RENAL 11ß-HYDROXYSTEROID DEHYDROGENASE ACTIVITIES: PHARMACOLOGICAL INHIBITION AND PUTATIVE REGULATORS

A thesis submitted
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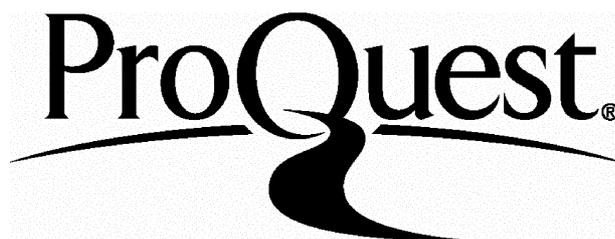
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Abstract

Isoforms of the enzyme 11ß-hydroxysteroid dehydrogenase (11ßHSD) catalyse the metabolism of glucocorticoids to inactive 11ketosteroids. In the kidney, the NAD^+-dependent type 2 isoform of 11ßHSD appears to confer aldosterone specificity on the mineralocorticoid receptor (MR). The physiological role for the $NADP^+$-dependent type 1 isoform of 11ßHSD, expressed in the proximal nephron of the rat, is uncertain.

Studies described in this thesis were designed to investigate: (a) the consequences of pharmacological inhibition of 11ßHSD for renal function, and (b) changes in 11ßHSD activity in response to dietary manipulations known to alter mineralocorticoid status.

Intravenous infusion of carbenoxolone (CBX; 6mg/hr) inhibited both $NADP^+$- and NAD^+-dependent 11ßHSD activities by >90% in the rat kidney. This inhibition was associated with reduced urinary excretion of sodium, although potassium excretion was unaffected. While sodium transport in the distal tubule was unaffected by CBX infusion, potassium secretion was significantly increased in this nephron segment. In a separate study, the administration of CBX to adrenalectomised rats inhibited $NADP^+$-dependent 11ßHSD activity by 80% while NAD^+-dependent glucocorticoid oxidation was unaffected, suggesting that adrenalectomy impairs the ability of CBX to inhibit renal glucocorticoid metabolism.

In the second series of studies, rats were maintained on either high potassium, low potassium or sodium diets, or NH_4Cl drinking water for 10 days. $NADP^+$-dependent 11ßHSD activities in renal homogenates were unchanged with any diet, whereas high potassium, low sodium and acidosis were associated with significant decreases in NAD^+-dependent glucocorticoid oxidation. Studies of the microdissected nephron demonstrated that dietary potassium loading tended to increase $NADP^+$-dependent glucocorticoid metabolism, but decreased NAD^+-dependent 11ßHSD activity in all nephron segments with the exception of the outer medullary collecting ducts: an important observation since the role of corticosteroids in this nephron segment is ill-defined. The data reported herein suggest important physiological roles for 11ßHSD activities in modulating renal electrolyte balance.

Acknowledgements

List of Abbreviations

11ßHSD	11ß-Hydroxysteroid dehydrogenase
11KSR	11keto-steroid dehydrogense
A	11 dehydro corticosterone
ACE	angiotensin converting enzyme
ACTH	adrenocorticotropic hormone
ADX	adrenalectomised
AME	apparent mineralocorticoid excess
aTL	thin ascending limb
AVP	vasopressin
B	corticosterone
CAMP	adenosine 3`,5`-cyclic monophosphate
CBG	corticosteroid binding globulin
CBX	carbenoxolone
CRH	corticotropin-releasing hormone
CCD	cortical collecting duct
cTAL	cortical thick ascending limb
DOC	deoxycorticosterone
DT	distal tubule
E	cortisone
ECF	extracellular fluid
ED	early distal tubule
EnaC	epithelial sodium channel
ER	endoplasmic reticulum
F	cortisol
GA	Glycyrrhizic acid
GE	Glcyrrhetinic acid
GFR	glomerular filtration rate
GR	glucocorticoid receptor
IMCDi	initial inner medullary collecting duct
IMCDt	terminal inner medullary collecting duct
LD	late distal tubule
MABP	mean arterial blood pressure
MR	mineralocorticoid
mTAL	medullary collecting duct
NAD^+	nicotinamide adenine dinucleotide
$NADP^+$	NAD^+ phosphate
OMCD	outer medullary collecting duct
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCT	proximal convoluted tubule
PGDH	prostaglandin dehydrogenase
PST	proximal straight tubule
RAS	renin-angiotensin-system
SCAD	short chain alcohol dehydrogense
TCO_2	plasma total carbon dioxide concentration
THE	tetrahydrocortisone
THF	tetrahydrocortisol
TLC	thin layer chromatography

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Chapter 1: Background

1. Introduction

Despite wide fluctuations in dietary intake, the composition of body fluids is maintained within narrow margins. The kidneys play a major role in such homeostasis by regulating the reabsorption of sodium and water (thus maintaining the extra cellular fluid (ECF) volume, the secretion of potassium (to regulate the resting potential of excitable cells) and proton secretion (to maintain optimum pH). In this context, tubular function itself is under the influence of circulating hormones and adrenal steroids have long been recognised for their important role in the regulation of renal electrolyte transport and acid-base balance.

The functional unit of the kidney, the nephron, consists of the glomerulus and its associated tubule (Figure 1.1). Nephrons are classified into superficial, midcortical and juxtamedullary nephrons according to the position of the glomerulus close to the surface, in the middle of the cortex or close to the medulla (Figure 1.1). Tubules are subdivided into different segments with respect to microanatomy and localisation within the kidney.

