ and positioned in lucite holders (World Precision Instruments, New Haven, Conn., USA.), each in contact with a silver wire. As shown in Figure 10, one micropipet is referred to as the current injection probe (labelled "a" in Figure 10), while the second micropipet was inserted onto the tip of an electrometer probe, forming a voltage sensing probe (labelled "b" in Figure 10).

Using a 5 nl constriction pipet, a 5 nl droplet of 1 M H_2SO_4 was pipetted

Figure 10. Diagrammatic representation of the apparatus used in the coulometric titration of nanoliter volumes of chloride. The current injection (a) and voltage sensing (b) probes are attached to micromanipulators. (c) 1 M H_2SO_4 droplet. (d) silver wire ground. (e) coaxial cable.



onto the silver wire immersed in water-saturated paraffin oil, and the tips of both probes were placed in the 1 M H₂SO₄ droplet. This droplet served as a supporting electrolyte, aiding in the adhesion of the drop to the silver wire. A constant current (-500 nA) flowed through the droplet such that a positive cation current left the silver wire. Chloride ions combined with silver ions at the ground (silver wire) forming silver chloride which precipitated to the bottom of the droplet, decreasing the conductivity of the droplet. After sufficient time had elapsed, the electromagnetic field fell rapidly due to chloride depletion from the droplet. The end point of titration (+ 250 mV) was chosen as the half-way point on the rapid transition phase of the voltage versus time recording.

A standard curve in the range 25-100 mM chloride was run daily, using duplicates of each concentration to confirm reproducibility of the apparatus and method. A typical standard curve obtained using the Microtitre ET-1 is shown in Figure 11. A standard curve with a correlation coefficient of 0.999 or better was considered acceptable. Perfusate and collected samples containing chloride were then pipetted into the droplet, using a 5 nl constriction pipet, and ran in duplicate. The coefficient of variation of pipetted samples was less than 0.5%. Standard concentrations of chloride were run periodically throughout the chloride analysis to check for drift.

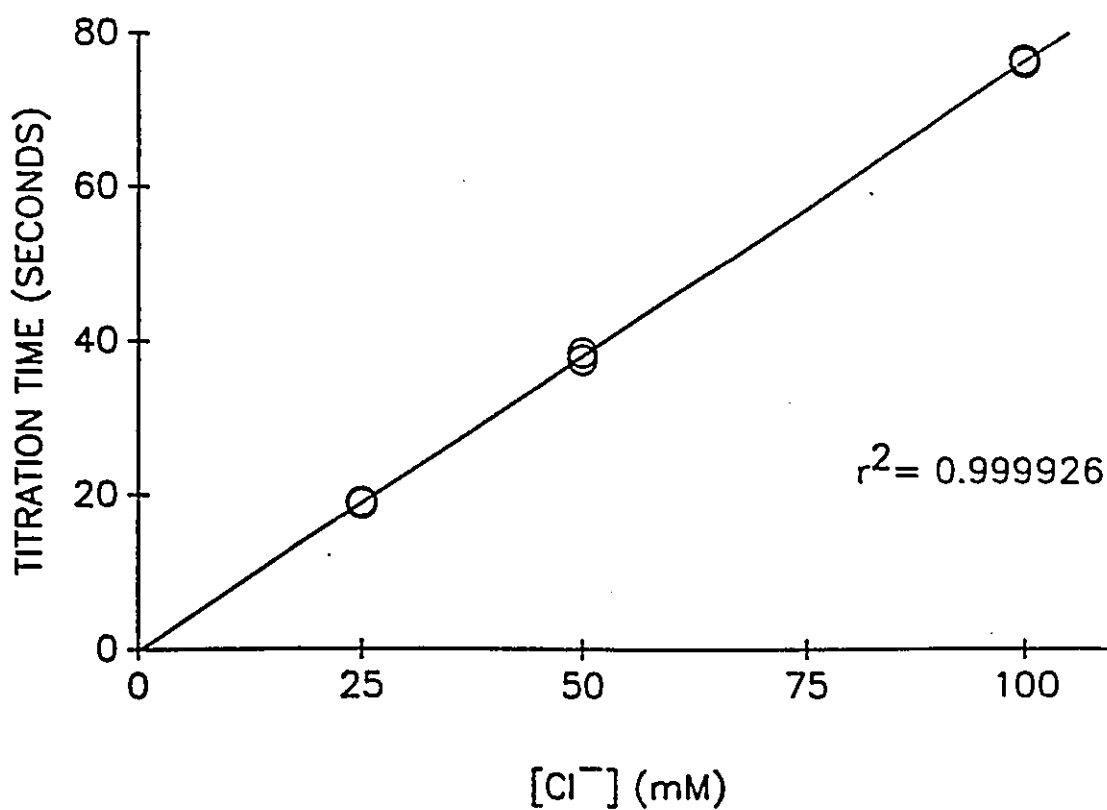


Figure 11. A typical standard curve obtained in the range 25-100 mM chloride using the method of coulometric titration. Duplicates of each concentration were performed to confirm reproducibility. The coefficient of correlation obtained for this curve was 0.999926.

2.1.8D Constriction Pipets

Aliquots of perfusate and collected samples were transferred using a constriction pipet during the coulometric titration of nanoliter volumes of chloride. The constriction pipet was made from capillary glass (outer diameter: 0.036", inner diameter: 0.0205") obtained from Drummond Scientific Company (Broomall, Penn., USA.). The glass capillary was pulled by hand over a small flame, to obtain a reasonably small internal diameter. The pulled end was then melted over the small flame to form a hook. The pulled glass capillary was then placed into the chuck of a vertical microfornice (custom made for Dr. D.Z. Levine, University of Ottawa). Using a heated wire, the internal constriction was formed. The internal dimensions of the constriction were determined using a Leitz stereomicroscope with a calibrated eye piece. To open the tip of the constriction pipet, a small weight was placed on the hook of the pipet. The wire was then heated until the weight dropped off, forming a small opening in the tip. Once the constriction pipet had cooled down, it was siliconized with 1% Prosil-28 (SCM Chemicals, Gainesville, Florida, USA.) to prevent samples from adhering to the inside and outside of the pipet. Constriction pipets were cleaned between experiments using distilled water, methanol and chromic acid. Constriction pipets generally had volumes in the 5 nl range.

2.1.9 Calculations

All perfusate and collected samples were analyzed in an identical manner. The in vivo calculated perfused rate (CPR), in nl/minute, was determined as shown by the following equation:

$$\text{CPR (nl/minute)} = \text{CR (nl/minute)} \times \text{CF/P [Inulin]}$$

where CPR (nl/minute) = calculated perfused rate in vivo

CR (nl/minute) = collected rate at the earliest surface distal site

CF/P [Inulin] = ratio of ^{14}C -inulin in the collected sample to the ^{14}C -inulin in the perfusate sample.

As previously indicated, data was only taken from experiments in which the CF/P [Inulin] ratio was between 0.95 and 1.15. This criterion excluded any samples obtained when the perfused system included more distal segments of the nephron (CF/P [Inulin] > 1.15). It also excluded any samples that were contaminated with surface fluid, which characteristically showed a low CF/P [Inulin] < 0.95. Samples were excluded from analysis if CPR was less than 19.8 nl/minute or greater than 24.2 nl/minute.

Fractional chloride reabsorption was calculated as a percentage (FR_{Cl} %) as shown below:

$$FR_{Cl} \% = \frac{([Cl^-]_{perf} \times CPR) - ([Cl^-]_{collected} \times CR)}{([Cl^-]_{perf} \times CPR)} \times 100$$

where $[Cl^-]_{perf}$ = $[Cl^-]$ in the perfusate sample
 $[Cl^-]_{collected}$ = $[Cl^-]$ in the collected sample
 CPR = calculated perfused rate

Chloride delivery to the TAL was calculated as $[Cl^-]_{perf} \times CPR$, while chloride remaining in the collected sample was determined by $[Cl^-]_{collected} \times CR$. Chloride flux (J_{Cl}) is the absolute rate of chloride reabsorption in the perfused segment in pmol/minute and was calculated as shown below:

$$J_{Cl} \text{ (pmol/minute)} = ([Cl^-]_{perf} \times CPR) - ([Cl^-]_{collected} \times CR)$$

Positive chloride fluxes indicate reabsorption while negative fluxes indicate secretion. A sample calculation, using the above equations, from an actual microperfusion experiment, is shown in Appendix A.

2.2 Method: Determination of the Effect of Dietary Potassium Depletion on Urine Concentrating Ability in Rats.

2.2.1 Animal Preparation and Diets

Studies were carried out using 66 male Sprague-Dawley rats (Charles River, Quebec, Canada) weighing between 200 and 303 grams. Animals were individually housed with free access to food and distilled water. Potassium depletion was induced in 33 rats by ad libitum feeding of the K-free diet. Control measurements were made in 33 rats fed the control diet. Complete diet information is given in section 2.1.1 and in Table 2.

Animals consumed either the control or K-free diet and drank distilled water for fourteen consecutive days. Animals were weighed daily to chart growth over the experimental period. In some studies, food intake and water intake were measured daily from day 1 to day 8 in all animals. Water, weight and food intake measurements were taken at the same time each day and measured gravitrimetrically, using a Toledo balance (Model 3115, Windsor, Ontario, Canada).

2.2.2 Urine Concentrating Tests

To determine the effect of chronic potassium depletion on maximal urine concentrating ability, 34 rats underwent urine concentrating tests on days 7, 10 and 13.

The night preceding the urine concentrating test, water bottles, which contained distilled water, were removed from the cages of all animals which were then water-restricted for the next sixteen hours. During this water restriction period, animals had free access to either the control or K-free diet. Following the sixteen-hour water deprivation period, the animals were then placed into individual, wire-bottom metabolic cages that contained funnels to direct urine output into water-saturated paraffin oil-filled urine collection bottles. Contamination of the collected urine by feces was minimized by the placement of wire traps into the urine collection funnels. Urine output was collected for four hours. At the end of the four-hour urine collection period, animals were returned to their cages and allowed to rehydrate with free access to distilled water and the appropriate diet (control or K-free diet). Collected urine was analyzed for urine osmolality to assess urine concentrating ability of the animals. Furthermore, the urine electrolytes $[Na^+]$, $[K^+]$ and $[Cl^-]$ were measured in the collected samples.

2.2.3 Blood Sampling

Animals were anaesthetized intraperitoneally with sodium pentobarbital (65 mg/Kg, Somnotol, MTM, Hamilton, Ontario, Canada) on day 14 and blood samples were drawn using the method of cardiac puncture.

To obtain the blood sample, the heart of the animal was located by its pulsating nature in the chest wall. A 23 gauge needle attached to a 3 ml syringe was inserted into the heart through the diaphragm at the xyphoid process. A 1.0 ml unheparinized blood sample was drawn for plasma [aldosterone] and immediately transferred to a 5 ml chilled EDTA vacutainer blood collection tube (Becton Dickinson, USA). A second 1.0-1.5 ml blood sample was then obtained from each animal, using an unheparinized syringe and needle, and immediately placed into a chilled 1.5 ml heparinized eppendorf tube for plasma osmolality and electrolyte analysis. Unheparinized blood samples were centrifuged, using a Beckman Model J2-21M Induction Drive centrifuge, at a speed of 3000 RPM (625 g) for twenty minutes at 4 °C. The plasma obtained from the unheparinized blood was used to measure plasma [aldosterone]. Heparinized blood samples were centrifuged for three minutes using a Fischer Micro-Centrifuge (Model 235A). These heparinized plasma samples were analyzed for plasma osmolality, as well as for plasma Na⁺, K⁺ and Cl⁻ content.

2.2.4 Left Kidney Wet Weight

In some studies, the left kidney was removed on day 14 to obtain a measurement of kidney wet weight in the control and potassium depleted (K-DEP) animals.

The abdomen of the animal was shaved from the sternum to the pubis. The initial skin incision was made using blunt suture scissors, and consisted of a middle laparotomy approximately six centimeters in length to expose the underlying peritoneal muscle layer. The peritoneum was cut along the linea alba which then allowed access to the left kidney. The kidney was gently dissected from the surrounding perirenal fat and excised. Wet weight of the kidney was quickly measured using a top-loading balance (Sartorius, 1219 MP).

2.3 Method: Determination of the Effect of a Potassium Load on Plasma [Aldosterone]

2.3.1 Animal Preparation and Diets

Studies were performed using 34 male Sprague-Dawley rats (Charles River, Quebec, Canada) weighing between 304 and 439 grams. Animals were individually housed for ten days with ad libitum access to food and distilled water. Potassium depletion was induced in 18 rats by ad libitum feeding of the

K-free diet whereas control measurements were made in 16 rats fed the control diet. The composition of the basal electrolyte free diet has been previously described in section 2.1.1. The chemical composition of the electrolytes added to the diet is given in Table 2.

2.3.2 Surgical Preparation

Rats were anaesthetized intraperitoneally with Inactin (100 mg/Kg, BYK Gulden Kontanz, FRG.) and prepared in an identical manner to animals used for microperfusion experiments as described in section 2.1.2. A complete description of the surgical preparation and neck surgery used in these studies is found in sections 2.1.2 and 2.1.2A.

2.3.3 Infusions Used During Potassium Loading Studies

All infusions used in these studies were identical to the infusions used in the microperfusion studies. A complete description of the infusions used is detailed in section 2.1.3.

2.3.4 Blood Sampling

An arterial blood sample was obtained from all animals following the completion of the neck surgery at $t = 15$ minutes. Heparinized arterial blood (0.5 ml) was collected from the left carotid artery. Collected blood was analyzed for $[K^+]$. K-DEP rats were excluded from the study if the initial measured plasma $[K^+]$ ($t = 15$ minutes) was greater than 2.50 mM. At $t = 45$, 75 and 120 minutes, additional 0.5 ml arterial blood samples were collected from the left carotid artery. The collected heparinized blood samples were then immediately analyzed for $[K^+]$. At the termination of the experiment ($t = 120$ minutes) unheparinized blood samples (2.0 ml) were obtained by cardiac puncture as described in detail in section 2.2.3. Unheparinized plasma was analyzed for [aldosterone].

2.4 Method: Determination of the Effect of Normalization of Total Body Potassium in Potassium Depleted Rats on Plasma [Aldosterone].

2.4.1 Animal Preparation and Diets

Studies were performed using 32 male Sprague-Dawley rats (Charles River, Quebec, Canada) weighing between 200 and 250 grams. Animals were individually housed for 13 days with ad libitum access to food and distilled

water. Potassium depletion was induced in 16 rats by ad libitum feeding the K-free diet. Control measurements were made in 16 rats fed the control diet. The composition of the basal electrolyte free diet is given in section 2.1.1. The chemical composition of the electrolytes added to the diets is given in Table 2.

2.4.2 0.5M KCl

On day 12, some rats (n=16) received an oral load of 0.5M KCl by gavage at 2 ml/100g body weight. All other animals (n=16) received an oral load of distilled water at 2 ml/100g body weight.

2.4.3 Blood Sampling

Animals were anaesthetized intraperitoneally with pentobarbital sodium (65 mg/Kg, Somnotol, MTM, Hamilton, Ontario, Canada) and blood samples were drawn by cardiac puncture as previously described in section 2.2.3.

A 1.0 ml unheparinized blood sample was drawn and immediately transferred to a 5 ml chilled EDTA vacutainer blood collection tube (Becton Dickinson, USA.). The plasma obtained from the unheparinized blood was used to measure plasma [aldosterone]. A second 1.0 - 1.5 ml heparinized blood sample was then obtained from each animal and immediately placed into a chilled 1.5 ml heparinized eppendorf tube. Heparinized blood samples were

analyzed for plasma osmolality, $[Na^+]$, $[K^+]$ and $[Cl^-]$.

2.5 Method: Determination of the Efficacy of Spironolactone and Aldosterone

2.5.1 Animal Preparation and Diets

Studies were carried out using 16 male Sprague-Dawley (Charles River, Quebec, Canada) rats weighing between 330 and 450 grams. Animals were individually housed with free access to standard rat chow (Purina, Canada) and tap water. Twenty-four hours following a bilateral adrenalectomy, all animals received ad libitum access to 155 mM saline drink and continued to feed on standard rat chow.

2.5.2 Surgical Protocol for Bilateral Adrenalectomy

Animals were weighed prior to the surgical procedure. All bilateral adrenalectomies were performed under sterile conditions, using the small animal surgical facilities provided by the Animal Care Service at the University of Ottawa. Appropriate sterile techniques were used throughout the entire surgical procedure which were carefully monitored by a trained Animal Health Technician or Veterinarian. Animals were studied 48 hours following the surgery.

2.5.2A Anaesthesia of Animals

All aspects of anaesthesia, including induction and maintenance, were monitored by a specially trained Animal Health Technician or Veterinarian from the University of Ottawa. Anaesthesia was induced in the animal by the use of a rodent anaesthetic box (21.5 cm X 15.0 cm X 13.5 cm) which was then placed into a scavenging box system (32.0 cm X 27.0 cm X 21.0 cm). The scavenging box system attached to a portable, small animal anaesthetic machine (Fluotec-3 Halothane Analyzer) which had a proper scavenging Bain circuit. A mixture of halothane (Fluothane, Ayerst) and oxygen was administered to the animal. Oxygen was administered at 1.0-1.5%. Initially, halothane was administered at 1% and then it was gradually increased to 3.5-4.0%. Once anaesthesia was induced in the animal, it was removed from the rodent anaesthetic box and placed on a sterile huck towel. At this point, anaesthesia was maintained using a small rodent anaesthetic mask (outer diameter: 30 mm, inner diameter: 28 mm) with oxygen maintained at 1.0-1.5% and halothane maintained at 1.5-2.5%. The exact mixture of gases used in these studies depended on the degree of anaesthesia induced in the animal and on the Animal Health Technician involved with the surgery.

2.5.2B Preparation of Skin

In preparation for the actual procedure, the left and right flank region of the rat was shaved from the bottom of each rib cage for approximately 5 centimeters. A vacuum was gently used over the shaved areas to remove any loose hair. The shaved areas were then cleansed with an antibacterial skin cleanser, Hibitane (chlorhexidine gluconate, 4% weight per volume) and rinsed with tap water. A topical germicide (1% free iodine) Betadine solution (10% providone-iodine) was swabbed over the shaved areas. The rat was then placed into a sterile stockinette (7.5 cm X 20 cm) to prevent contamination of sterility during the surgery, and transferred to a sterile huck towel-covered solid aluminum rodent ventilation operating table. The ventilation table was connected to a scavenging system which vented out any escaping halothane gas and minimized our exposure to the anaesthetic gas. Body temperature was maintained at approximately 37 °C by the use of a pump-driven hot water circulating heating pad (American Medical Systems, Aquamatic K, Model K-20-C) which was placed under the ventilation table. Anaesthesia was maintained by a rodent anaesthetic mask, with the gas mixture maintained with 1.5-2.5% halothane and 1.0-1.5% oxygen. The level of anaesthesia was carefully monitored throughout the surgical procedure by an Animal Health Technician who monitored depth of respiration and who administered periodic tail, toe and ear pinches to the animal.

2.5.2C Surgical Procedure

The sterile stockinette was cut open approximately 5 cm in length to expose the left shaved portion of the upper left flank. The initial skin incision consisted of a flank incision, approximately 2 cm in length, to expose the underlying peritoneal muscle layer. A blunt stab wound was made through the muscle layer, and the muscle was carefully cut open to allow direct access to the left adrenal gland. The left kidney was gently manipulated with sterile cotton swabs moistened with 0.9% sterile saline until the left adrenal gland was located. Small straight mosquito forceps were used to hold the left kidney by the surrounding perirenal fat, preventing damage to the kidney, while the left adrenal gland was clamped off using straight mosquito forceps. The left adrenal gland was then quickly excised from the upper pole of the left kidney, using Metzenbaum scissors. The left kidney was gently placed into the correct anatomical position within the abdominal cavity and the area moistened with sterile 0.9% NaCl. The peritoneal muscle layer was sutured closed, using a continuous stitch, using sterile, synthetic, absorbable 3-0 coated (polyglactin 910) Vicryl suture material (tapered edge, Ethicon). The skin incision was closed with three to five 9 mm stainless steel wound clips (Autoclips, Clay Adams). The procedure was repeated for the right adrenal gland. Completion of the surgery generally required between 20 and 30 minutes.

Upon closure of the skin wound, the halothane gas was turned off and

the animal was maintained on pure oxygen until signs of recovery, such as limb movement, were observed. At this point, the animal received a subcutaneous injection of the analgesic, Demerol (Meperidine Hydrochloride, Winthrop), at a dose of 0.1 ml/100g body weight as a 1 mg/ml solution. The animal was then placed into a warmed infant incubator, preset at 37 °C, for a minimum of 30 minutes prior to being returned to its cage.

2.5.3 Spironolactone

Two hours prior to the commencement of a two-hour urine collection, some animals (n = 11) received an oral load of the aldosterone antagonist, Spironolactone (Sigma). Spironolactone was gavaged at a dose of 3 mg/100 g body weight as a 3 mg/ml solution in 2.5% gum Tragacanth (Adam et al, 1987). Gum Tragacanth (Sigma) is a complex mixture of polysaccharides which includes bassarin and tragacanthin.

2.5.4 Aldosterone

Some animals (n = 10) received an intraperitoneal injection of aldosterone (Sigma) one hour prior to the commencement of the two-hour urine collection. All other animals (n = 6) received an intraperitoneal injection of the vehicle for aldosterone, one hour prior to the urine collection period.

Aldosterone was administered at a dose of 3 $\mu\text{g}/100$ g body weight as a 6 $\mu\text{g}/\text{ml}$ solution dissolved in 0.05% absolute alcohol-0.9% NaCl (Adam et al, 1987). Vehicle-injected animals received 0.05% absolute alcohol-0.9% NaCl.

2.5.5 Urine Collection

To facilitate collection of urine, all animals (n = 16) received an oral distilled water load by gavage at 2 ml/100 g body weight immediately prior to the initiation of the two-hour urine collection. Following the water load by gavage, animals were placed into individual, wire-bottom metabolic cages which contained funnels to direct the urine output into water-saturated paraffin oil-filled urine collection bottles. Contamination of collected urine output was minimized by the placement of wire traps into the base of the urine funnels. Urine output was collected for two hours. At the end of the urine collection period, animals were returned to their cages. Collected urine volume was measured gravitrimetrically using a top-loading balance (Sartorius, 1219 MP). Collected urine samples were analyzed for $[\text{Na}^+]$, $[\text{K}^+]$, $[\text{Cl}^-]$ and [creatinine].

2.5.6 Blood Sampling

Following the two-hour urine collection period, animals were anaesthetized intraperitoneally with pentobarbital sodium (65 mg/Kg, Somnotol,

MTM, Hamilton, Ontario, Canada) and blood samples were drawn by cardiac puncture as previously described in section 2.2.3. Heparinized plasma samples were analyzed for $[\text{Na}^+]$, $[\text{K}^+]$, $[\text{Cl}^-]$ and creatinine content.

2.6 Euthanasia

All animals used in these studies were sacrificed at the termination of the experiment with either an intraperitoneal overdose of pentobarbital sodium anaesthesia or an intravenous overdose of Inactin anaesthesia.

2.7 Analytical Methods

The osmolality of heparinized plasma samples was measured using a freezing point depression osmometer (μ osmette, Model 5004, Precision Science, MA.). Urine osmolality was analyzed using a vapour pressure osmometer (Model 5100C, Wescor Inc., Logan, UT.). In initial studies, heparinized plasma and urine $[\text{Na}^+]$ and $[\text{K}^+]$ were measured by flame photometry. In other studies, heparinized plasma and urine $[\text{Na}^+]$ and $[\text{K}^+]$ were measured by ion specific electrodes (Na^+/K^+ Analyzer, Model 614, Stat Analyzer, Ciba Corning Diagnostics Limited, Halstead, Essex, UK.). Urine and heparinized plasma $[\text{Cl}^-]$ were measured using a Corning 925 Chloride Analyzer (Corning Medical and Scientific, Halstead, Essex, UK.). Unheparinized plasma

[aldosterone] were measured by radioimmunoassay (Dr. Daniel Bichet, Hopital du Sacre-Coeur, Montreal, Quebec, Canada; Bichet et al, 1986). Plasma and urine [creatinine] were measured by the Jaffe Reaction Kinetic method using a Hitachi 705 Analyzer (Boehringer Mannheim, Dorval, Quebec, Canada; Children's Hospital of Eastern Ontario, Ottawa, Canada).

2.8 Statistical Analysis

All quantitative results are expressed as mean \pm SEM (standard error of the mean). The Student's unpaired t-test was used to compare two related groups. The Student's paired t-test was used to compare paired samples. For multiple comparisons of group means, a one-way analysis of variance (ANOVA) and the Tukey multiple comparison test or the Least Significant Difference (LSD) multiple comparison test was used to determine the statistical significance between group data. A probability value of $p < 0.05$ was considered statistically significant. The specific statistical test used for each comparison reported is indicated in the legend of each figure and table.

CHAPTER 3

The experimental work is reported in 6 major sections as described below. The first series of experiments examined the effects of dietary potassium deprivation on urine concentrating ability, body weight, kidney weight, water intake, and urine and potassium electrolytes in rats. The second series of results assessed the contribution of the TAL to net Cl^- reabsorption at the single nephron level in microperfused functionally isolated loop segments in vivo. The third series of experiments examined TAL Cl^- transport in control and K-DEP rats to determine whether an aldosterone deficiency and/or reduced extracellular fluid potassium concentration ($\text{ECF}[\text{K}^+]$) mediates inhibition of TAL Cl^- transport in K-DEP rats. The next series of experiments determined whether plasma [aldosterone] in K-DEP rats achieved control values in response to an acute potassium infusion (which partially replaced the potassium-deficit induced by the diet). The fifth series of experiments involved the measurement of plasma [aldosterone] to determine whether replacement of the total calculated potassium deficit in K-DEP rats would result in the normalization of this mineralocorticoid hormone to control values. The last series of results evaluated the efficacy of the aldosterone antagonist, spironolactone and aldosterone used in the microperfusion studies.

3.1 Results: Effects of Dietary Potassium Depletion in Rats.

The purpose of these studies was to determine the effect of dietary potassium depletion on urine concentrating ability. In addition, the effect of dietary potassium depletion on plasma and urine electrolytes, water and food intake, plasma [aldosterone], and kidney weight was examined. These studies were carried out using male Sprague-Dawley rats which were individually housed for 14 days with ad libitum access to either the control or K-free diet and distilled water.

3.1.1 Effect of Dietary Potassium Depletion on Food Intake, Growth, Kidney Weight and Water Intake.

Average food intake calculated over 8 days of a 14-day period of study showed no difference in terms of food consumption between control and K-DEP animals (control (n = 16): 14.68 ± 0.317 g/24 hrs/8 days per 100 g body weight versus K-DEP (n = 16): 13.97 ± 0.471 g/24 hrs/8 days per 100 g body weight, $p > 0.22$). Therefore, in all subsequent studies, control and K-DEP animals were allowed ad libitum access to the appropriate diet and pair feeding was not utilized. Despite similar food intakes over the same time period, growth in K-DEP animals was minimal, with these animals achieving only a 10% increase in body weight (over the 14 days of study) whereas control animals

achieved a 38% increase in body weight over the same time period ($p < 0.05$). At the end of the study, K-DEP animals demonstrated a hypertrophy in kidney weight compared to control animals (control ($n = 6$): 0.369 ± 0.017 g/100g body weight versus K-DEP ($n = 6$): 0.509 ± 0.024 g/100g body weight, $p < 0.0001$).

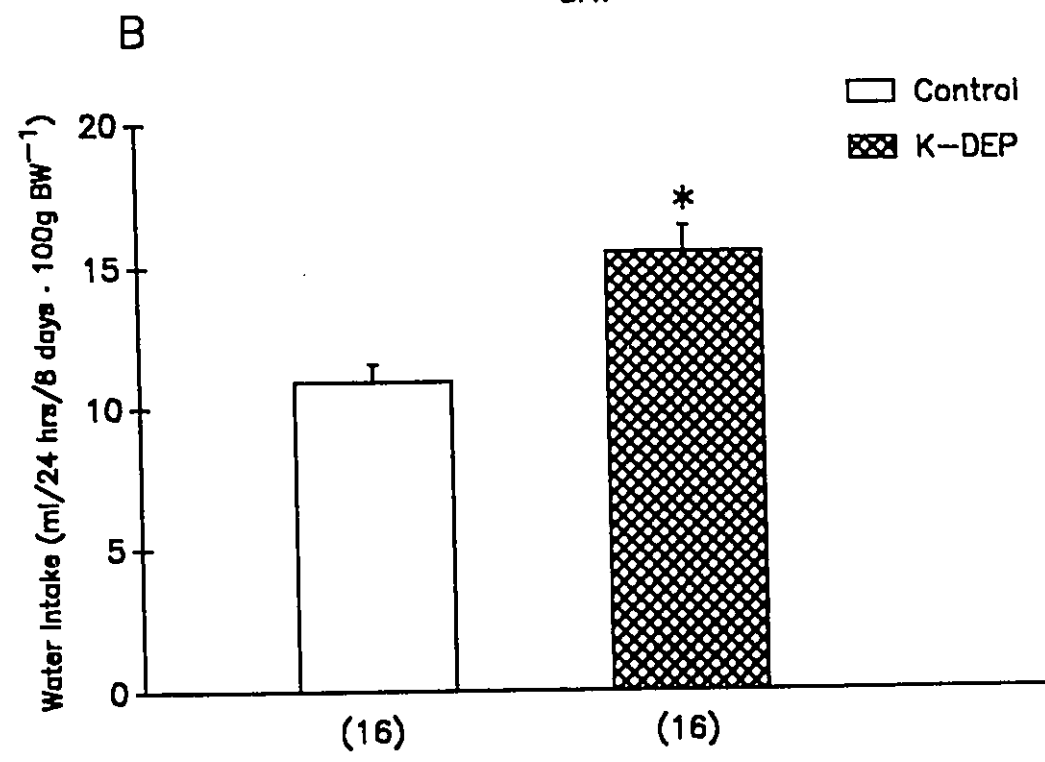
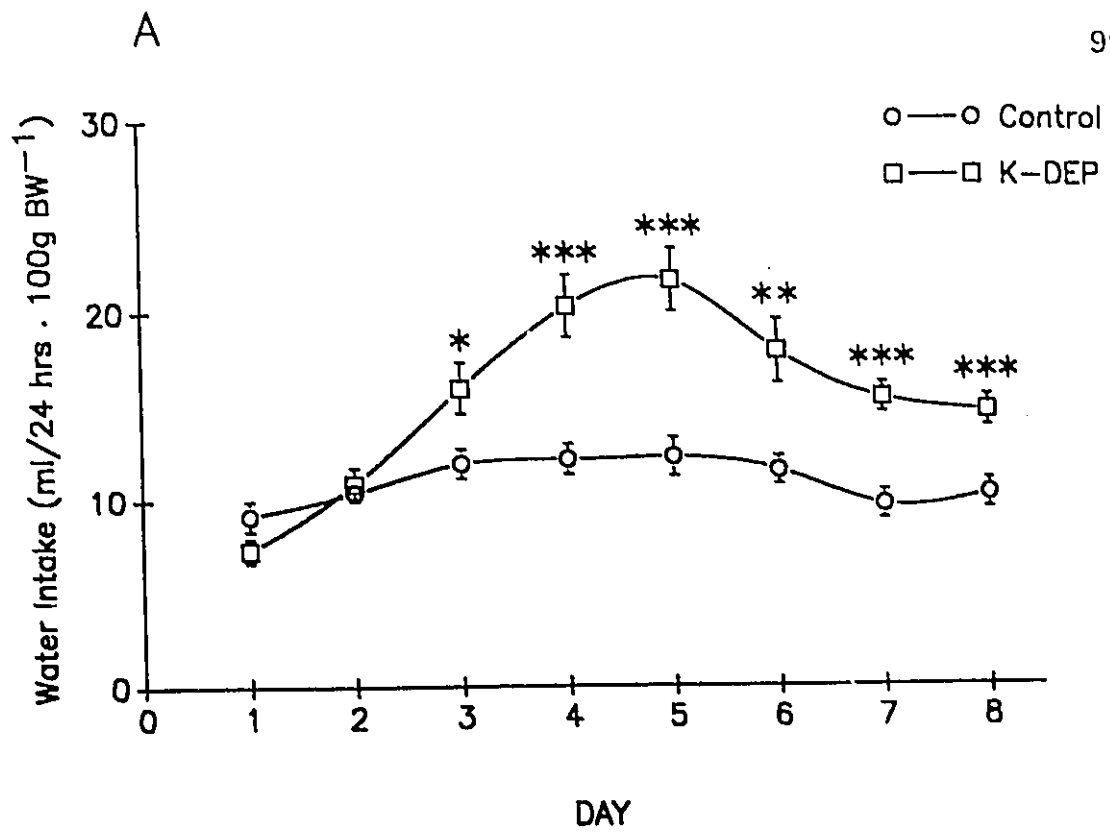
Daily water intake in rats consuming either the control diet or the K-free diet over an 8-day period of study is illustrated in Figure 12A. Daily water intake of K-DEP rats increased at day 3 (control: 11.91 ± 0.78 ml/24 hrs/100g body weight versus K-DEP: 15.92 ± 1.34 ml/24 hrs/100g body weight, $p < 0.05$), peaked at day 5 (control: 12.24 ± 1.02 ml/24 hrs/100g body weight versus K-DEP: 21.61 ± 1.66 ml/24 hrs/100g body weight, $p < 0.0001$) and remained elevated for the remainder of the study. Average water intake calculated over the 8-day period of study was 42% greater in K-DEP animals compared to controls (Figure 12B).

3.1.2 Effect of Dietary Potassium Depletion on Urine Electrolytes and Urine Concentrating Ability.

Following a 16-hour water deprivation period, urine osmolality (U_{osm}) was measured in collected urine samples and used as an index of urine concentrating ability. The U_{osm} of control and K-DEP rats on days 7, 10 and 13 is shown in Figure 13. An impairment in urine concentrating ability was

Figure 12A. Daily water intake of rats consuming control or K-free diet throughout the first 8 days of a 14-day experimental period. Number of animals in each group is 16. Results are expressed as mean \pm SEM. * $p < 0.05$ vs control. ** $p < 0.01$ vs control. *** $p < 0.001$ vs control animals on the same day using the Student's unpaired t-test.

Figure 12B. Average water intake of rats on control or K-free diet calculated over the 8 day period of study. Number of animals in each group is shown in parentheses. Results are expressed as mean \pm SEM. * $p < 0.001$ compared with control animals using the Student's unpaired t-test.



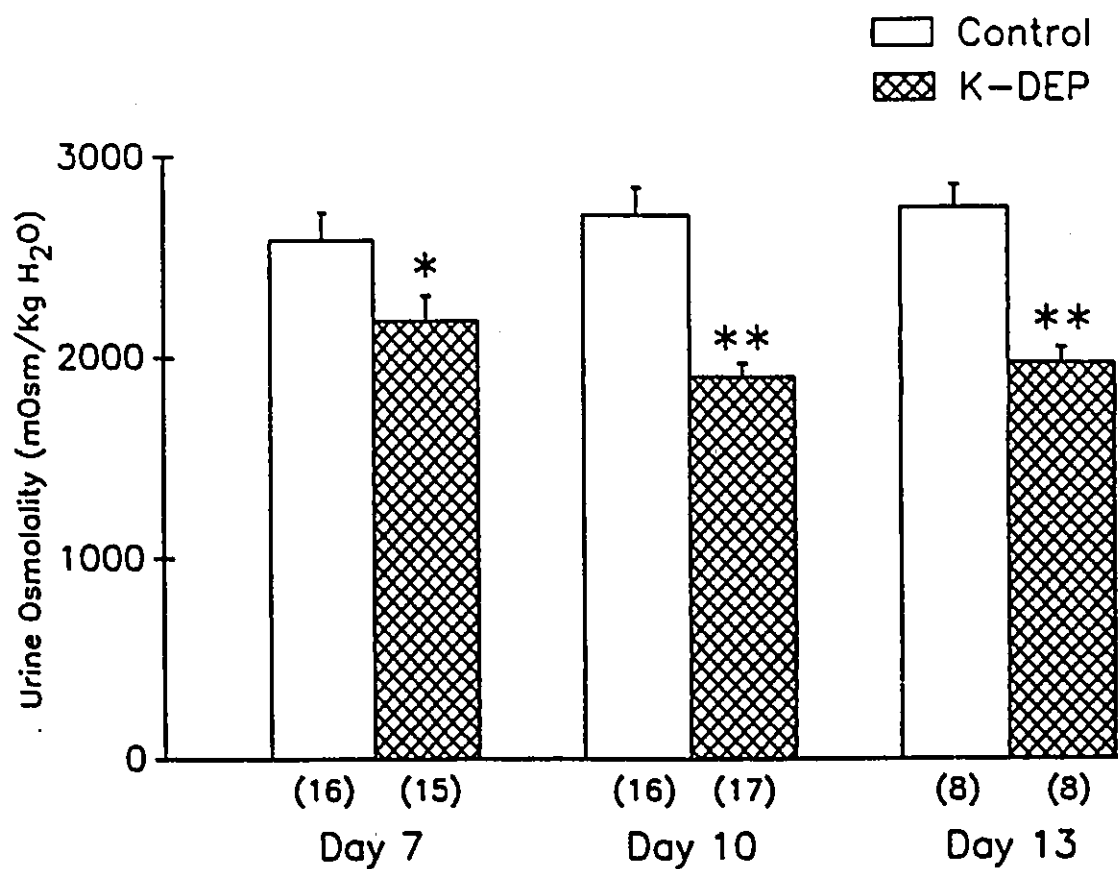


Figure 13. Urine osmolality of rats consuming control or K-free diet measured on day 7, 10 and 13 after an overnight 16 hour water deprivation period. Number of animals in each group is shown in parentheses. Results are expressed as mean \pm SEM. * $p < 0.05$ vs control. ** $p < 0.0001$ compared to control on the same day; using the Student's unpaired t-test.

observed as early as day 7 in K-DEP rats where U_{osm} was reduced compared to control rats (control: 2583 ± 134 mmol/Kg H_2O versus K-DEP: 2179 ± 126 mmol/Kg H_2O , $p < 0.05$). K-DEP animals demonstrated a further decrease in urine concentrating ability on day 10 (K-DEP, day 7 ($n = 15$): 2179 ± 126 mOsm/Kg H_2O versus K-DEP, day 10 ($n = 17$): 1900 ± 69.4 , $p = 0.0541$) as measured by a reduction ($p < 0.05$) in the maximal urine osmolality achieved in comparison to normal control values. Although the urine concentrating defect was present on day 13 in K-DEP rats, the impairment in maximum urine osmolality attained had not worsened from day 10 with prolonged dietary induced potassium depletion. These results taken together with water intake measurements show that the polydipsia in K-DEP rats preceded the development of a renal concentrating defect. Since the impairment in urine concentrating ability in K-DEP was maximal at day 10, all subsequent studies examined the defect in renal concentrating ability observed during potassium depletion at this time.

Urine electrolytes measured from control and K-DEP animals are summarized in Table 5. As expected, urine $[\text{K}^+]$ was reduced by day 7 in K-DEP animals (control: 449 ± 39.9 mM versus K-DEP: 8.23 ± 0.743 mM, $p < 0.0001$) and remained suppressed for the duration of study. In K-DEP rats, urine $[\text{Na}^+]$ was decreased on day 10 (control: 265 ± 27.1 mM versus K-DEP: 175 ± 37.2 mM, $p < 0.06$) and reduced ($p < 0.05$) on day 13 compared to control urine $[\text{Na}^+]$. No significant difference was measured in urine $[\text{Cl}^-]$ in the

Table 5. Urine electrolytes of control and K-DEP rats collected on day 7, 10 and 13 following an overnight 16-hour water deprivation period. Number of animals in each group is shown in parentheses. Results are expressed as mean \pm SEM. * $p < 0.0001$ versus control animals on the same day using the Student's unpaired t-test. ** $p < 0.05$ versus control animals on the same day using the Student's unpaired t-test.

	URINE [Na ⁺] (mM)	URINE [K ⁺] (mM)	URINE [Cl ⁻] (mM)
CONTROL (Day 7)	223 \pm 29.8 (14)	449 \pm 39.9 (14)	251 \pm 26.6 (13)
K-DEP (Day 7)	211 \pm 39.6 (13)	8.23 \pm 0.743* (13)	296 \pm 32.4 (13)
CONTROL (Day 10)	265 \pm 27.1 (13)	517 \pm 69.0 (13)	301 \pm 22.8 (13)
K-DEP (Day 10)	175 \pm 37.2 (11)	6.82 \pm 1.16* (11)	255 \pm 34.8 (12)
CONTROL (Day 13)	327 \pm 36.4 (6)	528 \pm 88.3 (6)	348 \pm 27.9 (6)
K-DEP (Day 13)	203 \pm 36.9** (7)	6.39 \pm 0.763* (7)	301 \pm 32.2 (6)

urine samples collected from control and K-DEP animals.

3.1.3 Effect of Dietary Potassium Depletion on Plasma Osmolality and Electrolytes.

Plasma osmolality and electrolytes measured in blood samples obtained by cardiac puncture, after 14 days of animals consuming either the control or K-free diet, are summarized in Table 6. K-DEP rats were strikingly hypokalemic on day 14 with plasma $[K^+]$ reduced from control values (control: 4.27 ± 0.153 mM versus K-DEP: 2.31 ± 0.0157 mM, $p < 0.0001$). Hypochloremia was also observed in K-DEP rats (control: 104 ± 0.904 mM versus K-DEP: 95 ± 0.960 mM, $p < 0.0001$). No significant differences were measured in plasma osmolality or plasma $[Na^+]$ between control and K-DEP rats.

3.1.4 Effect of Dietary Potassium Depletion on Plasma [Aldosterone].

Plasma [aldosterone] in control and K-DEP animals after 14 days of consuming either the control diet or the K-free diet is summarized in Figure 14. Plasma [aldosterone] was reduced in K-DEP rats compared to control animals (K-DEP: 19.2 ± 3.32 ng/dl versus control: 56.4 ± 8.05 ng/dl, $p < 0.001$).

Table 6. Plasma electrolytes and osmolality in control and K-DEP rats after 14 days of study. Number of animals in each group is shown in parentheses. Results are expressed as mean \pm SEM. * $p < 0.0001$ compared with control by the Student's unpaired t-test.

Plasma	Control	K-DEP
Osmolality (mOsm/Kg H ₂ O)	304 \pm 1.95 (8)	301 \pm 2.70 (10)
[Na ⁺] (mM)	140 \pm 0.467 (10)	141 \pm 0.666 (11)
[K ⁺] (mM)	4.27 \pm 0.153 (10)	2.31 \pm .0157* (11)
[Cl ⁻] (mM)	104 \pm 0.904 (9)	95 \pm 0.960* (10)

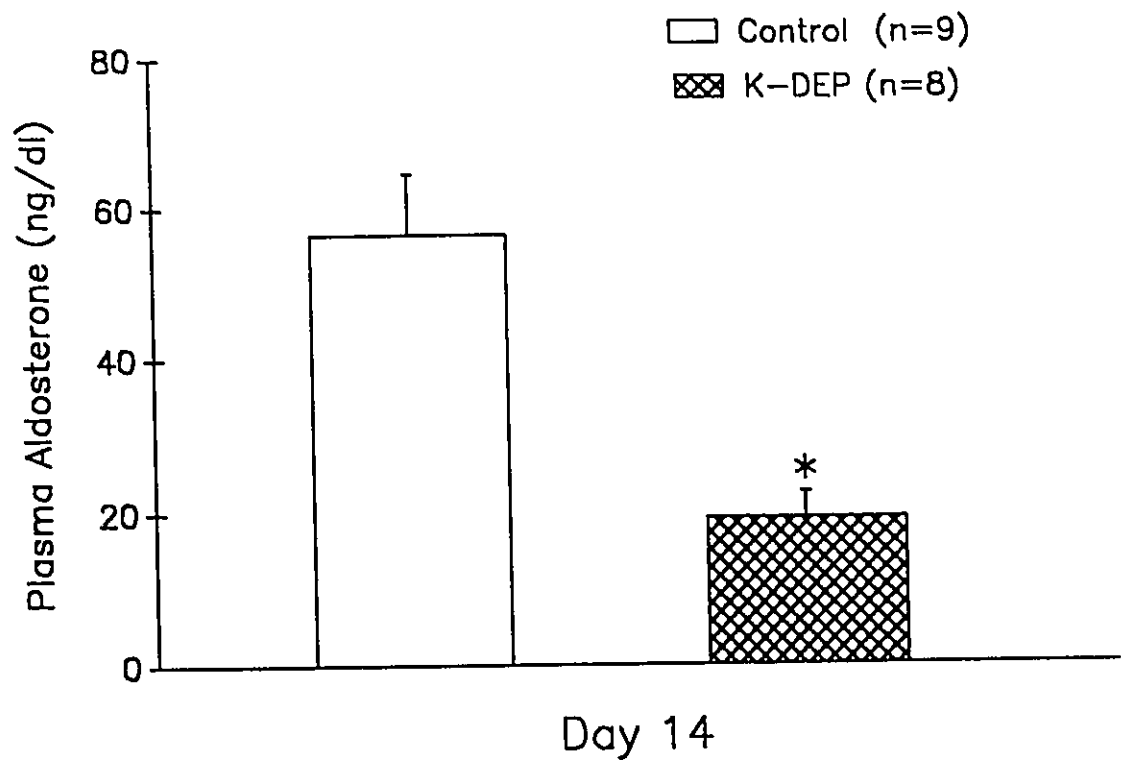


Figure 14. Plasma aldosterone levels measured in rats after 14 days of consuming either the control or K-free diet. Number of animals in each group is shown in parentheses. Results are expressed as mean \pm SEM. * $p < 0.001$ compared with control animals using the Student's unpaired t-test.

3.2 Results: Evaluation of In Vivo Microperfusion Conditions to Assess TAL Cl⁻ Transport.

The purpose of these studies was to determine the contribution of the TAL to net Cl⁻ reabsorption measured during perfusion of the loop segment in vivo. Furosemide-sensitive J_{Cl} was used as an index of TAL chloride reabsorption. These studies were carried out using male Sprague-Dawley rats bred from our colony at the University of Ottawa. All rats used in these studies consumed standard rat chow and drank tap water ad libitum. TAL Cl⁻ transport was measured in functionally isolated microperfused loop of Henle segments in vivo.

3.2.1 Effect of Furosemide on Loop Segment Cl⁻ Reabsorption.

Furosemide (10^{-3} M), a loop diuretic, was added to the luminal perfusate to determine the contribution of the TAL to net Cl⁻ reabsorption in the loop segment. A significant reduction in chloride flux (J_{Cl}) was observed in the presence of furosemide (Figure 15). Control loop segments reabsorbed 1325 ± 104.8 pmol/min of Cl⁻ compared to loop segments perfused with furosemide, which secreted -198 ± 56.1 pmol/min of Cl⁻ ($p < 0.0001$). To correct the data for differences in Cl⁻ delivery, the fraction of Cl⁻ reabsorbed was also expressed as a percentage of the delivered Cl⁻ load (fractional chloride

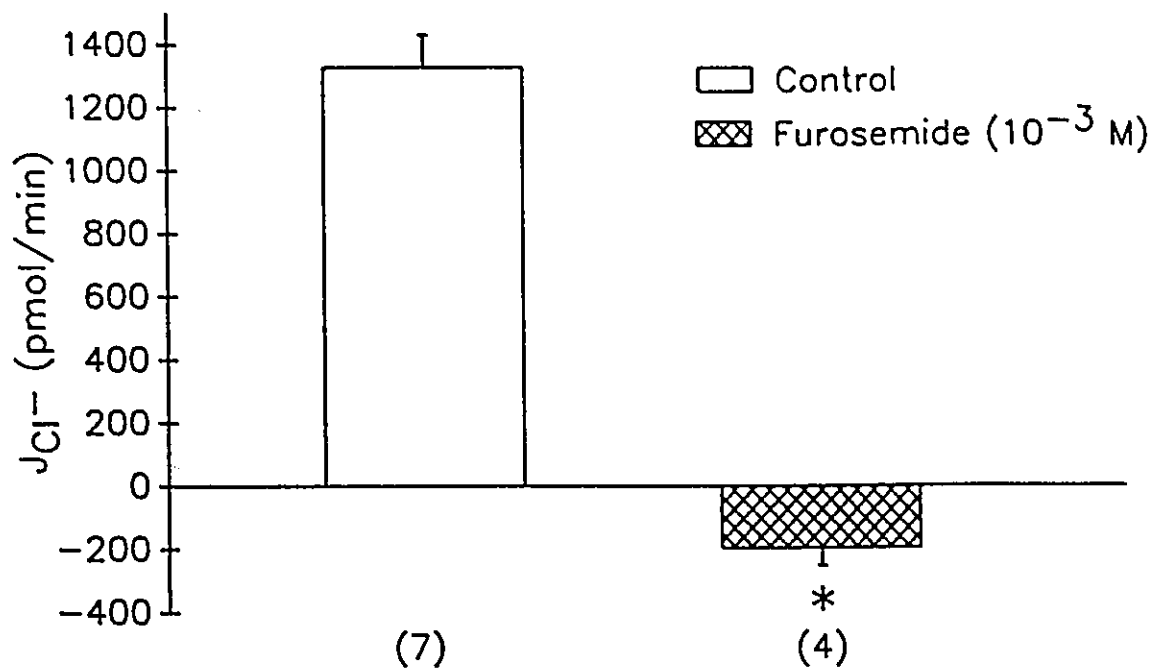


Figure 15. The chloride flux (J_{Cl^-}) in perfused functionally isolated loop segments in control and furosemide-treated animals. Furosemide (10^{-3} M) was added to the luminal perfusate. Number of tubules in each group is shown in parentheses. Number of animals in each group is 4. Results are expressed as mean \pm SEM. * $p < 0.0001$ versus control animals using the Student's unpaired t-test.

reabsorption). In these experiments, control loops of Henle reabsorbed $60 \pm 3.6\%$ of the delivered Cl^- compared to the net secretion of $-8.6 \pm 2.2\%$ ($p < 0.0001$) in loops of Henle perfused with furosemide (Figure 16).

There was no difference ($p > 0.05$) in the calculated Cl^- delivery in the absence and presence of furosemide (Figure 17, Table 7). However, the amount of Cl^- collected at the early distal site was elevated in furosemide-perfused loop segments compared to control segments (control ($n = 7$ tubules): 860 ± 65.2 pmol/min versus furosemide ($n = 4$ tubules): 2266 ± 147.9 pmol/min, $p < 0.0001$). Further evidence of impaired Cl^- reabsorption was shown by the failure of furosemide-perfused segments to reduce $[\text{Cl}^-]$ to that measured in control tubules (Table 7). The $[\text{Cl}^-]$ remaining in the collected fluid in control loop segments was 41.1 ± 3.56 mM compared to 104.1 ± 4.44 mM ($p < 0.001$) in furosemide-perfused loop segments. The collected $[\text{Cl}^-]$ from furosemide-perfused loop segments did not differ significantly from the $[\text{Cl}^-]$ in the initial perfusate used in these studies (Table 7). Taken together, these results demonstrate that virtually all Cl^- reabsorption by the loop segment can be inhibited by the addition of furosemide (10^{-3} M) to the luminal perfusate. As expected, plasma electrolytes were not different between the two groups of rats (Table 8).

The calculated perfusion rate in vivo and the measured collection rate in control and furosemide-perfused loop segments are summarized in Table 7. There were no significant differences between control and furosemide-perfused

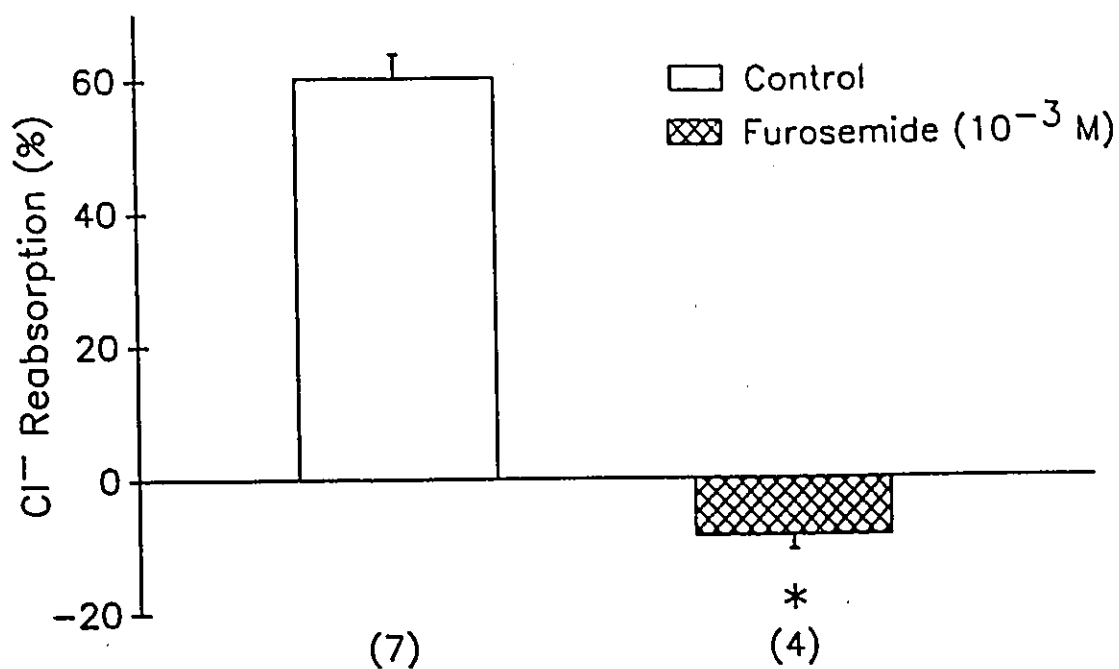


Figure 16. Fractional chloride reabsorption expressed as a percentage of the delivered chloride load in functionally isolated perfused loop segments *in vivo* measured in control and furosemide-treated animals. Furosemide (10^{-3} M) was added to the luminal perfusate. Number of tubules in each group is shown in parentheses. Number of animals in each group is 4. Results are expressed as mean \pm SEM. * $p < 0.0001$ versus control animals using the Student's unpaired t-test.

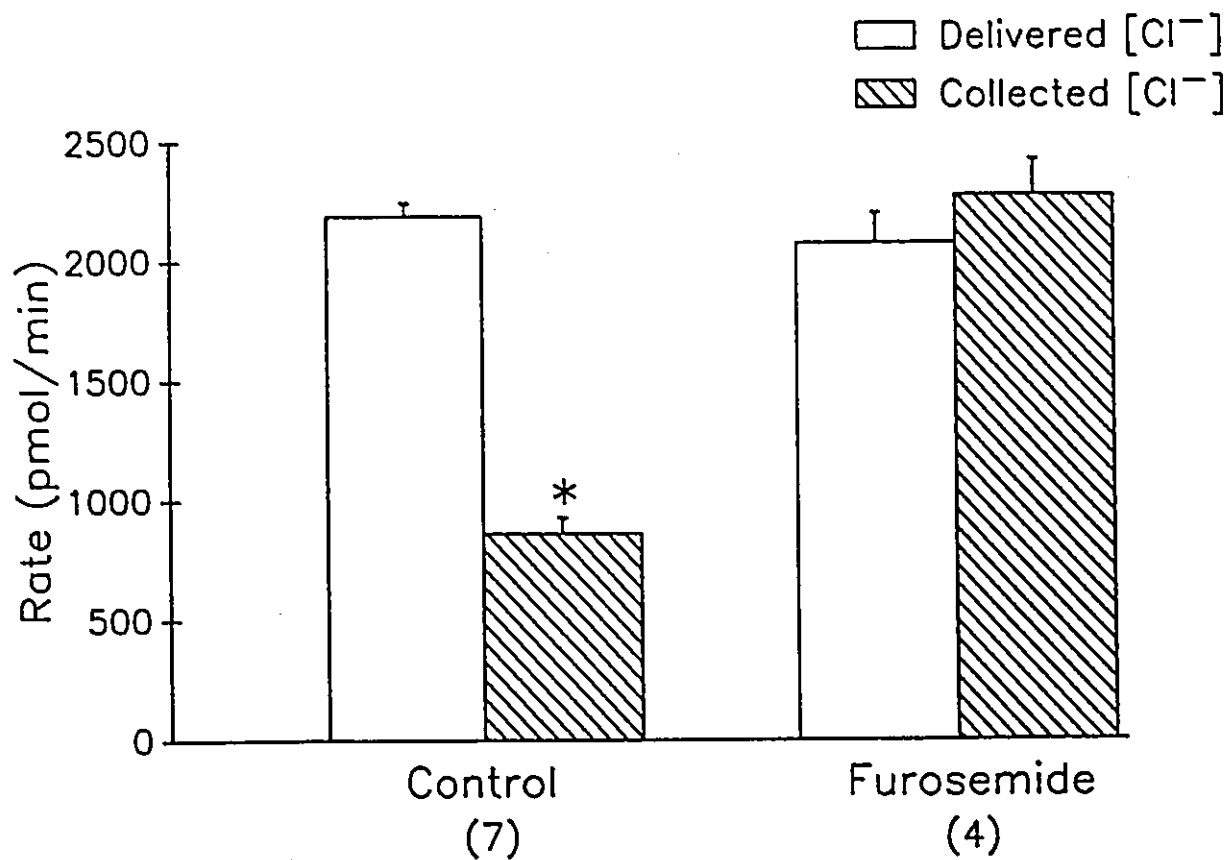


Figure 17. The calculated rate of delivered and collected chloride (pmol/min) in functionally isolated microperfused tubules taken from control and furosemide-treated animals. Furosemide (10^{-3} M) was added to the luminal perfusate. Number of tubules in each group is shown in parenthesis. Number of animals in each group is 4. Results are expressed as mean \pm SEM. * $p < 0.05$ versus furosemide-treated animals using the Student's unpaired t-test.

Table 7. Effect of furosemide, added to the luminal perfusate, on chloride reabsorption in functionally isolated microperfused TAL loops of Henle segments *in vivo* in standard chow fed rats. Furosemide (10^{-3} M) was added to the luminal perfusate. Number of tubules in each group is shown in parentheses. Number of animals in each group is 4. Results are expressed as mean \pm SEM. * $p < 0.05$ versus control animals using the Student's unpaired t-test. PERF = perfusion. COLL = collected. CF/P = ratio of collected fluid [inulin] to perfusate [inulin]. DEL = delivered.

	CONTROL	FUROSEMIDE (10^{-3} M)
PERF RATE (nl/min)	22.2 \pm 0.52 (7)	21.3 \pm 0.84 (4)
COLL RATE (nl/min)	21.1 \pm 0.48 (7)	21.7 \pm 0.48 (4)
CF/P [INULIN]	1.07 \pm 0.013 (7)	0.98 \pm 0.015* (4)
PERF [Cl ⁻] (mM)	96.8 \pm 0.83 (7)	96.8 \pm 2.12 (4)
COLL [Cl ⁻] (mM)	41.1 \pm 3.56 (7)	104.1 \pm 4.44* (4)
DEL [Cl ⁻] (pmol/min)	2185 \pm 55.1 (7)	2068 \pm 126.6 (4)

Table 8. Plasma electrolytes measured following the completion of the neck surgery in rats divided into either the control or furosemide-treated group (prior to the perfusion of the tubules with 10^{-3} M furosemide). Number of animals in each group is shown in parentheses. Results are expressed as mean \pm SEM.

	CONTROL	FUROSEMIDE (10^{-3} M)
PLASMA [Na ⁺] (mM)	153 \pm 2.96 (4)	150 \pm 4.05 (4)
PLASMA [K ⁺] (mM)	3.64 \pm 0.106 (4)	3.62 \pm 0.258 (4)
PLASMA [Cl ⁻] (mM)	125 \pm 5.12 (4)	125 \pm 6.10 (4)

loop segments in terms of the actual perfusion and collection rates, which closely represented the delivered rate of 22 nl/min set on the calibrated Hampel nanoliter pump (control (7 tubules) : 22.2 ± 0.52 nl/min ; control + furosemide (4 tubules) : 21.3 ± 0.84 nl/min). A significant difference in the concentration ratio of ^{14}C -inulin in the collected and perfused fluid was measured between control and furosemide-perfused loop segments (Table 7). However, the ratio calculated for each group clearly fell within the previously described inclusion range (see Section 2.1.5).

3.3 Results: Examination of the Impairment in TAL Cl^- Transport in K-DEP Rats.

The purpose of these studies was to assess whether TAL transport in K-DEP rats was defective, in the presence of axial flow, and whether this inhibition in Cl^- transport by this loop segment could be reversed by the administration of potassium and/or aldosterone in K-DEP rats. Animals were individually housed for 10 days with ad libitum access to food (control or K-free diet) and distilled water. TAL Cl^- transport was measured in functionally isolated microperfused loop of Henle segments in vivo (for description, see Section 2.1).

3.3.1 Microperfusion Data Derived From Control and K-DEP Rats.

To familiarize the reader with the location of the data, the results are organized as follows: (a) chloride flux (J_{Cl}) is illustrated in Figure 18 and Table 12, (b) fractional Cl^- reabsorption, expressed as a percentage of the delivered Cl^- load, is reported in Figure 19 and Table 12, (c) a summary of the microperfusion data is shown in Tables 9 and 12, (d) all plasma electrolytes are reported in Tables 10 and 11, and (e) a summary of perfusion and collection rates, delivered and collected $[Cl^-]$ and collected fluid [Inulin]/Perfusate [Inulin] are shown in Table 9. As indicated in Section 2.8, all quantitative results are expressed as the mean \pm SEM. All data derived from the microperfusion studies underwent multiple comparisons of group means, using an ANOVA and either the Tukey or the LSD multiple comparison test to determine the statistical significance between group data. A probability value of $p < 0.05$ was considered statistically significant. The specific statistical test used for each comparison has been indicated in the legend of each figure and table.

No significant differences were detected in either the actual perfusion rate or the collection rate between control and K-DEP rats (Table 9). In fact, the calculated or actual perfusion rate closely resembled the delivered rate of 22 nl/min set on the calibrated Hampel nanoliter pump.

The $[Cl^-]$ in the luminal perfusate and the $[Cl^-]$ delivered to the functionally isolated loop segments in control and K-DEP rats are represented

Table 9. Effect of potassium and aldosterone on microperfusion data collected from functionally isolated loop of Henle segments microperfused *in vivo* in control and K-DEP rats on day 10. Results are expressed as mean \pm SEM. ¹p < 0.05 versus K-DEP + K; ¹p < 0.05 versus K-DEP + S + K using a one-way ANOVA and the Tukey multiple comparison test.

GROUP	PERF RATE (nl/min)	COLL RATE (nl/min)	CF/P [INULIN]	PERF [Cl] (mM)
Control	21.9 \pm 0.368 (11)	21.2 \pm 0.478 (11)	1.03 \pm 0.017 (11)	100.4 \pm 0.735 (11)
Control + K	22.7 \pm 0.429 (10)	21.3 \pm 0.656 (10)	1.07 \pm 0.019 (10)	98.47 \pm 1.42 (10)
K-DEP	22.2 \pm 0.547 (6)	21.5 \pm 0.455 (6)	1.04 \pm 0.010 (6)	99.03 \pm 1.41 (6)
K-DEP + K	21.8 \pm 0.406 (8)	21.3 \pm 0.608 (8)	1.03 \pm 0.016 (8)	99.59 \pm 0.961 (8)
K-DEP + S + K	22.2 \pm 0.494 (9)	20.3 \pm 0.380 (9)	1.09 ¹ \pm 0.016(9)	96.74 \pm 0.770 (9)
K-DEP + A	22.0 \pm 0.452 (9)	21.6 \pm 0.512 (9)	1.02 \pm 0.014(9)	102.0 \pm 0.591 (9)
K-DEP + V	20.7 \pm 0.494 (5)	19.6 \pm 1.135 (5)	1.06 \pm 0.035 (5)	103.8 ¹ \pm 0.913 (5)

in Tables 9 and 12. There was no difference in the perfusate $[Cl^-]$ between the groups except for the aldosterone vehicle-infused K-DEP animals. In this group, luminal perfusate $[Cl^-]$ was higher compared to spironolactone-treated K-DEP animals infused with potassium. Although perfusate $[Cl^-]$ was higher in the aldosterone vehicle-infused K-DEP animals, there was no significant difference observed in the Cl^- delivery to the loop segments in control or K-DEP animals (Table 9).

A significant difference in the concentration ratio of ^{14}C -inulin in the collected and perfused fluid (Table 9) was measured between spironolactone-treated K-DEP animals infused with potassium (1.09 ± 0.016 , 9 tubules) and K-DEP rats infused with potassium (1.03 ± 0.016 , 8 tubules). However, the ratio calculated for each group clearly fell within the acceptable range as described in detail in Section 2.1.5.

3.3.2 Effect of Potassium Depletion on TAL Cl^- Reabsorption.

The aim of these initial microperfusion studies was to test the hypothesis that potassium depletion causes a quantitatively significant impairment in TAL Cl^- reabsorption in K-DEP rats.

Chloride flux (J_{Cl}) was reduced ($p < 0.05$) in K-DEP rats compared to control animals (Figure 18 and Table 12). Loop Cl^- delivery was similar in both experimental groups. Control loop segments reabsorbed Cl^- at 1249 ± 69.57

pmol/min compared to K-DEP loop segments which reabsorbed only 718 ± 31.4 pmol/min ($p < 0.05$) (Figure 18). As expected, fractional Cl^- reabsorption was also reduced (41%, $p < 0.05$) in K-DEP rats compared to control segments which reabsorbed 59% of the delivered Cl^- (Figure 19 and Table 12). As stated previously, there was no difference in the calculated Cl^- delivery in these two groups of animals. The reduction in Cl^- reabsorption in K-DEP loop segments was due to the failure to reduce luminal $[\text{Cl}^-]$. The $[\text{Cl}^-]$ in the fluid collected from K-DEP loop segments at an early distal tubule site was significantly higher (68.85 ± 3.30 mM) compared to the $[\text{Cl}^-]$ measured in collected fluid obtained in control loop segments (45.76 ± 2.98 mM). Plasma electrolyte measurements showed that K-DEP rats were strikingly hypokalemic (Table 10).

Acute potassium infusion in K-DEP animals restored plasma $[\text{K}^+]$ to 3.84 ± 0.32 mM (Table 11). In response to an acute potassium infusion in K-DEP rats (K-DEP + K), J_{Cl} increased to 1123 ± 62.13 pmol/min ($n = 8$ tubules). This value was higher ($p < 0.05$) than the J_{Cl} measured in untreated K-DEP rats, but was not significantly different from that measured in control loop segments (Figure 18, Table 12). The equivalent fractional rate of TAL Cl^- reabsorption in K-DEP + K rats was $51.5 \pm 2.48\%$ (Figure 19). Chloride delivery in K-DEP + K animals was similar to that measured in both control and K-DEP loop segments (Table 12). Normalization of Cl^- reabsorption in K-DEP + K rats was associated with a significant reduction in both the $[\text{Cl}^-]$ of the collected fluid and the amount of Cl^- remaining at the early distal tubule site (Table 12),

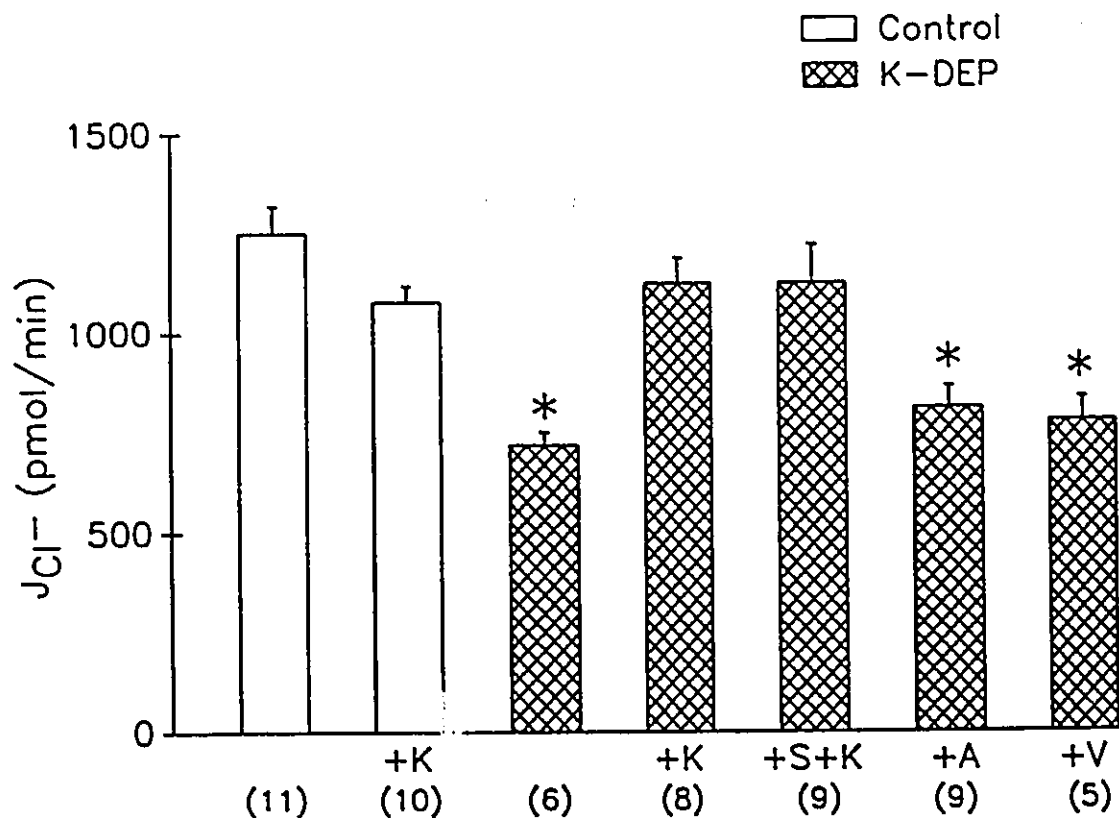


Figure 18. The measured chloride flux (J_{Cl}) in perfused functionally isolated loop segments in control and K-DEP rats on day 10. Number of tubules is shown in parentheses. Minimum number of rats in each group is 6 except for K-DEP+V where the number of rats is 3. +K = 150mM KCl. +S = spironolactone (3mg/100g). +A = aldosterone (0.3 μ g/100g body weight per hour). +V = aldosterone vehicle (0.05% absolute alcohol-0.9% NaCl). Results are expressed as mean \pm SEM. * $p < 0.05$ versus unmarked groups using a one-way ANOVA and the Tukey multiple comparison test.

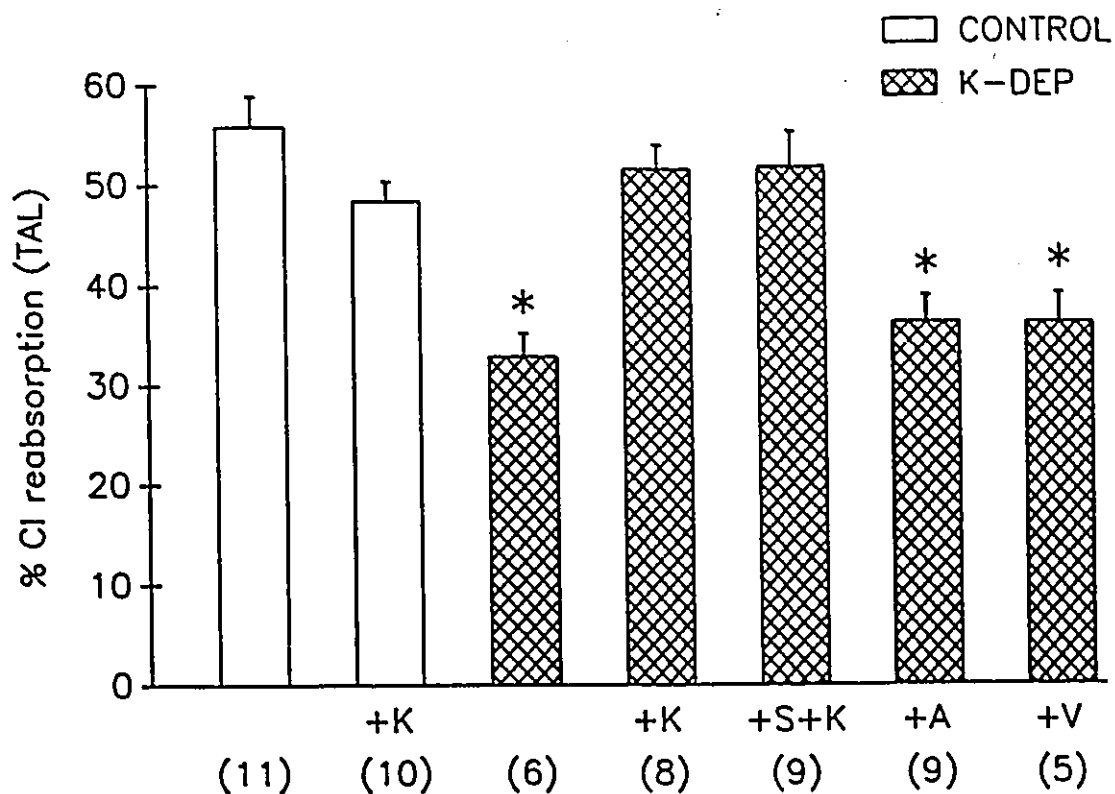


Figure 19. Fractional chloride reabsorption expressed as a % of the delivered chloride load in isolated loop segments in control and K-DEP rats on day 10. Number of tubules is shown in parentheses. Minimum number of animals in each group is 6 except for K-DEP+V where the number of rats is 3. +K = 150mM KCl. +S = spironolactone (3mg/100g). +A = aldosterone (0.3 μ g/100g body weight per hour). +V = aldosterone vehicle (0.05% absolute alcohol-0.9% NaCl). Results are expressed as mean \pm SEM. * $p < 0.05$ versus unmarked groups using a one-way ANOVA and the Tukey multiple comparison test.

Table 10. Plasma electrolytes and body weight measured in control and K-DEP rats on day 10. Plasma electrolytes were measured upon completion of the neck surgery. Number of animals in each group is shown in parentheses. Results are expressed as mean \pm SEM. * $p < 0.05$ versus unmarked groups using a one-way ANOVA and the Tukey multiple comparison test.

GROUP	Plasma [Na ⁺] (mM)	Plasma [K ⁺] (mM)	Plasma [Cl ⁻] (mM)	Body Weight (g)
Control	146 \pm 1.72 (7)	3.63 \pm 0.17 (7)	120 \pm 2.34 (7)	353 \pm 20.4 (7)
Control +K	147 \pm 2.03 (6)	3.76 \pm 0.11 (6)	122 \pm 5.72 (6)	338 \pm 5.90 (6)
K-DEP	144 \pm 2.50 (6)	2.16 \pm 0.12* (6)	113 \pm 3.12 (6)	310 \pm 17.4 (6)
K-DEP +K	144 \pm 0.75 (5)	2.08 \pm 0.13* (5)	120 \pm 2.48 (5)	324 \pm 16.0 (6)
K-DEP +S+K	150 \pm 4.14 (6)	2.26 \pm 0.16* (6)	118 \pm 4.36 (6)	326 \pm 10.5 (6)
K-DEP +A	146 \pm 0.65 (6)	1.90 \pm 0.10* (6)	111 \pm 2.62 (6)	337 \pm 8.28 (6)
K-DEP +V	149 \pm 3.21 (3)	2.19 \pm 0.19* (3)	106 \pm 3.61 (3)	285 \pm 18.0 (3)

Table 11. Plasma electrolytes measured in control and K-DEP rats on day 10 following (a) the completion of the neck surgery ([I₁]) and (b) the completion of the microperfusion experiment ([I₂]). Number of animals in each group is shown in parentheses. Results are expressed as mean \pm SEM. * $p < 0.05$ versus its [I₁] sample using the Student's paired t-test.

PLASMA	[Na ⁺] ₁ (mM)	[Na ⁺] ₂ (mM)	[K ⁺] ₁ (mM)	[K ⁺] ₂ (mM)	[Cl] ₁ (mM)	[Cl] ₂ (mM)
CONTROL	146 ± 1.72 (7)	---	3.63 ± 0.17 (7)	---	120 ± 2.34 (7)	---
CONTROL +K	147 ± 2.03 (6)	143 ± 3.06 (6)	3.76 ± 0.11 (6)	4.93* ± 0.31 (6)	122 ± 5.72 (6)	123 ± 5.37 (6)
K-DEP	144 ± 2.50 (6)	---	2.16 ± 0.12 (6)	---	113 ± 3.12 (6)	---
K-DEP +K	144 ± 0.75 (5)	142 ± 1.28 (6)	2.08 ± 0.13 (5)	3.84* ± 0.32 (6)	120 ± 2.48 (5)	125 ± 5.09 (5)
K-DEP +S+K	150 ± 4.14 (6)	153 ± 6.68 (6)	2.26 ± 0.16 (6)	4.12* ± 0.25 (6)	118 ± 4.36 (6)	127 ± 7.71 (6)
K-DEP+A	146 ± 0.65 (6)	149 ± 1.10 (6)	1.90 ± 0.10 (6)	1.98 ± 0.09 (6)	111 ± 2.62 (6)	112 ± 4.77 (6)
K-DEP+V	149 ± 3.21 (3)	153 ± 5.24 (3)	2.19 ± 0.19 (3)	2.37 ± 0.33 (3)	106 ± 3.61 (3)	111 ± 5.29 (3)

Table 12. Effect of potassium and aldosterone on microperfusion data collected from functionally isolated loop of Henle segments microperfused *in vivo* in control and K-DEP rats on day 10. Results are expressed as mean \pm SEM. * $p < 0.05$ versus unmarked groups using a one-way ANOVA and the Tukey multiple comparison test.

GROUP	DEL [Cl ⁻] (pmol/min)	COLL [Cl ⁻] (mM)	J _{Cl⁻} (pmol/min)	Fr Cl ⁻ (%)	COLL [Cl ⁻] (pmol/min)
Control	2239 ± 52.92 (11)	45.76 ± 2.98 (11)	1249 ± 69.57 (11)	55.8 ± 2.98 (11)	976 ± 73.1 (11)
Control + K	2234 ± 60.39 (10)	54.01 ± 1.73 (10)	1076 ± 40.73 (10)	48.4 ± 1.97 (10)	1158 ± 66.8 (10)
K-DEP	2204 ± 67.14 (6)	68.85 $\pm 3.30^*$ (6)	718 $\pm 31.40^*$ (6)	32.9 $\pm 2.33^*$ (6)	1485 $\pm 98.4^*$ (6)
K-DEP + K	2175 ± 39.10 (8)	49.45 ± 2.06 (8)	1123 ± 62.13 (8)	51.5 ± 2.48 (8)	1052 ± 54.4 (8)
K-DEP +S+K	2144 ± 47.19 (9)	50.67 ± 3.47 (9)	1123 ± 95.67 (9)	51.9 ± 3.58 (9)	1021 ± 60.6 (9)
K-DEP + A	2239 ± 40.83 (9)	65.72 $\pm 2.13^*$ (9)	809 $\pm 54.12^*$ (9)	36.3 $\pm 2.66^*$ (9)	1429 $\pm 76.1^*$ (9)
K-DEP + V	2152 ± 62.24 (5)	69.96 $\pm 1.72^*$ (5)	777 $\pm 57.98^*$ (5)	36.3 $\pm 2.97^*$ (5)	1374 $\pm 93.8^*$ (5)

In contrast to the effect of potassium infusion in K-DEP rats, the acute administration of potassium in control rats (control + K) did not alter J_{Cl} ($p > 0.05$, Table 12, Figure 18) despite an elevation in plasma $[K^+]$ to 4.93 ± 0.31 mM ($p < 0.05$, Table 11). Control + K loop segments did not show any alteration ($p > 0.05$) in TAL Cl^- transport from normal control loop segments (Figure 19). The acute infusion of potassium in control rats (ie. control + K) did not alter Cl^- delivery to and collected from the loop segments and in the collected fluid, compared to control loop segments ($p > 0.05$, Table 12).

3.3.3 Effect of Aldosterone Infusion on TAL Cl^- Reabsorption in K-DEP Rats.

The purpose of these experiments was to test the hypothesis that an aldosterone deficiency mediates defective TAL Cl^- transport in K-DEP rats.

As shown in Figure 18, aldosterone infusion in K-DEP animals failed to correct the impairment in TAL J_{Cl} . Chloride reabsorption (J_{Cl}) in K-DEP rats treated with aldosterone (A) or its vehicle (V), was significantly reduced (K-DEP + A ($n = 9$ tubules): 809 ± 54.12 pmol/min; K-DEP + V ($n = 5$ tubules): 777 ± 57.98 pmol/min) compared to control animals and did not differ from values measured in untreated K-DEP rats (Figure 18, Table 12). Similarly, fractional Cl^- reabsorption was reduced (35%, $p < 0.05$) in both aldosterone-infused K-DEP rats and in vehicle-treated K-DEP rats compared to control animals (Figure 19). A reduction in TAL Cl^- reabsorption in K-DEP rats infused

with aldosterone (A) or its vehicle (V) was associated with an elevation ($p < 0.05$) in the $[Cl^-]$ of the collected fluid (Table 12). As expected, both groups of K-DEP animals (K-DEP + A, K-DEP + V) were hypokalemic (Tables 10 and 11)

3.3.4 Effect of Potassium Infusion in Repairing Defective TAL Cl^- Reabsorption in K-DEP Rats in the Absence of Increased Aldosterone Action.

The aim of these experiments was to test the hypothesis that the acute infusion of potassium and not increased aldosterone action rapidly corrects defective TAL Cl^- transport in K-DEP rats.

As expected, the acute infusion of potassium in K-DEP animals pretreated with the aldosterone antagonist spironolactone (K-DEP + S + K) restored plasma $[K^+]$ to 4.12 ± 0.25 mM and achieved normal control values (Tables 10 and 11). In response to the potassium infusion in K-DEP rats pretreated with spironolactone (ie. K-DEP + S + K), J_{Cl} increased to 1123 ± 95.67 pmol/min ($n = 9$ tubules). This value was significantly higher than the J_{Cl} measured in untreated K-DEP rats, but was not different ($p > 0.05$) from that measured in control loop segments (Figure 18, Table 12). The rate of TAL Cl^- reabsorption in K-DEP + S + K rats was equivalent to a fractional Cl^- reabsorption of $51.9 \pm 3.58\%$ (Figure 19). Chloride delivery in these rats (ie. K-DEP + S + K) was similar to control and K-DEP loop segments (Table 12).

Normalization of Cl^- reabsorption in K-DEP + S + K rats was associated with a reduction ($p < 0.05$) in the $[\text{Cl}^-]$ of the collected fluid and the amount of Cl^- remaining at the early distal tubule site.

3.4 Results: Determination of the Effect of a Potassium Infusion on Plasma [Aldosterone].

The hypothesis tested in these experiments was that an acute potassium infusion in K-DEP rats would cause a parallel elevation in plasma [aldosterone]. All K-DEP and control animals were treated in an identical manner to the previously described microperfusion protocol (see Section 2.1). Approximately half of the animals were infused with potassium (1% body weight per hour for minimum 75 min) while the remaining animals were infused with 0.9% NaCl (1% body weight per hour). At $t = 120$ min, an arterial blood sample was obtained and analyzed for plasma [aldosterone].

Data collected from control and K-DEP animals infused with either 0.9% NaCl or potassium are summarized as indicated: (a) plasma $[\text{K}^+]$ at $t = 75$ min and 120 min are reported in Figures 20A and B; and (b) plasma [aldosterone] are reported in Figure 21.

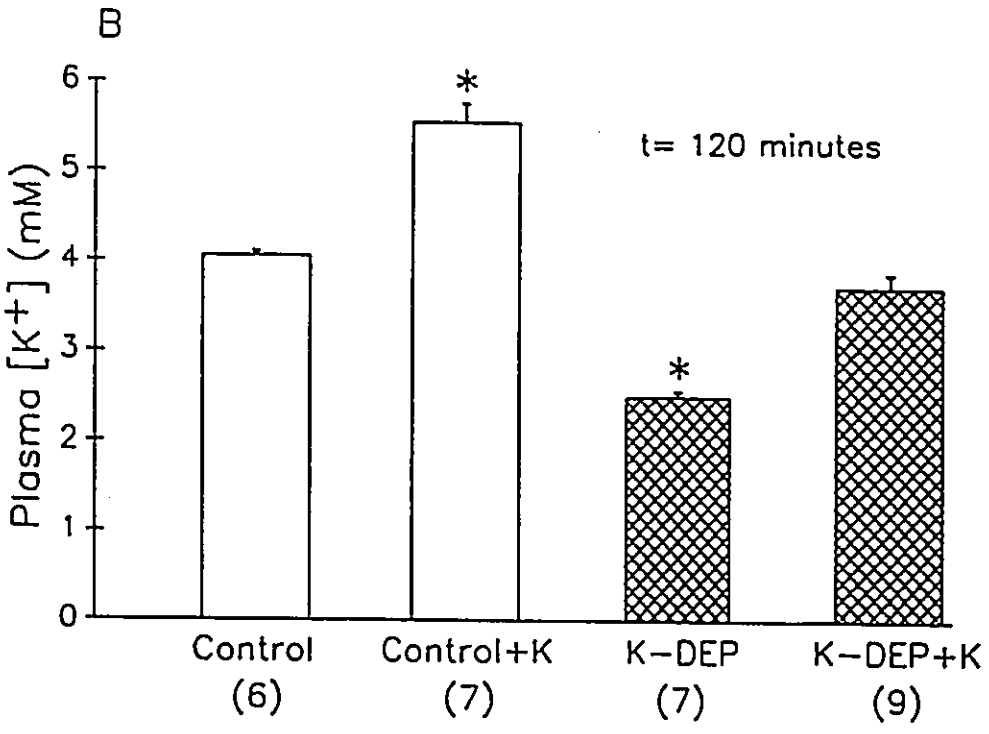
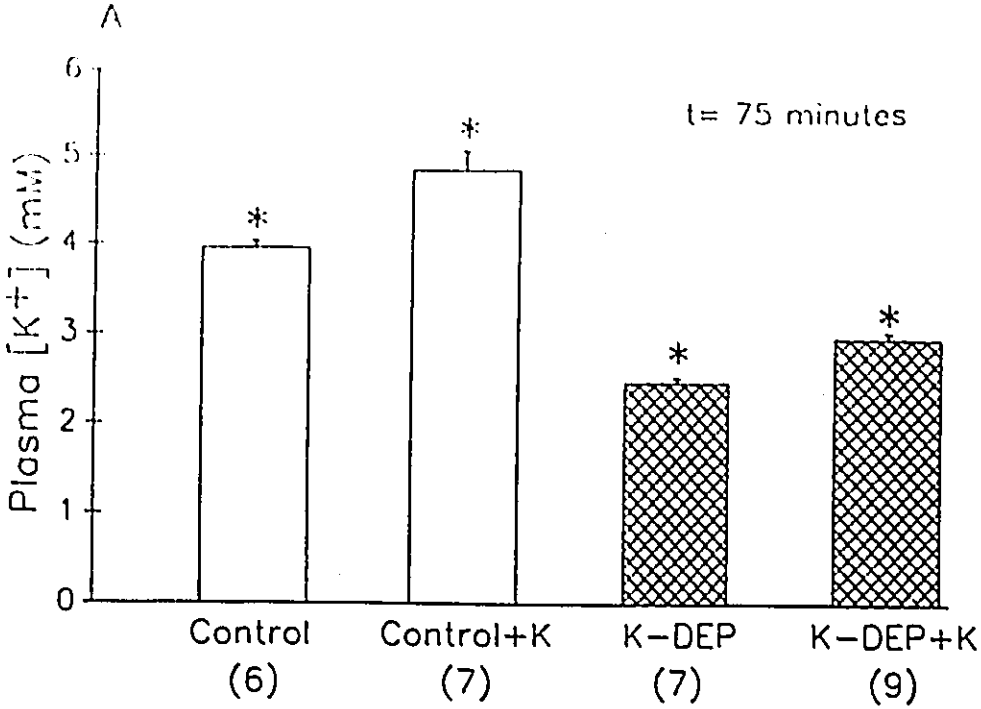
All animals consuming the K-free diet were hypokalemic as indicated by the plasma K^+ concentrations measured upon the completion of the neck surgery ($t = 15$ min). These values were lower than those measured in control

animals (K-DEP (n = 16): 2.23 ± 0.10 mM versus control (n = 14): 3.89 ± 0.038 mM, $p < 0.0001$). At t = 75 min, plasma $[K^+]$ in control animals infused with potassium was slightly elevated ($p < 0.05$) compared to control rats administered 0.9% NaCl and K-DEP animals infused with either K^+ or 0.9% NaCl (Figure 20A). Similarly, K-DEP animals infused with potassium showed a small increase ($p < 0.05$) in plasma $[K^+]$ compared to K-DEP rats administered 0.9% NaCl.

As mentioned previously, although plasma $[K^+]$ increased in K-DEP rats given potassium, the measured plasma $[K^+]$ was still lower compared to the control groups. Plasma $[K^+]$ was reduced ($p < 0.05$) in K-DEP animals at t = 120 min compared to rats consuming the control diet or K-DEP animals infused with potassium (Figure 20B). In response to the potassium infusion, control rats showed an elevation ($p < 0.05$) in plasma $[K^+]$ at t = 120 min compared to (i) control rats infused with 0.9% NaCl and (ii) K-DEP rats infused with either 0.9% NaCl or potassium (Figure 20B). Plasma $[K^+]$ reached control values in K-DEP animals infused with potassium at t = 120 min (K-DEP + K: 3.71 ± 0.152 mM versus control: 4.05 ± 0.0542 mM, $p > 0.05$).

Figure 20A. Plasma $[K^+]$ measured at $t = 75$ min in animals consuming either the control or K-free diet for 10 days. +K represents animals infused intravenously with 150 mM KCl at 1% body weight per hour at $t = 45$ min. All other animals were infused with 0.9% NaCl at 1% body weight per hour. Number of animals in each group is shown in parentheses. Results are expressed as mean \pm SEM. * $p < 0.05$ versus all other groups using a one-way ANOVA and the LSD multiple comparison test.

Figure 20B. Plasma $[K^+]$ measured at $t = 120$ min in animals consuming either the control or K-free diet on day 10. +K represents animals infused intravenously with 150 mM KCl (1% body weight per hour) at $t = 45$ min. All other animals were infused with 0.9% NaCl at 1% body weight per hour. Number of animals in each group is shown in parentheses. Results are expressed as mean \pm SEM. * $p < 0.05$ versus unmarked groups using a one-way ANOVA and the LSD multiple comparison test.



3.4.1 Effect of an Acute Potassium Infusion on Plasma [Aldosterone].

Plasma [aldosterone] was reduced at $t = 120$ min ($p < 0.05$) in animals consuming the K-free diet (with or without the potassium infusion) compared to control animals (with or without the potassium infusion) as shown in Figure 21. Acute infusion of potassium elevated ($p < 0.05$) plasma [aldosterone] in control rats. However, an acute infusion of potassium in K-DEP rats failed to stimulate an increase in plasma [aldosterone] despite attainment of a normal plasma $[K^+]$ at $t = 120$ min.

These studies demonstrated that the restoration of plasma $[K^+]$ by a potassium infusion in K-DEP rats is not immediately associated with a parallel rise in plasma [aldosterone].

3.5 Results: Determination of the Effect of Normalization of Total Body Potassium on Plasma [Aldosterone] in K-DEP Rats.

The hypothesis tested in these studies was that complete replacement of the potassium-deficit in K-DEP rats is associated with the restoration of plasma [aldosterone] to control values. Experiments were carried out in male Sprague-Dawley rats which were individually housed for 13 days with ad libitum access to the appropriate diet (control or K-free) and distilled water. On day 12, animals received either distilled water or 0.5 M KCl at 2 ml/100g body

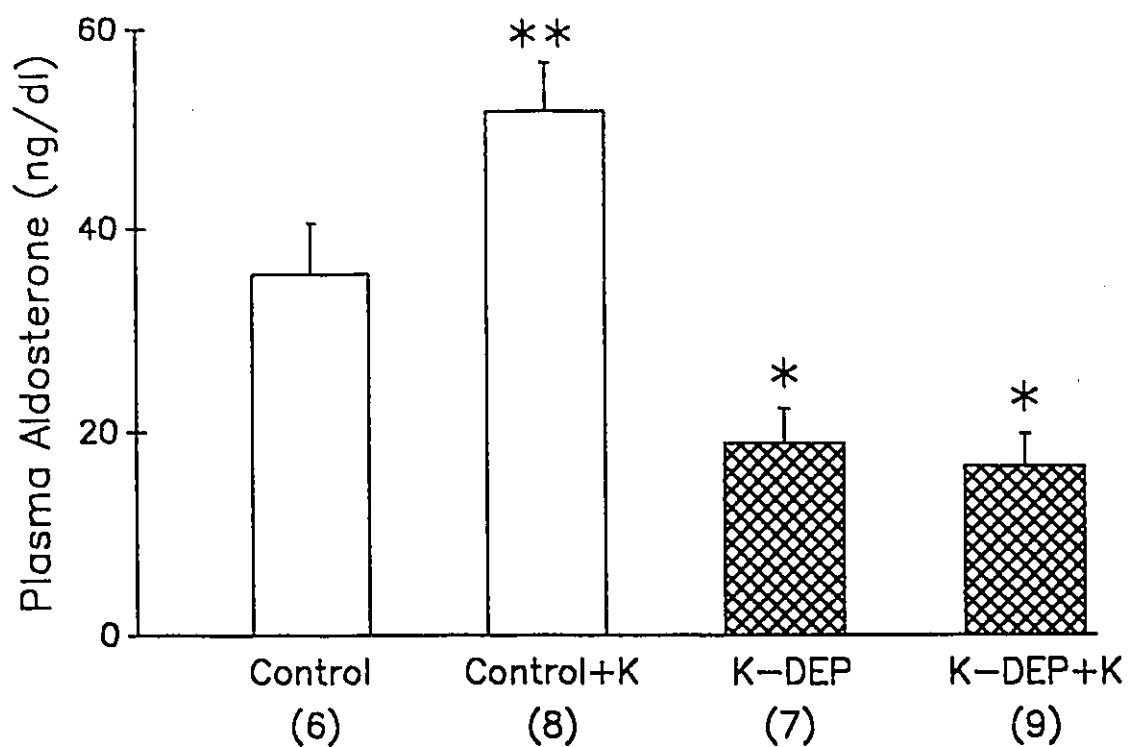


Figure 21. Plasma [aldosterone] measured at $t = 120$ min in control and K-free diet fed animals on day 10. +K represents animals infused intravenously at $t = 45$ min with 150 mM KCl at 1% body weight per hour. All other animals were infused intravenously with 0.9% NaCl at 1% body weight per hour. Number of animals in each group is shown in parentheses. Results are expressed as mean \pm SEM. * $p < 0.05$ versus control K animals. ** $p < 0.05$ versus all groups using a one-way ANOVA and the LSD multiple comparison test.

weight by gavage. Plasma [aldosterone] was measured 28 hours later.

3.5.1 Effect of Normalization of Total Body Potassium on Plasma [K⁺].

As shown in Figure 22, plasma [K⁺] was reduced ($p < 0.05$) in K-DEP animals given distilled water. However, plasma [K⁺] in K-DEP rats given potassium (K-DEP + K) increased ($p < 0.05$) compared to K-DEP animals given distilled water. Moreover, plasma [K⁺] in K-DEP + K rats reached values measured in control rats.

3.5.2 Effect of Normalization of Total Body Potassium on Plasma [Aldosterone].

Plasma [aldosterone] was elevated ($p < 0.05$) in both control and K-DEP rats given an oral load of potassium compared to K-DEP animals given water (Figure 23). However, the plasma [aldosterone] measured in both groups of animals given potassium was not different from control animals given distilled water (Figure 23).

Therefore, upon restoration of a potassium-deficit in K-DEP rats, there is also a restoration of plasma [aldosterone] to control values.

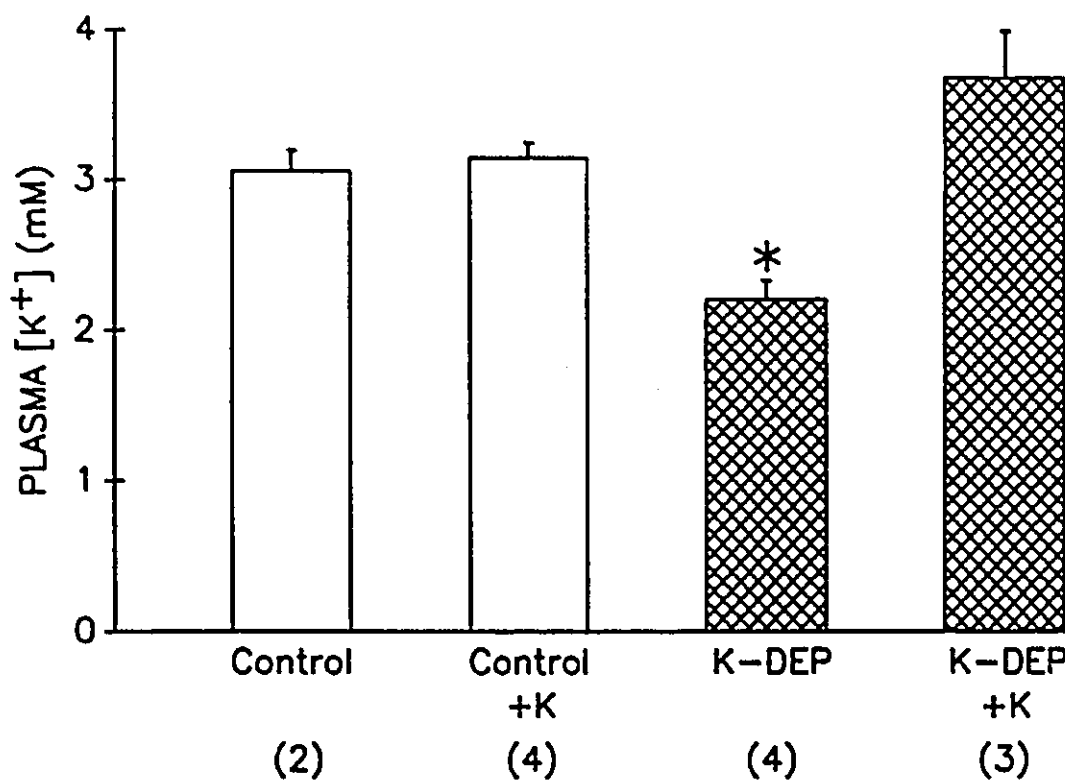


Figure 22. Plasma [K⁺] measured in animals consuming either the control or K-free diet on day 13, 28 hours after oral administration of potassium or distilled water. Number of rats in each group is shown in parentheses. +K = 0.5 M KCl. dH₂O = distilled water. Results are expressed as mean ± SEM. *p < 0.05 versus unmarked groups using a one-way ANOVA and the Tukey multiple comparison test.

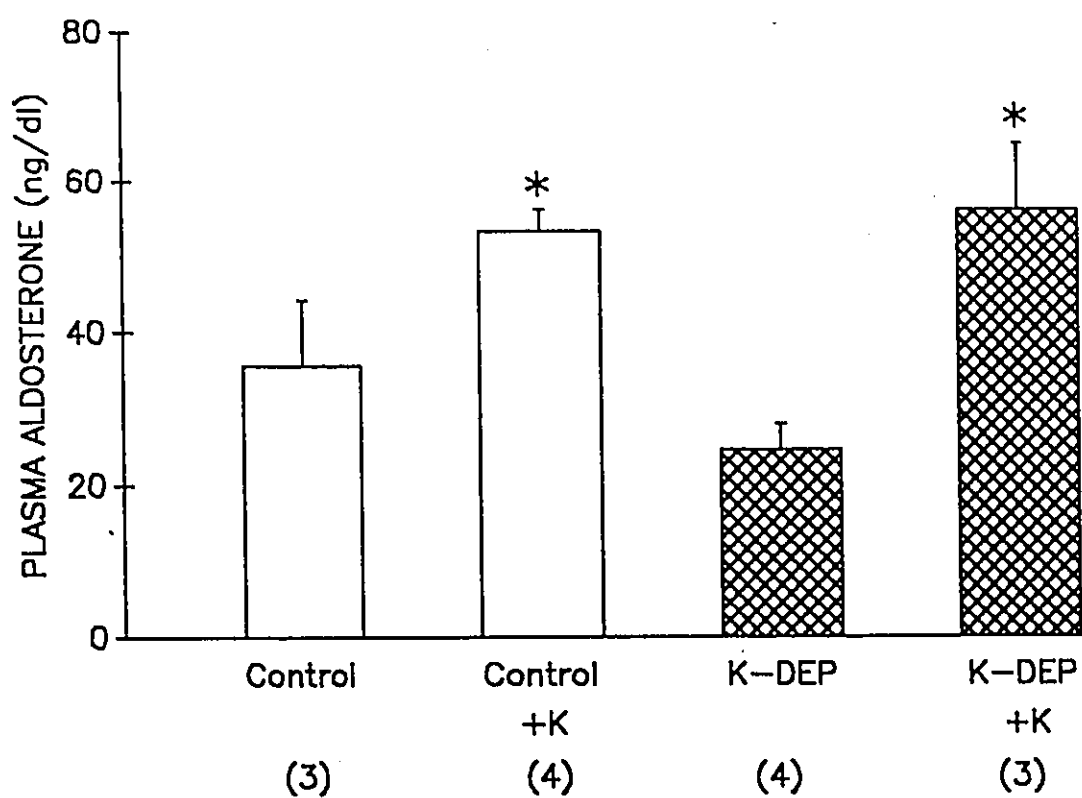


Figure 23. Plasma [aldosterone] measured on day 13, 28 hours after oral administration of potassium or distilled water, in animals consuming either the control or K-free diet. Number of animals in each group is shown in parentheses. Results are expressed as mean \pm SEM. * $p < 0.05$ versus K-DEP using a one-way ANOVA and the Tukey multiple comparison test.

3.6 Results: Efficacy of Spironolactone and Aldosterone.

The purpose of these studies was to test the efficacy of spironolactone and aldosterone used in the microperfusion studies. Stimulation of urinary K^+ excretion was used as an index of aldosterone bioactivity. The bioactivity of spironolactone was defined as its ability to decrease the response to aldosterone. Studies were carried out in adrenalectomized male Sprague-Dawley rats given standard rat chow and tap water. Some animals received an oral load of the aldosterone antagonist, spironolactone (3 mg/100g body weight), 2 hours prior to the commencement of a two-hour urine collection. One hour prior to the urine collection period, some animals received an intraperitoneal injection of aldosterone (3 μ g/100g body weight). Urine and plasma was collected as described in Sections 2.2.2 and 2.2.3.

3.6.1 The Efficacy of Spironolactone.

Urinary K^+ excretion ($U_K = \mu\text{mol}/100\text{g}$ per 2 hours) was corrected for the simultaneous excretion of creatinine ($U_{Cr} = \mu\text{mol}/100\text{g}$ per 2 hours) in each animal and the data are reported as the ratio, U_K/U_{Cr} . The U_K/U_{Cr} ratio observed in bilaterally adrenalectomized rats treated with either spironolactone or aldosterone alone or in combination are summarized in Figure 24. Adrenalectomized rats given aldosterone had a higher ratio (54%, $p < 0.05$)

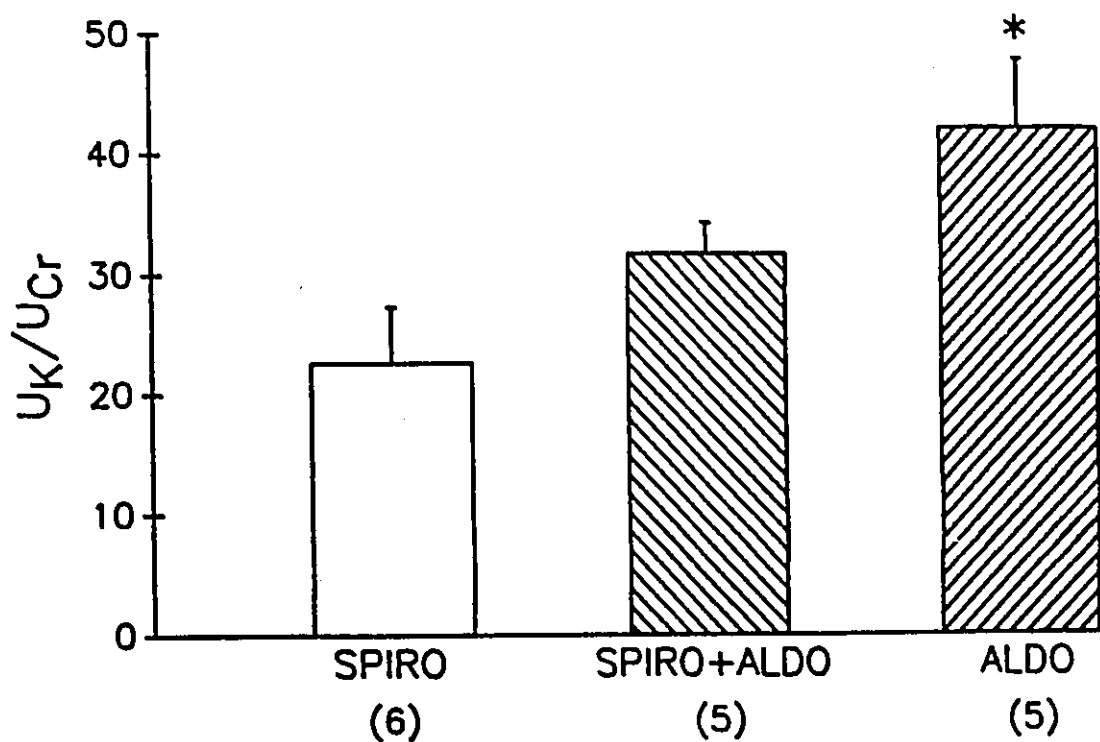


Figure 24. The ratio of urinary potassium to urinary creatinine (U_K/U_{Cr}) excretion in Purina Chow fed, adrenalectomized rats treated with either spironolactone and/or aldosterone. Number of animals in each group is shown in parentheses. SPIRO = spironolactone (3mg/100g). ALDO = aldosterone (3 μ g/100g). Results are expressed as mean \pm SEM. * $p < 0.05$ versus SPIRO using a one-way ANOVA and the Tukey multiple comparison test.

compared to spironolactone-treated adrenalectomized rats. Spironolactone and aldosterone in combination reduced slightly (25%, $p > 0.05$) the U_K/U_{Cr} measured in aldosterone-treated adrenalectomized animals.

Other indices of aldosterone bioactivity are summarized in Table 13. These data, including urine $[Na^+]/[K^+]$ ratio, show that aldosterone was biologically active while spironolactone partially blunted the effect of aldosterone.

There were no differences ($p > 0.05$) in any of the factors used to calculate GFR, GFR itself or body weight in rats treated with either spironolactone or aldosterone alone or in combination (Table 14).

TABLE 13. Urinary volume, electrolyte excretion, and Na^+/K^+ ratio in Purina Chow fed, adrenalectomized rats treated with either spironolactone and/or aldosterone. Number of animals in each group is shown in parentheses. SPIRO = spironolactone (3mg/100g). ALDO = aldosterone (3 μ g/100g). Results are expressed as mean \pm SEM. * $p < 0.05$ versus SPIRO using a one-way ANOVA and the Tukey multiple comparison test.

	SPIRO (3mg/100g)	SPIRO + ALDO	ALDO (3 μ g/100g)
Urine Volume (ml/100g)	1.66 \pm 0.20 (6)	1.35 \pm 0.18 (5)	1.32 \pm 0.13 (5)
$\text{U}_{\text{Cr}}\text{V}$ (μ mol/100g)	1.82 \pm 0.14 (6)	1.52 \pm 0.13 (5)	1.53 \pm 0.21 (5)
$\text{U}_{\text{K}}\text{V}$ (μ mol/100g)	39.15 \pm 7.24 (6)	46.95 \pm 2.11 (5)	61.67 \pm 7.08 (5)
$\text{U}_{\text{Na}}\text{V}$ (μ mol/100g)	85 \pm 14.7 (6)	37 \pm 9.89* (5)	43 \pm 12.3 (5)
Na^+/K^+ Ratio	2.43 \pm 0.50 (6)	1.15 \pm 0.41 (5)	0.716 \pm 0.21* (5)

TABLE 14. Plasma and urine creatinine content, glomerular filtration rate (GFR) and body weight in Purina Chow fed, adrenalectomized rats treated with either spironolactone and/or aldosterone. Number of animals in each group is shown in parentheses. SPIRO = spironolactone (3mg/100g). ALDO = aldosterone (3 μ g/100g). Results are expressed as mean \pm SEM.

	SPIRO (3mg/100g)	SPIRO + ALDO	ALDO (3 μ g/100g)
Plasma [Creatinine] (μ mol/l)	35 \pm 3.65 (6)	37 \pm 2.06 (5)	32 \pm 2.77 (5)
Urine [Creatinine] (μ mol/l)	1196 \pm 186.2 (6)	1165 \pm 97.30 (5)	1177 \pm 149.6 (5)
Body Weight (g)	356 \pm 12.1 (6)	358 \pm 10.3 (5)	360 \pm 8.53 (5)
GFR (ml/min/100g)	0.441 \pm 0.0325 (6)	0.343 \pm 0.270 (5)	0.393 \pm 0.033 (5)

CHAPTER 4

The results of the present study, using the technique of continuous microperfusion of functionally isolated single loops of Henle, show that the defect in TAL Cl⁻ reabsorption in K-DEP rats is quantitatively significant and can be rapidly reversed by the acute systemic infusion of potassium. These studies demonstrated that acute administration of aldosterone, in the presence of reduced ECF[K⁺], cannot reverse the impairment in TAL Cl⁻ reabsorption. In addition, acute administration of potassium in the presence of an aldosterone antagonist in K-DEP rats rapidly reversed the defect. Further studies showed that despite restoration of ECF[K⁺] by acute potassium infusion in K-DEP rats, the plasma [aldosterone] failed to increase within this time period. In control rats, plasma [aldosterone] increased 46%, however, TAL Cl⁻ reabsorption remained unchanged. Thus, although a critical plasma [aldosterone] may be required for normal TAL Cl⁻ reabsorption, this hormone does not regulate TAL Cl⁻ transport. Therefore, this study provides conclusive evidence that in K-DEP rats, the rapid reversal of defective TAL Cl⁻ reabsorption in response to an acute potassium infusion occurs via an aldosterone independent mechanism. The discussion which follows will address four main issues:

- (1) Overview of the effects of potassium depletion.
- (2) Assessment of TAL Cl⁻ reabsorption in vivo.
- (3) Potassium infusion, not aldosterone, rapidly corrects defective TAL Cl⁻ transport in K-DEP rats.
- (4) Hypotheses regarding the mediator of impaired TAL Cl⁻ transport in potassium depletion.

4.1 Overview of the Effects of Potassium Depletion.

It is well-established that chronic dietary potassium depletion is associated with a number of effects which include: polydipsia, impaired concentrating ability, and hypertrophy of the kidney (Brokaw, 1953; Toback et al, 1976; Ordonez et al, 1977; Linas et al, 1979; Peterson, 1984; Saikaley et al, 1986; Peterson et al, 1987; McKay et al, 1990).

The onset of polydipsia measured in K-DEP rats in the present study occurred within the first three days of the consumption of the potassium deficient diet and was maintained over the duration of the study. These results are in agreement with earlier studies (Brokaw, 1953; Saikaley et al, 1986). An elevation in water intake is used as an index of thirst. Although water intake increased in K-DEP rats, it was not as large a response as has been described when NaCl depletion is simultaneously induced. A number of studies have demonstrated that polydipsia induced by a low NaCl, K-free diet (Saikaley et al,

1986; McKay et al, 1990) is nearly two-fold higher than that induced by a potassium deficient diet. In fact, the added stimulus of NaCl depletion results in a nearly three-fold increase in water intake (Saikaley et al, 1986; McKay et al, 1990) compared to the 1.5 to 2-fold increase in water intake observed in the present study.

The elevation in water intake in response to potassium depletion occurs in the presence of a normal plasma osmolality. Earlier studies have shown that the onset of polydipsia occurred in the presence of normal or reduced plasma osmolality and was associated with a significant elevation in plasma renin activity (PRA) and [angiotensin I] (Saikaley et al, 1986; McKay et al, 1990). Furthermore, polydipsia could be abolished in rats consuming a low NaCl, K-free diet by (a) electrolytic destruction of the subfornical organ and (b) by chronic enalapril (converting enzyme inhibitor) treatment (Saikaley et al, 1986; McKay et al, 1990). Binding of the peptide hormone, angiotensin II, to its receptors in the subfornical organ has been postulated to stimulate the enhanced water intake (Saikaley et al, 1986). Based on these previous studies, polydipsia induced by feeding a potassium deficient diet is most likely mediated by increased [angiotensin II] and not potassium depletion per se.

Dietary potassium depletion was also associated with a significant impairment in renal concentrating ability, as measured by a reduction in U_{osm}^{max} (defined in this thesis as the maximal urine osmolality measured in a urine sample collected after 16 hours of water deprivation) as early as day 7.

$U_{osm,max}$ was further reduced by day 10. The onset of polydipsia occurred (a) prior to the impairment in urine concentrating ability in K-DEP rats and (b) in the presence of normal plasma osmolality. Berl (1980) has shown in rats consuming a potassium deficient diet that after six days, there was a significant increase in water intake. However, at this time (day 6) there was no difference in concentrating ability. Therefore, in agreement with Berl (1980), the results of the present study demonstrate that polydipsia associated with potassium depletion precedes the development of a urine concentrating defect. Although the elevation in water intake may contribute to the reduction in $U_{osm,max}$ measured in K-DEP rats (Berl, 1980), the major factor responsible for the renal concentrating defect appears to involve an impairment in TAL NaCl transport (Eknoyan et al, 1970; Gutsche et al, 1984).

In the present study, renal mass in K-DEP rats increased 72% after 14 days of consuming the K-free diet. These findings confirm earlier studies (Brokaw, 1953; Toback et al, 1976; Ordonez et al, 1977; Peterson, 1984; Peterson et al, 1987) which demonstrated that potassium depletion stimulates renal growth. The increase in kidney growth in K-DEP animals appears to represent a true increase in kidney mass as Peterson et al (1987) reported there was no significant difference in tissue water content between K-DEP and control animals. The enhanced growth stimulated by potassium depletion is localized to the outer medullary region such that this region is disproportionately greater than that observed in the papillary or cortical regions

(Toback et al, 1976; Ordonez et al, 1977). Peterson et al (1987) also showed that dietary potassium depletion enhanced renal mass significantly in partially nephrectomized rats. Earlier studies from our laboratory have shown that there is hypertrophy of the kidney obtained from rats given a low NaCl, K-free diet (McKay et al, 1990). Furthermore, we found that the hypertrophy was not as marked in rats given the same low NaCl, K-free diet but who were chronically treated with the converting enzyme inhibitor, enalapril (40 mg/Kg/day). These findings suggest that angiotensin II may be involved in inducing hypertrophy of the kidneys in K-DEP animals. It is clear that more work is required to provide conclusive evidence that angiotensin II induces renal hypertrophy in response to dietary potassium depletion. The mechanism of renal hypertrophy was not the focus of the current study.

In summary, the results of the present study confirmed previous findings that potassium depletion stimulates water intake which precedes the development of a urine concentrating defect. Ten days of dietary potassium depletion was associated with (a) hypokalemia, (b) suppression of plasma [aldosterone] and (c) enhanced renal growth. To improve survival of the K-DEP animals during the microperfusion experiments and to avoid further complications associated with more prolonged dietary treatment (Peterson, 1987), all animals used in the microperfusion studies were given the K-free diet for 10 days.

4.2 Assessment of TAL Cl⁻ Reabsorption In Vivo.

In the current study, TAL Cl⁻ reabsorption was assessed in vivo using the technique of continuous microperfusion of functionally isolated segments of the loop of Henle. This nephron segment is both anatomically and histologically variable (reviewed in Capasso et al, 1991; McKay and Peterson, 1993; Peterson et al, 1993). Microperfusion allows Cl⁻ transport to be measured in the presence of axial flow while the nephron segment remains in its natural location within the renal interstitium. Moreover, in vivo microperfusion of the loop segment allows the electrolyte composition and flow rate of the luminal perfusate to be controlled by the investigator.

In these studies, superficial loops of Henle were perfused from a late proximal site and timed collections were obtained from an early distal site. Therefore, several regions of the nephron with specific transport properties were perfused. These regions include: (a) a portion of the late proximal convoluted segment (S2) which is also referred to as the pars convoluta, (b) the proximal straight segment (S3) also called the pars recta, (c) the thin descending limb, (d) the medullary and cortical TAL of Henle and (e) an early portion of the distal convoluted tubule. The reader will note the absence of the thin ascending limb of Henle in the above list. Nephrons examined in these studies were primarily cortical or superficial nephrons and not the deep juxtamedullary nephrons. It is well-established that approximately 70% of all

outer cortical nephron loops of Henle turn at the junction of the outer and inner medulla (Wahl and Schnermann, 1969). The loops of Henle of the remaining outer cortical nephrons penetrate the outer medulla to varying degrees. It is of interest that although the loops of Henle of juxtamedullary nephrons extend into the inner medulla, the TAL of both nephron populations begins at the junction of the inner and outer medulla. In the present study, both the medullary and cortical regions of the TAL were perfused. The contribution of the cortical and medullary TAL to changes in Cl⁻ transport cannot be discerned.

The loop perfusate used in the present studies was modified in comparison to that used by other investigators. Mannitol, a non-reabsorbable solute, was added to the luminal perfusate to impair water reabsorption in tubule segments proximal to the TAL. The proximal tubule actively transports sodium from the lumen into the cell, however, it cannot do this against any sizeable concentration gradient. If the [Na⁺] in the luminal fluid presented to the proximal tubules falls to 100 mM, then net sodium reabsorption will be abolished. At this point, the rate of active transport of sodium into the cell will be equal to the backflux of sodium from the lumen, and hence, net reabsorption of sodium is virtually eliminated. Giebisch et al (1964) have shown that a luminal [NaCl] of 95 mEq/l in rats will result in net inhibition of NaCl reabsorption. Thus, at this concentration, there is no net movement of NaCl and water in the rat proximal tubule (Giebisch et al, 1964). In the current studies, the loop perfusate was designed to minimize proximal nephron

transport by approximating the equilibrium concentration of NaCl in the proximal tubule. The equilibrium concentration of NaCl was achieved by limiting the perfusate $[\text{Na}^+]$ to 110 mM and the perfusate $[\text{Cl}^-]$ to 100 mM. An additional 10 mM HCO_3^- accompanied the balance of the $[\text{Na}^+]$ in the luminal perfusate. Under these conditions, the composition of the luminal fluid presented to the TAL is essentially identical to the composition of the perfusate.

The data reported in this thesis were derived from tubules perfused at a rate of 22 nl/min. This rate is minimally higher than that measured in single short loops of Henle in the rat kidney during free-flow micropuncture experiments. Schnermann (1968) has shown that normal tubular fluid flow rate through the loop of Henle is approximately 15-20 nl/min.

Samples collected from perfused loop segments were considered acceptable only when the collected fluid [inulin] to perfusate [inulin] ratio (CF/P [inulin]) was between 0.95 and 1.15. The ideal ratio for the CF/P [inulin] would theoretically be 1.0 in the TAL, reflecting no net movement of water. However, the acceptance ratio was set 5% below the ideal ratio of 1.0, to 0.95, to compensate for any surface fluid that may have been aspirated into the collected sample as the collection pipet was removed from the early distal site. The upper end of the acceptable ratio limit was set at 1.15 which excluded more distal segments of the nephron. As well, others in this laboratory have found a significant correlation between a high CF/P [inulin] ratio greater than

1.50 and higher rates of Cl^- reabsorption when samples were deliberately collected from sites in the late distal nephron.

Since there was variation in Cl^- delivery to the loop segments, Cl^- reabsorption was also expressed as a percentage of the delivered Cl^- load. This expression corrects the amount of Cl^- reabsorbed to the amount of Cl^- delivered to the perfused segment. The results of all microperfusion experiments were the same whether the data was expressed as J_{Cl} or fractional Cl^- reabsorption. This finding is consistent with the earlier work by Wahl and Schnermann (1969) that most outer cortical nephrons loop of Henle turn at the junction of the outer and inner medulla, and suggests that the loop segments perfused in the current studies were of similar length.

Using this modified luminal perfusate and the described exclusion criteria, fluid reabsorption by the perfused loop segment was prevented as shown by the virtually identical rates of collection and calculated perfusion rates in all control and experimental groups. In fact, the perfusion and collection rates closely approximated 22 nl/min, the perfusion rate selected on the calibrated Hampel nanoliter pump. A comparison of collection and perfusion rates in all groups of animals showed that these rates differed by less than 2 nl/min. The successful blockade of fluid reabsorption by our modified perfusate and exclusion criteria contrasts with other studies in which the measured net water flux (J_v) in the perfused loop of Henle is approximately 50% of the perfusion rate (Galla et al, 1986; Capasso et al, 1991).

The $[\text{Cl}^-]$ measured in samples collected from the early distal tubule of control animals in this study was 41 mM. This value corresponds very closely to the limiting $[\text{Na}^+]$ established following 15 seconds of stop-flow measured in previous studies (Gutsche et al, 1984; Peterson et al, 1987a) using the technique of microstop-flow conductivity developed by Gutsche et al (1980). However, this value is significantly lower than the $[\text{Cl}^-]$ of 74 mM measured in the early distal tubular fluid by Galla et al (1986). This higher $[\text{Cl}^-]$ (Galla et al, 1986) likely reflects the higher rate of water reabsorption by the proximal nephron since J_w was 50% of the perfusion rate. As well, this higher value will also reflect the higher $[\text{Cl}^-]$ (approximately 135 mM) found in the luminal perfusate used in those studies (Galla et al, 1986). In the present study, the elimination of net Cl^- reabsorption in the perfused loop segment by the loop diuretic, furosemide (10^{-3} M), in the luminal perfusate demonstrates that J_{Cl^-} measured across the loop segment can be attributed to Cl^- transport by the TAL.

Jung and Endou (1990) have shown that loop diuretics, such as furosemide and bumetanide, act on the TAL and induce very striking effects of diuresis and natriuresis. The main action of furosemide is to inhibit carrier-mediated transport of Cl^- by the TAL which was originally demonstrated by Burg et al (1973), and subsequently confirmed by others (Hebert et al, 1981; Greger, 1985; reviewed by Jung and Endou, 1990). Recently, it has been suggested that furosemide may inhibit ATP consumption in thin descending

limbs of outer cortical nephrons; however, Jung and Endou (1990) measured cellular ATP within isolated rat thin descending limbs and found that the presence of the loop diuretic did not alter the level of cellular ATP from control values. Kondo et al (1987) have shown that furosemide (10^{-3} M) when added to the luminal perfusate had no effect on thin ascending limb of Henle's loop function, in vitro. However, when this loop diuretic was added to the bath at the same concentration, a 50% reduction in voltage was observed (Kondo et al, 1987). From these findings, Peterson et al (1993) suggested that when furosemide is present only on the luminal side, as in the experiments described in this thesis, it may also not alter ATP consumption or NaCl reabsorption in the thin descending limb. In addition, the amount of NaCl reabsorbed by the thin descending and ascending limbs is small in comparison to the TAL which reabsorbs approximately 25% of the filtered NaCl.

In the current study, the addition of furosemide (10^{-3} M) to the luminal perfusate may have impaired carbonic anhydrase activity in the proximal tubule and TAL (Radtke et al, 1972; Capasso et al, 1991) such that NaHCO_3 reabsorption was impaired. However, recently Capasso et al (1991) found that 10^{-4} M furosemide had no effect on bicarbonate transport in the loop of Henle in vivo. In vitro studies using the isolated rat cortical TAL showed an elevation in bicarbonate reabsorption in the presence of furosemide, which is most likely due to enhanced luminal Na^+ - H^+ exchange (Good, 1985). Furthermore, in recent studies by Capasso et al (1991), the presence of luminal bumetanide

(10^{-6} M), a more specific loop diuretic, was also associated with a significant elevation in loop bicarbonate reabsorption. These findings suggest that the addition of furosemide to the luminal perfusate may have caused an overestimation of the contribution of the TAL due to enhanced NaHCO_3 reabsorption if sodium had been measured. However, in the present study, Cl^- reabsorption in the perfused loop segment was used as an index of transport in the TAL and therefore, an overestimation of the contribution of the TAL was not possible. In summary, the abolishment of Cl^- reabsorption (J_{Cl} and fractional Cl^- reabsorption) in loop segments perfused in vivo with furosemide (10^{-3} M) in the present study can be attributed to furosemide-sensitive inhibition of carrier-mediated Cl^- transport. Therefore, net Cl^- uptake in these studies can be attributed primarily to carrier-mediated Cl^- reabsorption by the TAL.

4.3 Potassium Infusion, Not Aldosterone, Rapidly Corrects Defective TAL Cl^- Transport in K-DEP Rats.

The technique of microstop-flow conductivity (Gutsche et al, 1984) allowed the estimation of equilibrium TAL $[\text{NaCl}]$ to be measured during periods of stop-flow of 10-60 seconds. This method enabled the estimation of $[\text{NaCl}]$ in TAL tubular fluid as it is presented to an early distal collection site. Using this method, earlier studies from our laboratory have shown an inability of K-DEP rats to lower $[\text{NaCl}]$ in tubular fluid to values measured in control animals

(Gutsche et al, 1984). In fact, the K-DEP rats in that study showed a [NaCl] at equilibrium which was nearly two-fold higher than that measured in control animals. Moreover, a striking correlation between the severity of the impairment in TAL NaCl transport and the degree of reduced ECF[K⁺] was reported (Gutsche et al, 1984). Thus, it could be predicted that TAL NaCl transport would be impaired if plasma [K⁺] fell below 2.9 mM.

In the current study, animals given the K-free diet for 10 days had a significant reduction in plasma [K⁺] which was well below the value of 2.9 mM. Associated with this fall in plasma [K⁺] was a significant reduction in Cl⁻ reabsorption (J_{Cl} and fractional Cl⁻ reabsorption) by the TAL. Since water reabsorption was prevented in these studies, the decrease in Cl⁻ reabsorption was due to the failure to lower luminal [Cl⁻] in K-DEP rats. The results of the present study clearly showed a reduction in TAL Cl⁻ transport associated with sustained hypokalemia and thus, established the quantitative significance of this defect in vivo.

Acute potassium administration in K-DEP rats restored plasma [K⁺] to control values and completely reversed the impairment in TAL Cl⁻ reabsorption (J_{Cl} and fractional Cl⁻ reabsorption) to control values in the present study. These findings provided evidence that ECF[K⁺] may play a role in regulating TAL Cl⁻ reabsorption. Acute systemic infusion of potassium in control rats increased plasma [K⁺] by 1.5 mM, but had no effect on TAL transport. This finding was interesting because large elevations in bath [K⁺] had previously

been associated with impaired Cl^- reabsorption in both the mouse and rabbit TALs (Stokes, 1982). These results are consistent with earlier studies by Stokes (1982) which showed that increasing lumen and bath $[\text{K}^+]$ from 5 mM to 10 mM had no effect on medullary TAL Cl^- reabsorption in rabbits. However, increasing bath and lumen $[\text{K}^+]$ to 10 mM in mouse medullary TAL was associated with a 90% reduction in Cl^- reabsorption (Stokes, 1982). It has also been shown that increasing bath and lumen $[\text{K}^+]$ from 5 mM to 15 mM or 25 mM is associated with a significant impairment in medullary TAL Cl^- reabsorption in both the rabbit and mouse (Stokes, 1982). The lack of inhibition of TAL Cl^- transport observed in control rats infused with potassium in the present study may suggest that the increment plasma $[\text{K}^+]$ was too small to induce a significant effect on TAL Cl^- transport.

Gutsche et al (1984) have shown that (a) acute systemic infusion of potassium to partially replace the potassium deficit and (b) restoration of total body potassium in K-DEP rats was associated with the ability of the TAL to reduce estimated $[\text{NaCl}]$ of the luminal fluid to control values. The findings of the present study, observed in the presence of axial flow, clearly demonstrated that the defect in TAL Cl^- reabsorption in K-DEP rats can be rapidly reversed by the acute administration of potassium. How does potassium repletion correct this defect in TAL Cl^- reabsorption?

It has been well-established that transcellular TAL NaCl transport is driven by the basolateral Na^+/K^+ -ATPase enzyme. Doucet et al (1979) have

shown, in vitro, that maximal activity of this enzyme occurs at a bath $[K^+]$ of 5 mM. Increasing bath $[K^+]$ to 20 mM did not alter the maximal activity of this enzyme. However, as bath $[K^+]$ was reduced, a corresponding reduction in enzyme activity was observed. There was a marked reduction in Na^+/K^+ -ATPase activity when bath $[K^+]$ fell below 2.5 mM. Based on these findings, Gutsche et al (1984) postulated that $ECF[K^+]$ may be rate-limiting for TAL NaCl transport via inhibition of basolateral Na^+/K^+ -ATPase activity. Gutsche et al (1984) demonstrated that defective TAL NaCl transport was not restored when luminal K^+ delivery was increased in K-DEP rats to that measured in control rats. However, it is possible in these studies that insufficient K^+ entered the cell via the luminal membrane to significantly alter intracellular $[K^+]$. The results of the studies by Gutsche et al (1984) did not address whether the impairment in TAL NaCl transport in K-DEP involves the luminal entry step and/or the basolateral exit step. The rapid reversal of the defect within 30 minutes (Gutsche et al, 1984) and the results of the current study provide evidence that major changes in the synthesis of transport proteins are probably not involved in this defect in Cl^- transport by the TAL.

In vitro studies using rat isolated and perfused segments of TAL (Burg and Bourdeau, 1978; Greger, 1981) have shown inhibition of NaCl transport when K^+ is removed from the bath and lumen. However, since K^+ was removed from the bath and lumen simultaneously, the direct effect of changes in luminal and basolateral $[K^+]$ on TAL NaCl transport could not be determined

(Burg and Bourdeau, 1978; Greger, 1981).

Although reductions in $\text{ECF}[\text{K}^+]$ may be rate-limiting for the Na^+/K^+ -ATPase enzyme and therefore, account for impaired TAL NaCl transport in K-DEP rats, recent in vitro studies have demonstrated that bath $[\text{K}^+]$ must be less than 1.5 mM before a significant impairment in NaCl reabsorption by the rat cortical TAL could be detected (Greger and Velasquez, 1987). Thus, inhibition of TAL NaCl reabsorption may not be mediated directly by reductions in $\text{ECF}[\text{K}^+]$ but by some factor which is regulated by $\text{ECF}[\text{K}^+]$.

A number of studies have also provided evidence to support a role for aldosterone in affecting NaCl transport by the TAL. Earlier studies by Green et al (1970) observed a significant reduction in free water clearance in adrenalectomized animals which could be increased to control levels upon aldosterone repletion. It has also been shown, in both rabbits and rats, that physiological aldosterone replacement in adrenalectomized animals significantly enhanced their ability to maximally concentrate their urine (Sigler et al, 1965; Schwartz and Kokko, 1980). In addition, Farman and Bonvalet (1983) have found binding sites for aldosterone in the rat TAL.

Recent in vivo microperfusion (Stanton, 1986) and in vitro perfusion (Work and Jamison, 1987) studies in adrenalectomized rats have shown that NaCl reabsorption by the loop of Henle appears to be dependent upon a normal plasma [aldosterone]. These studies demonstrated an effect of aldosterone on Na^+ transport by the loop of Henle in adrenalectomized rats such that chronic

aldosterone replacement (via implanted mini-pumps) restored Na^+ transport to control levels in adrenalectomized rats (Stanton, 1986; Work and Jamison, 1987). Unfortunately, neither of these investigators examined the direct effect of aldosterone on loop of Henle transport. To clearly establish an effect of aldosterone on TAL NaCl transport requires in vitro investigation of loop of Henle segments taken from adrenalectomized rats in the absence and presence of aldosterone.

It has been well-established that aldosterone release from the adrenal gland is regulated by $\text{ECF}[\text{K}^+]$ (Boyd et al, 1971). Linas et al (1979) provide evidence that following 24 hours of dietary potassium depletion in rats, there is a significant reduction in both plasma $[\text{K}^+]$ and plasma [aldosterone] compared to control animals. Furthermore, after 72 hours of dietary potassium deprivation, both plasma $[\text{K}^+]$ and plasma [aldosterone] were further reduced such that plasma [aldosterone] was significantly suppressed and paralleled decreases in plasma $[\text{K}^+]$ (Linas et al, 1979).

In the present study, plasma [aldosterone] was significantly suppressed on day 10 when (a) TAL Cl^- reabsorption (both J_{Cl} and fractional Cl^- reabsorption) was impaired and (b) plasma $[\text{K}^+]$ was reduced in K-DEP animals. In fact, aldosterone release in K-DEP rats will remain suppressed even in the presence of the added stimulus of NaCl depletion which has previously been shown to enhance the levels of circulating plasma renin activity (McKay et al, 1990). The finding of decreased plasma [aldosterone] in hypokalemic rats in

the current study suggests that the impairment in TAL Cl⁻ reabsorption in K-DEP may be due directly to an aldosterone deficiency and not to reduced ECF[K⁺]. As noted previously, there is evidence that aldosterone appears to be necessary for normal rates of TAL Cl⁻ reabsorption.

To test the hypothesis that an aldosterone deficiency mediates inhibition in TAL Cl⁻ reabsorption, K-DEP rats were infused with aldosterone for a minimum of 75 min. The failure of aldosterone to reverse defective TAL Cl⁻ transport in K-DEP rats cannot be attributed to the possibility that the dose of this hormone was too small. The aldosterone infusion protocol was based on previous studies (Martin et al, 1983; Stanton et al, 1985) which have demonstrated that this rate of infusion produced hormone levels similar to that measured in awake, unstressed rats. Based on these earlier studies, K-DEP rats were infused with aldosterone at a similar rate in the present study. Since the K-DEP rats had measurable plasma [aldosterone] already, one would have predicted that the plasma [aldosterone] achieved in these rats would have been significantly higher compared to untreated control rats. However, the acute systemic infusion of aldosterone, in the presence of sustained hypokalemia, failed to correct the impairment in TAL Cl⁻ reabsorption in K-DEP rats.

It was possible that the aldosterone infused in the present study was not biologically active. Thus, the efficacy of the aldosterone was tested in a separate series of experiments. Stimulation of urinary K⁺ excretion was used as an index of aldosterone bioactivity. Urinary K⁺ excretion was divided by the

simultaneous excretion of creatinine in each animal to correct for incomplete collections. Biological activity of aldosterone was clearly demonstrated in adrenalectomized rats treated with aldosterone who showed a significant enhancement (54%) in urinary K^+ excretion.

Another well-established index of aldosterone activity is the urinary Na^+/K^+ ratio. In adrenalectomized rats, the presence of aldosterone significantly lowered the measured Na^+/K^+ ratio, providing further evidence that in these studies, the hormone was biologically active. Therefore, the lack of effect of aldosterone in correcting impaired TAL Cl^- reabsorption in potassium depletion cannot be attributed to biological inactivity.

The time course of infusion of aldosterone prior to the collection of samples (75 min) was of sufficient duration to allow the effects of this hormone to occur. The results of the current studies provide evidence that although a minimal plasma [aldosterone] is required for normal TAL Cl^- reabsorption, an aldosterone deficiency cannot account for the impairment in TAL Cl^- reabsorption in K-DEP animals.

The potent regulatory effect of $ECF[K^+]$ on aldosterone production and release from the adrenal gland is well-established. Boyd et al (1971) showed that potassium (a) influences the synthesis of aldosterone at the level of the conversion of corticosterone to aldosterone, and (b) stimulates widening of the zona glomerulosa of the adrenal gland. In rats, aldosterone release was suppressed in response to reduced $ECF[K^+]$ induced by dietary potassium

depletion (Boyd et al, 1971). Furthermore, oral potassium loading, to replace the total potassium deficit induced by the potassium deficient diet, significantly enhanced aldosterone release which could be measured as early as 4 hours following the oral potassium load (Boyd et al, 1971). In our rats given a K-free diet, both plasma $[K^+]$ and plasma [aldosterone] were significantly reduced. To further determine the role of aldosterone and $ECF[K^+]$ on impaired TAL Cl^- transport in K-DEP, the effect of potassium infusion in repairing defective TAL Cl^- reabsorption, in the absence of increased aldosterone action, in K-DEP rats was investigated.

The aldosterone antagonist, spironolactone, was administered 2 hours prior to the potassium infusion to prevent increased aldosterone action in the presence of increasing plasma $[K^+]$. In these studies, the acute administration of potassium, in the presence of spironolactone, rapidly reversed the defect in TAL Cl^- reabsorption in K-DEP rats.

Is it possible that the rapid reversal in defective TAL Cl^- reabsorption in these studies is due to ineffective blockade of aldosterone action such that increased plasma $[K^+]$ in K-DEP animals resulted in a significant elevation in plasma [aldosterone]? The bioactivity of the aldosterone antagonist, spironolactone, was examined and defined as its ability to decrease the response to aldosterone. Efficacy studies showed only slight blockade (25%) of aldosterone actions in terms of both urinary K^+ excretion and urinary Na^+/K^+ ratio in adrenalectomized rats. These findings indicated that there may have

been enhanced aldosterone actions in K-DEP rats infused with potassium. Thus, the rapid reversal of the impairment may have been due to elevated plasma [aldosterone] and normalization of ECF[K⁺] in K-DEP rats. The time course of partial potassium repletion in K-DEP rats was of sufficient duration (minimum 75 min) to allow these effects of aldosterone to occur.

Linas et al (1979) have demonstrated that complete replacement of the potassium deficit in K-DEP rats (potassium chloride by gavage) restored plasma [K⁺] to control levels (Linas et al, 1979). Although plasma [aldosterone] was not measured in their study, it was assumed that the restoration of plasma [K⁺] to control values was associated with a comparable restoration of plasma [aldosterone] to reported control values. Based on the study of Boyd et al (1971), these investigators expected the rate of rise in plasma [K⁺] induced by the administration of potassium to be paralleled by a similar rise in plasma [aldosterone]. The reported measurements of Boyd et al (1971) were taken 3 hours following the administration of potassium such that there was sufficient time for the effects of aldosterone to occur.

As discussed earlier, our rats were hypokalemic with reduced plasma [aldosterone] after 10 days of dietary potassium depletion in the present study. Based on previous studies (Boyd et al, 1971; Linas et al, 1979), it was hypothesized that an acute infusion of potassium (as potassium chloride) on day 10, to partially replace the potassium deficit induced by the potassium deficient diet would (a) restore plasma [K⁺] in K-DEP rats and (b) restore plasma

[aldosterone], such that both these variables would rise in a parallel manner.

In the present study, plasma [aldosterone] in both K-DEP and control rats was measured following partial acute replacement (30%) of the potassium deficit in K-DEP rats. Following 30 minutes of potassium infusion, K-DEP rats showed a small but significant increase of 0.7 mM in plasma $[K^+]$, increasing plasma $[K^+]$ above 2.9 mM. Based on the work of Gutsche et al (1984), this elevation in plasma $[K^+]$ in potassium-infused K-DEP animals would be associated with the complete restoration of TAL Cl^- reabsorption. At this same time point, plasma $[K^+]$ increased 1.5mM in control animals infused with potassium compared to untreated control rats. However, the rise in the plasma $[K^+]$ in control animals was much greater than the rise in plasma $[K^+]$ measured in the potassium-infused K-DEP animals. Following 75 min of the potassium infusion, plasma [aldosterone] was still suppressed in K-DEP rats despite restoration of plasma $[K^+]$ to control values. These results showed that the rate of rise in plasma $[K^+]$ in K-DEP rats was much slower than that measured in control rats. Furthermore, the rise and restoration of plasma $[K^+]$ in K-DEP rats was not associated with a parallel increase in plasma [aldosterone]. However, the acute infusion of potassium significantly increased (46%) plasma [aldosterone] in control animals. This is the first study to demonstrate in K-DEP rats, that the restoration of plasma $[K^+]$ is not immediately associated with a parallel rise in plasma [aldosterone], suggesting that the cellular potassium deficit must be replaced before aldosterone release returns to normal.

The failure of plasma [aldosterone] to rise in K-DEP rats infused with potassium may be associated with changes in insulin secretion by pancreatic beta cells. Potassium deficiency has been associated with reduced insulin secretion (Gabow and Peterson, 1992). Under normal conditions, insulin is required for K^+ uptake into cells. Increased plasma [K^+] would presumably be associated with increased plasma [insulin], plasma [aldosterone] and K^+ uptake into cells. The failure of K-DEP rats infused with potassium to significantly elevate plasma [aldosterone] may be due to reduced entry of K^+ into cells secondary to an insulin deficiency. It is also possible that aldosterone release in K-DEP rats may be delayed due to the synthesis of enzymes in the aldosterone biosynthetic pathway. It can be proposed that once ECF[K^+] (or intracellular [K^+]) has reached a 'normalized' value, one would expect elevations in plasma [K^+] to be associated with a parallel rise in plasma [aldosterone].

Further studies were subsequently performed to determine the effect of complete restoration of the induced potassium deficit on plasma [aldosterone] in K-DEP rats. In these studies, the entire potassium deficit was replenished by a gavage of potassium chloride. Plasma [aldosterone] in K-DEP rats measured 28 hours after the oral potassium loading achieved control values.

Although there are differences in (a) the route of the administration of potassium (gavage versus intravenous potassium administration), (b) the degree of replenishment of the potassium deficit in K-DEP rats, (complete or partial

replacement), and (c) the time when plasma [aldosterone] was measured, these findings suggest that the rate of rise of plasma [aldosterone] is much slower (ie. does not parallel) than the rate of rise in plasma $[K^+]$. Linas et al (1979) have shown that total repletion of tissue potassium in K-DEP rats occurred within three hours following a gavage of potassium chloride. One could hypothesize that at this time point, plasma [aldosterone] was also normalized to control values, although this was not measured by Linas et al (1979). From this work, it was assumed that a similar time course and parallel rise in restoration of ECF $[K^+]$ and plasma [aldosterone] would result.

The results of the current series of experiments with spironolactone are the first to show that restoration of plasma [aldosterone] does not parallel the rise and normalization of ECF $[K^+]$ in K-DEP rats. In these studies, TAL Cl^- reabsorption was corrected when ECF $[K^+]$ increased towards normal despite sustained aldosterone deficiency. In control animals, the acute administration of potassium increased (46%) plasma [aldosterone], but did not alter TAL Cl^- reabsorption. Therefore, these results provide further evidence that an aldosterone deficiency does not contribute to the impairment in TAL Cl^- reabsorption in K-DEP rats. In fact, the failure of the aldosterone infusion to correct impaired TAL Cl^- reabsorption in K-DEP rats is the first study to demonstrate that aldosterone does not have a regulatory role in Cl^- transport by the TAL. Furthermore, based on the results of the present study, it is possible to conclude that normal TAL Cl^- reabsorption will occur as long as a critical

level of aldosterone is present. These results suggest that the required minimum hormone level is at most only 20% of the normal circulating concentration. Future studies will need to examine the time course effect of potassium on increasing plasma [aldosterone] in K-DEP rats.

4.4 Hypotheses Regarding the Mediator of Impaired TAL Cl⁻ Transport in Potassium Depletion.

Since these studies demonstrated that an aldosterone deficiency in K-DEP animals cannot account for the impairment in TAL Cl⁻ reabsorption, what factor is responsible for this defect in TAL Cl⁻ transport? Is it possible that the impairment in TAL Cl⁻ reabsorption in K-DEP could be due directly to (a) a reduction in ECF[K⁺] which may be rate-limiting for Cl⁻ transport or (b) due to a change in a mediator whose production may be affected by changes in intracellular [K⁺]?

It is possible, as Gutsche et al (1984) have previously suggested, that reduced ECF[K⁺] may be rate-limiting for TAL NaCl transport in potassium depletion via inhibition of basolateral Na⁺/K⁺-ATPase activity. However, the more recent in vitro study of Greger and Velasquez (1987) showed that bath [K⁺] must fall below 1.5 mM before an impairment in rat cortical TAL NaCl transport can be detected. Although there was a significant reduction in ECF[K⁺] in K-DEP animals in the present study, plasma levels did not fall below

1.5 mM. In contrast to the results of Greger and Velasquez (1987), Doucet et al (1979) have shown using rat medullary TAL that reductions in bath $[K^+]$ below 2.5 mM were associated with significant inhibition in basolateral Na^+/K^+ -ATPase enzyme activity. It is highly unlikely that medullary interstitial K^+ will fall significantly below 5 mM in K-DEP rats. Therefore, it is unlikely that the reduction in $ECF[K^+]$ measured in K-DEP rats can directly explain the inhibition in TAL $NaCl$ transport. What then mediates impaired TAL Cl^- reabsorption in potassium depletion?

A number of recent studies have provided some clues regarding the nature of the inhibitor which may ultimately be involved in the impairment of TAL Cl^- reabsorption observed in K-DEP rats in the present study. Renal outer medullary cells, including those of the medullary TAL, have been shown to synthesize arachidonic acid metabolites via three pathways: (1) prostaglandins via the cyclooxygenase enzyme complex, (2) epoxides via the cytochrome P-450 pathway, and (3) leukotrienes via the 5-lipoxygenase enzyme pathway. Stimulation of renal arachidonic acid metabolites has been shown to inhibit Na^+/K^+ -ATPase enzyme activity in guinea-pig taenia coli (Coburn and Soltoff, 1977), rat and rabbit medulla (Zusman and Keiser, 1980; Wald et al, 1990) and rat isolated medullary TAL cells (Beck and Shaw, 1981; Carroll et al, 1990; Carroll et al, 1991). As well, arachidonic acid metabolite pathways are stimulated by chronic potassium depletion in vivo and reductions in bath $[K^+]$ in vitro (Düsing et al, 1978; Beck and Shaw, 1981; Güllner et al, 1983; Carroll

et al, 1990). The major sites of prostaglandin production in the mammalian kidney primarily involve the medullary collecting duct and medullary interstitial cells, although the TAL does have a limited capacity for prostaglandin synthesis (reviewed by Bonvalet et al, 1987). From these findings, it can be hypothesized that the link between reduced ECF[K⁺] in K-DEP rats and altered TAL Cl⁻ reabsorption in K-DEP observed in the present study may involve an arachidonic acid metabolite.

A number of investigators have shown that renal production of the arachidonic acid cyclooxygenase metabolite, PGE₂, is altered in the presence of potassium depletion (Coburn and Soltoff, 1977; Düsing et al, 1978; Beck and Shaw, 1981; Güllner et al, 1983). Early studies have shown a significant enhancement in urinary PGE₂ excretion in K-DEP dogs and rabbits (but not rats) which could be reversed by inhibiting prostaglandin synthesis, using the prostaglandin synthetase inhibitor, indomethacin (Galvez et al, 1976). Galvez et al (1977) also found enhanced PGE₂ urinary excretion in K-DEP dogs following acute hemodialysis with a low potassium dialysate. In fact, prostaglandin synthesis inhibition by indomethacin was associated with normalization of renal concentrating ability in K-DEP dogs (Galvez et al, 1976). Dietary potassium depletion has also been shown to stimulate PGE₂ biosynthesis in rabbit renomedullary interstitial cells *in vitro* such that PGE₂ synthesis was inversely related to the [K⁺] in the incubation medium, suggesting a putative role for potassium in controlling PGE₂ synthesis (Galvez

et al, 1976; Zusman and Keiser, 1977; Güllner et al, 1983). Enhanced urinary PGE₂ excretion and potassium depletion are also features of Bartter's syndrome (Gill et al, 1976), and of patients with chronic vomiting (Gill and Bartter, 1978). It was assumed in these studies that any change in urinary PGE₂ excretion would indicate an alteration in PGE₂ biosynthesis. These data suggest that prostaglandin metabolism is stimulated by potassium depletion. In contrast to the studies involving K-DEP dogs and rabbits, other studies have been unable to show an enhanced urinary PGE₂ excretion in K-DEP rats (Hood and Dunn, 1978; reviewed by Beck and Shaw, 1981). Furthermore, these studies failed to measure an alteration in PGE₂ content in the rat renal medulla (Hood and Dunn, 1978; reviewed by Beck and Shaw, 1981).

The arachidonic acid cyclooxygenase metabolite, PGE₂, has also been shown to inhibit Na⁺/K⁺-ATPase enzyme activity (Coburn and Soltoff, 1977; Rubinger et al, 1986; Wald et al, 1990). Na⁺/K⁺-ATPase enzyme activity in (a) microsomes isolated from the renal medulla and (b) isolated medullary TAL segments from rats pretreated with indomethacin have been found to have a significant elevation in enzymatic activity (Rubinger et al, 1986; Wald et al, 1990). Recently, Wald et al (1990) showed that exposure of isolated medullary TAL segments to increasing concentrations of PGE₂ in indomethacin pretreated rats *in vitro*, reduced the activity of the Na⁺/K⁺-ATPase enzyme in a dose-dependent manner. In fact, the addition of 100 μM PGE₂ to the incubation medium completely blocked the indomethacin-induced stimulation

of Na^+/K^+ -ATPase activity. One would predict from the results of Wald et al (1990) that the elevation in Na^+/K^+ -ATPase activity observed by indomethacin pretreatment *in vivo* would occur as a result of reduced PGE_2 . *In vitro* studies have shown that PGE_2 can directly reduce the net rate of NaCl reabsorption by the medullary TAL in the rat and rabbit (reviewed by Hebert et al, 1987). Our laboratory has recently shown in control rats that exogenous PGE_2 added to the luminal perfusate can impair TAL Cl^- reabsorption *in vivo* (Peterson et al, 1993). More importantly, in the same study, we were able to demonstrate for the first time that endogenous PGE_2 accounts for inhibition of TAL Cl^- reabsorption in chronic hypercalcemia.

Beck and Shaw (1981) proposed that other arachidonic acid metabolites, such as TXA_2 (vasoconstrictor), may be involved in hemodynamic and metabolic changes associated with potassium depletion since it has been shown that its renal biosynthesis is altered by chronic potassium depletion. In these studies, TXB_2 was measured as an index of TXA_2 , since TXA_2 has a short half-life and is chemically labile (Beck and Shaw, 1981). These investigators showed that TXB_2 production in papillary slices taken from K-DEP rats was increased compared to control values. As well, enhanced concentrations of TXB_2 were measured in homogenates of renal cortical slices taken from K-DEP rats (Beck and Shaw, 1981). These elevated concentrations of the vasoconstrictor TXA_2 may account for the reduction in inner medullary plasma flow which has been previously shown in K-DEP rats (Whinnery and Kunau,

1979; Peterson, 1984). To my knowledge, it does not appear that the effect of arachidonic acid metabolites on TAL Cl^- reabsorption has been investigated in potassium depletion under *in vivo* conditions. It seems likely that one of the arachidonic acid cyclooxygenase metabolites, whose production in the renal medulla is stimulated by K-DEP, may be responsible for the impairment in TAL J_{Cl} in potassium depletion.

Recent studies report that rabbit medullary TAL cells have a significant ability to generate arachidonic acid P-450 metabolites and 5-lipoxygenase metabolites (Ferreri et al, 1984; Capdevila et al, 1988; Carroll et al, 1990; Carroll et al, 1991; Cantley et al, 1991; Escalante et al, 1991). The epoxides synthesized from the arachidonic acid P-450 pathway and the other eicosanoids generated from the arachidonic acid 5-lipoxygenase pathways have been shown to inhibit ouabain-sensitive oxygen consumption (Cantley et al, 1991; Ferreri et al, 1984; Capdevila et al, 1988; Escalante et al, 1990), which has been suggested to be due to direct inhibition of Na^+/K^+ -ATPase activity. These findings suggest that these metabolites may be involved in altering oxygen demands and thus, NaCl transport by the TAL under *in vivo* conditions (Cantley et al, 1991).

Reverse phase high pressure liquid chromatography (HPLC) has shown two peaks (P_1 and P_2) to be present in rabbit medullary TAL cells (Carroll et al, 1990; Carroll et al, 1991). While P_1 appears to have mainly vasodilator activity, the second peak, P_2 , has been associated with cytochrome P-450

metabolites capable of inhibiting the activity of the Na^+/K^+ -ATPase enzyme (Carroll et al, 1990; Carroll et al, 1991). As well, P_2 has been shown to be a much more potent inhibitor of the Na^+/K^+ -ATPase enzyme compared to the first peak, P_1 (Carroll et al, 1990; Carroll et al, 1991). Recent structural identification of these two peaks indicates that the principal component of P_1 was 19-HETE and 20-HETE, while P_2 was mainly composed of 20-COOH-AA (Carroll et al, 1991).

In addition to dietary potassium depletion induced enhancement of arachidonic acid cyclooxygenase metabolites in the renal medulla, Carroll et al (1991) measured a significant increase in the generation of arachidonic acid cytochrome P-450 metabolites in the presence of reduced external potassium. Repletion of potassium in K-DEP rabbits reversed the enhancement of arachidonic acid cytochrome P-450 metabolites to control values. It is possible that defective TAL Cl^- reabsorption in K-DEF could be mediated by arachidonic acid P-450 metabolites.

The results of the current study examining defective TAL Cl^- transport in K-DEP rats leaves questions which require further investigation. Does the impairment in TAL J_{Cl} observed in potassium depletion involve the basolateral exit step and/or luminal entry step?

Numerous speculations can be proposed to describe how control mechanisms, involved with TAL Cl^- transport, may be altered in potassium depletion. At the present time, these proposals must remain pure speculation

since there is no evidence to provide support for these views within the current literature. Clearly, these kinds of studies must be undertaken by other laboratories who have previously demonstrated an ability to analyse alterations in transport mechanisms at the membrane level. However, the potential alterations in TAL NaCl transport in potassium depletion are discussed below.

It is possible that elevated levels of arachidonic acid metabolites may inhibit the generation of intracellular second messengers such as cyclic AMP (cAMP) and protein kinase C. Potassium depletion has been shown to reduce TAL adenylyl cyclase activity in rats. This inhibition may cause down-regulation of the basolateral Na^+/K^+ -ATPase pump, a well-documented effect observed in rat distal tubular segments during chronic potassium depletion (Stanton, 1985). It is also possible that potassium depletion may alter the activity of the putative basolateral KCl symport or the basolateral K^+ conductance channel of TAL cells. However, there is currently no evidence to demonstrate these pathways are altered in K-DEP animals.

The reduced generation of second messengers (in response to potassium depletion) may also alter the activity of luminal transport proteins and/or luminal K^+ conductance channels. The luminal cotransporter (of TAL cells) may continue to function in the high efficiency, K^+ -dependent mode, such that net NaCl reabsorption will continue via both transcellular and paracellular pathways. Reduced activity (ie. number or affinity) of the high efficiency, K^+ -dependent mode of the luminal cotransporter will impair net TAL NaCl reabsorption.

Chronic potassium depletion may also result in the conversion of the high efficiency, K^+ -dependent cotransporter to the low efficiency, K^+ -independent mode. The effect of reduced $ECF[K^+]$ or intracellular $[K^+]$ on the mode or affinity or number of luminal cotransporters is currently unknown. If potassium depletion alters the activity of the luminal cotransporter to the low efficiency mode, then net Cl^- reabsorption by the TAL would only occur via a transcellular pathway. In this hypothetical situation, a 50% reduction in net TAL Cl^- would be expected. Clearly, the mode of the TAL luminal cotransporter (ie. low versus high efficiency) during potassium depletion must be determined. Does reductions in $ECF[K^+]$ or intracellular $[K^+]$ cause alterations in the mode from a high efficiency to a low efficiency form? What percentage of the cotransporters, if any, are altered (ie. mode or affinity or number) in response to potassium depletion? It is also possible that potassium depletion may alter the activity of luminal K^+ conductance channels. These are essential questions that must be investigated if the mechanism(s) controlling TAL $NaCl$ transport are to be understood.

Is potassium depletion associated with down-regulation of basolateral pumps only, while luminal cotransporters continue to transport in the high and/or low efficiency mode? Does potassium depletion alter the activity (ie. number, affinity, mode) of luminal cotransporters alone, without altering the activity of the basolateral pump? Alternatively, is the activity (ie. number, affinity, mode [luminal cotransporter only]) of both basolateral and luminal

transport pathways reduced in a parallel manner during chronic potassium depletion? These are important questions which may account for the defect in TAL Cl^- reabsorption measured in K-DEP rats in the current study. Clearly, more work is required at the cellular level, using isolated medullary TAL segments, medullary cells and membranes, to elucidate the control mechanism(s) of the NaCl transport system in the TAL.

4.5 Future Studies:

Since the impairment in TAL Cl^- reabsorption in K-DEP rats is a rapid and reversible defect (by an aldosterone-independent mechanism), future work will need to study this defect to further our current understanding and to elucidate the control mechanisms of NaCl transport by the TAL. It remains to be determined whether the impairment in TAL J_{Cl} is due directly to (a) a reduction in $\text{ECF}[\text{K}^+]$ which may be rate-limiting for NaCl transport or (b) due to a change in a cellular mediator whose production may be affected by changes in intracellular $[\text{K}^+]$. Using the model of potassium depletion, the arachidonic acid metabolite responsible for impaired TAL J_{Cl} needs to be identified.

These specific objectives can be investigated using control and K-DEP rats. Functionally isolated loop segments would be perfused in vivo, using continuous microperfusion to measure furosemide-sensitive J_{Cl} . These studies would allow the examination of each arachidonic acid pathway and its possible

link between potassium depletion and TAL J_{Cl} inhibition. J_{Cl} would be measured following inhibition of the specific arachidonic acid pathway.

Initial studies should examine the effect of potassium depletion to impair TAL J_{Cl} in the presence of arachidonic acid cyclooxygenase inhibition. These preliminary studies would use (i) the microperfusion technique, (ii) modified luminal perfusate and (iii) exclusion criteria described in this thesis (see Chapter 2, Table 3) to measure TAL J_{Cl} (*in vivo*) in functionally isolated Loop of Henle segments. The arachidonic acid pathway would be investigated initially since there is convincing evidence that low K^+ stimulates prostaglandin release in renomedullary interstitial cells, renal medulla and extrarenal tissues (for review, see Section 4.4). Indomethacin should be used to inhibit arachidonic acid cyclooxygenase metabolism since it has been well-documented to be a potent inhibitor of the cyclooxygenase system (Greger and Schlatter, 1981; Capdevila et al, 1988; Wald et al, 1990; Cantley et al, 1991). This pharmacological agent also inhibits, to a lesser degree, the P-450 cytochrome and 5-lipoxygenase pathways (Capdevila et al, 1988). In these studies, control and K-DEP rats would receive an intravenous infusion of indomethacin or its vehicle on day 10 and TAL J_{Cl} would be measured (following 10 days of dietary treatment). This laboratory has recently shown in hypercalcemic rats that there is a significant reduction in TAL J_{Cl} which was reversed to control values following the acute administration of indomethacin (Peterson et al, 1993). Multivariate analysis of variance of both hypercalcemic and control rats

demonstrated that (i) TAL J_{Cl} is impaired in chronic hypercalcemia and (ii) indomethacin increases TAL J_{Cl} in hypercalcemic rats only. Based on these findings, it would be predicted that in control rats treated with indomethacin, Cl^- reabsorption by the TAL would not be affected. If an arachidonic acid cyclooxygenase product (prostaglandin or thromboxane) induced by potassium depletion inhibits TAL J_{Cl} , then K-DEP rats treated with indomethacin, in the presence of sustained hypokalemia, should achieve restoration of TAL Cl^- reabsorption to control values. If the impairment in TAL J_{Cl} reabsorption in K-DEP rats is reversed in the presence of indomethacin, despite sustained hypokalemia, then one will have established a link between potassium depletion and TAL Cl^- reabsorption. The source and identity of the cellular mediator would not be determined by these in vivo microperfusion experiments, but this would be the first study to provide evidence that an endogenous cyclooxygenase metabolite can mediate inhibition of NaCl transport in potassium depletion.

If the presence of indomethacin corrects the defect in TAL J_{Cl} in K-DEP rats, then future studies should begin to investigate which cyclooxygenase products (prostaglandins or thromboxanes) are involved. Imidazole, would be added to the luminal perfusate to inhibit thromboxane synthesis and TAL J_{Cl} would be measured in control and K-DEP rats. To ensure that the effect of imidazole in antagonizing the action of TXA_2 on TAL J_{Cl} , it would be essential that all microperfusion studies performed in control rats had both imidazole and

TXA₂ added to the luminal perfusate. If TXA₂ induced by potassium depletion inhibits TAL J_{Cl}, then K-DEP rats treated with imidazole will show a complete reversal of the defect to normal control values. If the defect in TAL J_{Cl} is reversed in the absence of thromboxane, despite sustained hypokalemia, then the arachidonic acid cyclooxygenase metabolite responsible for impaired TAL Cl⁻ reabsorption in K-DEP rats will have been identified. However, if the defect is still present, then this would suggest an important role for a prostaglandin metabolite in mediating defective TAL J_{Cl} in potassium depletion.

To further test this hypothesis that an arachidonic acid metabolite is responsible for impaired TAL J_{Cl} in potassium depletion, control loops of Henle must be perfused with TXA₂ directly and transport measurements made. This laboratory has previously shown in indomethacin-treated control rats that TAL J_{Cl} can be impaired in vivo by acute exposure to exogenous PGE₂ (Peterson et al, 1993). The ability of exogenous PGE₂ or TXA₂ to impair TAL J_{Cl} in control rats will provide convincing evidence that indomethacin does not impair the mechanism by which prostaglandins or TXA₂ induce their effect on the TAL.

Simultaneously with the microperfusion studies, the putative mediator PGE₂ or TXB₂ (the more stable form of TXA₂) could be extracted from the outer medulla of kidneys obtained from control and K-DEP rats. These animals would be treated with indomethacin or its vehicle and the putative mediator (PGE₂ or TXB₂) measured by radioimmunoassay. This laboratory has successfully measured PGE₂ levels in renal outer medulla in control and hypercalemic rats

(Peterson et al, 1993). This technical ability contrasts others who have not been successful in this endeavour. If PGE₂ or TXB₂ is significantly elevated in K-DEP rats and normalized in the presence of indomethacin, then these studies will provide convincing evidence that impaired TAL J_{Cl} in potassium depletion is due to endogenous PGE₂ or TXA₂ and not due to reduced ECF[K⁺] per se.

If the above studies have a negative outcome, then additional studies could be undertaken to determine TAL J_{Cl} in K-DEP rats in the presence of inhibition of either the cytochrome P-450 or the 5-lipoxygenase pathways. These studies would be designed in a manner identical to the microperfusion studies previously described. Ketoconazole would be used to inhibit the arachidonic acid P-450 pathway, and FPL 55712 to impair the 5-lipoxygenase pathway. If cytochrome P-450 metabolites induced by potassium depletion inhibit TAL J_{Cl}, then K-DEP rats treated with ketoconazole will show a complete reversal of defective TAL J_{Cl} to control values. This finding would establish the link between potassium depletion and TAL J_{Cl} inhibition. Although the source and identity of the cellular metabolite would not be determined by these experiments, this would be the first study to identify that an endogenous P-450 metabolite can mediate inhibition of TAL NaCl transport in potassium depletion. However, if the presence of ketoconazole does not correct the defect in TAL J_{Cl} in potassium depletion, then the 5-lipoxygenase pathway should be investigated. If lipoxides induced by potassium depletion impair TAL J_{Cl}, then K-DEP rats treated with FPL 55712 should show a restoration of J_{Cl} to control

levels. If the defect in J_{Cl} is reversed in the presence of 5-lipoxygenase inhibition, then this study would be the first to establish that an arachidonic acid 5-lipoxygenase pathway can mediate inhibition of TAL NaCl transport in potassium depletion.

The above described future work will allow the investigation of the hypothesis that arachidonic acid metabolites mediate impaired TAL J_{Cl} in potassium depletion. However, the studies will not be able to identify the source of the arachidonic acid metabolite responsible for this defect in TAL transport in vivo because of the presence of a number of cell types which are capable of metabolizing arachidonic acid.

The continuation of this work will allow the most comprehensive and precise analysis of TAL Cl^- reabsorption possible in potassium depletion. These studies will establish whether the impairment in TAL J_{Cl} is due directly to a reduction in $ECF[K^+]$ or due to an arachidonic acid metabolite whose production is affected by changes in intracellular $[K^+]$. These future studies will be essential in extending both our current understanding of TAL transport and elucidating the control mechanisms of NaCl transport in the TAL.

4.6 Conclusion:

The present study confirmed earlier work that dietary potassium depletion is associated with a renal concentrating defect. Urine concentrating ability was maximally reduced in K-DEP rats following 10 days of dietary potassium depletion.

Modification of the luminal perfusate composition used in the microperfusion studies allowed the measurements of furosemide-sensitive Cl^- reabsorption in the perfused loop segments such that net Cl^- uptake in the current studies could be attributed primarily to carrier-mediated Cl^- transport by the TAL. Using the technique of continuous microperfusion, this study demonstrated that the defect in TAL Cl^- reabsorption in K-DEP rats is quantitatively significant and can be rapidly reversed by the acute administration of potassium. The acute administration of aldosterone, in the presence of sustained potassium depletion, failed to reverse the impairment in TAL Cl^- reabsorption. In addition, the acute administration of potassium, in the presence of an aldosterone antagonist, rapidly reversed the defect in TAL Cl^- reabsorption in K-DEP rats to control levels. Additional studies demonstrated that, despite restoration of $\text{ECF}[\text{K}^+]$ by systemic K^+ infusion in K-DEP rats, plasma [aldosterone] failed to increase within this time period. In control animals, the acute administration of potassium increased (46%) plasma [aldosterone], but did not alter TAL Cl^- transport. Therefore, although a minimal

level of aldosterone may be required for normal TAL Cl^- reabsorption, this hormone does not regulate Cl^- transport by the TAL. In fact, the present studies suggest that only approximately 20% of the normal circulating plasma [aldosterone] is required for normal TAL Cl^- reabsorption. Therefore, these studies are the first to provide conclusive evidence that the rapid reversal of defective TAL Cl^- reabsorption seen with acute potassium infusion in K-DEP rats occurs via an aldosterone independent mechanism.

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APPENDIX A

APPENDIX A

A sample calculation from an actual microperfusion experiment is shown below, using the equations described in section 2.1.9.

Date:	March 20, 1990.
Rat Identification:	J18
Tubule Microperfused:	1
Collected Sample:	2
Volume of Collected sample:	65.11 nL/3 minutes
Collected rate of sample:	21.703 nL/minute
CF/P [Inulin]:	1.01
$[Cl^-]_{\text{collected}}$:	69.7153 mM
$[Cl^-]_{\text{perf}}$:	102.0725 mM

(1) In vivo calculated perfused rate (CPR):

$$\text{CPR (nL/minute)} = \text{CR (nL/minute)} \times \text{CF/P [Inulin]}$$

where CPR (nL/minute) = calculated perfused rate in vivo

CR (nL/minute) = collected rate at the earliest surface
distal site

CF/P [Inulin] = ratio ^{14}C -inulin in the collected sample to the ^{14}C -
inulin in the perfusate sample

$$\begin{aligned} \therefore \text{CPR (nL/minute)} &= (21.701 \text{ nL/minute}) \times 1.01 \\ &= 21.92 \text{ nL/minute} \end{aligned}$$

Therefore, the calculated perfused rate in vivo falls within the established parameters of 22 ± 2 nL/minute, and thus, is acceptable for the experiment.

(2) Chloride flux (J_{Cl}) is the absolute rate of chloride reabsorption in the perfused segment in pmol/minute.

$$J_{Cl} \text{ (pmol/minute)} = ([Cl^-]_{perf} \times CPR) - ([Cl^-]_{collected} \times CR)$$

where $[Cl^-]$ (mM) = $[Cl^-]$ in the perfusate sample

$[Cl^-]$ (mM) = $[Cl^-]$ in the collected sample

CPR (nL/minute) = calculated perfused rate in vivo

CR (nL/minute) = collected rate in vivo

$$\begin{aligned} \therefore J_{Cl} &= (102.0725 \times 21.92) - (69.7153 \times 21.703) \\ &= 724.4 \text{ pmol/minute} \end{aligned}$$

(3) Fractional Cl⁻ reabsorption was calculated as a percentage (FR_{Cl⁻} %).

$$\begin{aligned} \text{FR}_{\text{Cl}^-} \% &= \frac{([\text{Cl}^-]_{\text{perf}} \times \text{CPR}) - ([\text{Cl}^-]_{\text{collected}} \times \text{CR})}{([\text{Cl}^-]_{\text{perf}} \times \text{CPRF})} \times 100 \\ &= \frac{([102.0725 \times 21.92] - [69.7153 \times 21.703])}{(102.0725 \times 21.92)} \times 100 \\ &= 32.4\% \end{aligned}$$