Advances in knowledge of renal transport mechanisms have arisen from the application of a wide variety of experimental techniques. The modern period of investigation into modes of electrolyte transport by the kidney began in the 1940's with the use of clearance methods. In such studies, comparisons are made between the filtered and excreted amounts of a substance, enabling examination of whole kidney function. The development of techniques for the collection and analysis of nanolitre samples of tubular fluid has allowed the direct study of single-nephron function, thus resolving some of the fundamental principles of renal physiology. During *in vivo* micropuncture studies, native tubular fluid is collected and compared between two specified tubule segments. With *in vivo* microperfusion studies, the tubule segment of interest is perfused with fluid of known composition and subsequent analysis of fluid collected at a more distal site will yield precise information about electrolyte handling at the tubular level. This technique can also be applied to isolated nephron segments otherwise inaccessible *in vivo*.

Figure 1.1: The structure of the nephron. 1-glomerulus; 2-proximal convoluted tubule; 3-thick ascending limb; 4-distal tubule; 5, 6 and 7-cortical, outer medullary, and inner medullary collecting ducts, respectively.

Superficial nephron

Juxtamedullary nephron

2

4

1

2

Cortex

4

5

1

3

Outer

6

Medulla

3

7

Inner

Medulla
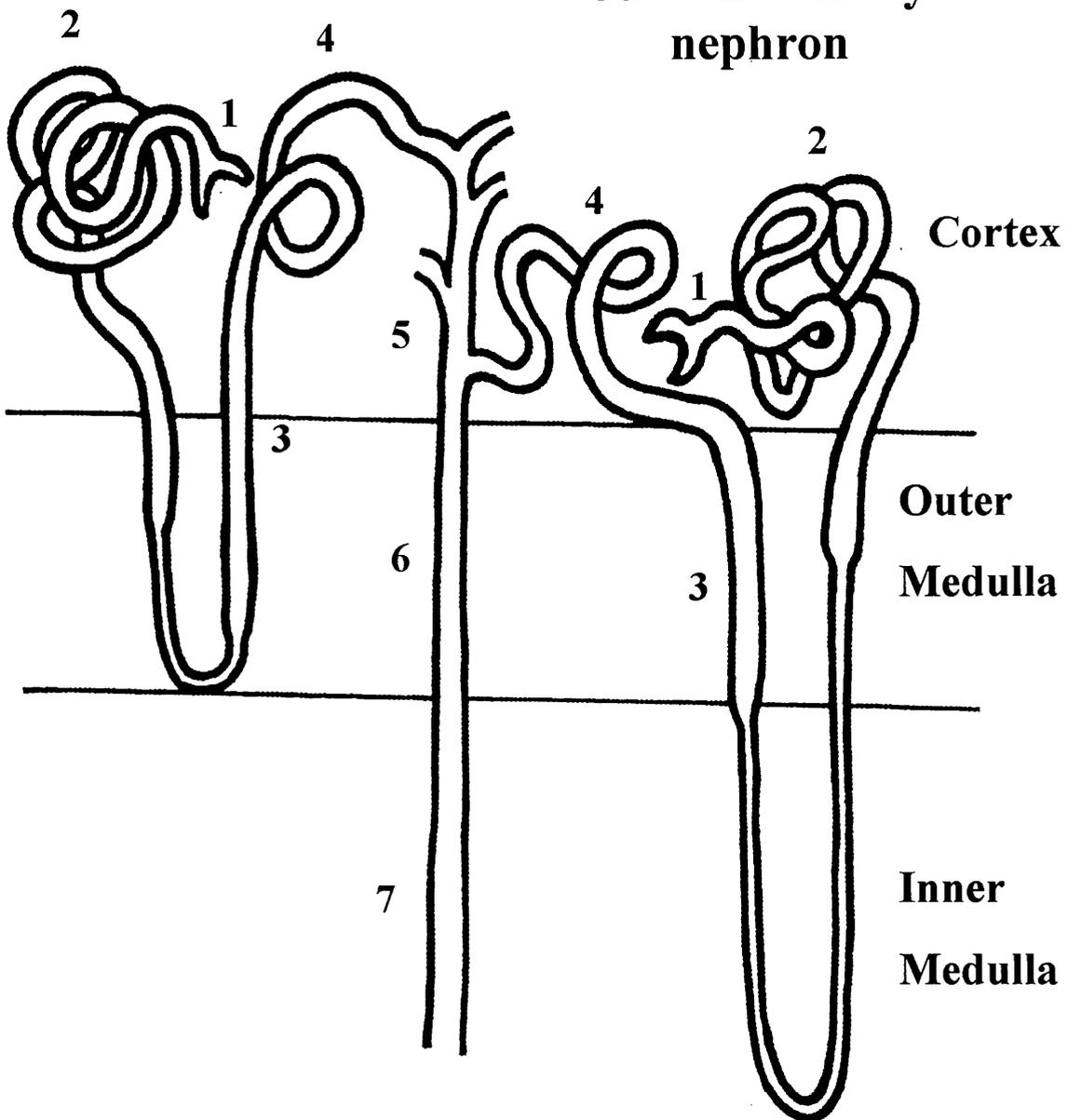
Figure 1.2:
Major electrolyte transport
mechanisms in the PCT.
'X' = glucose, amino acids
and sulphate.

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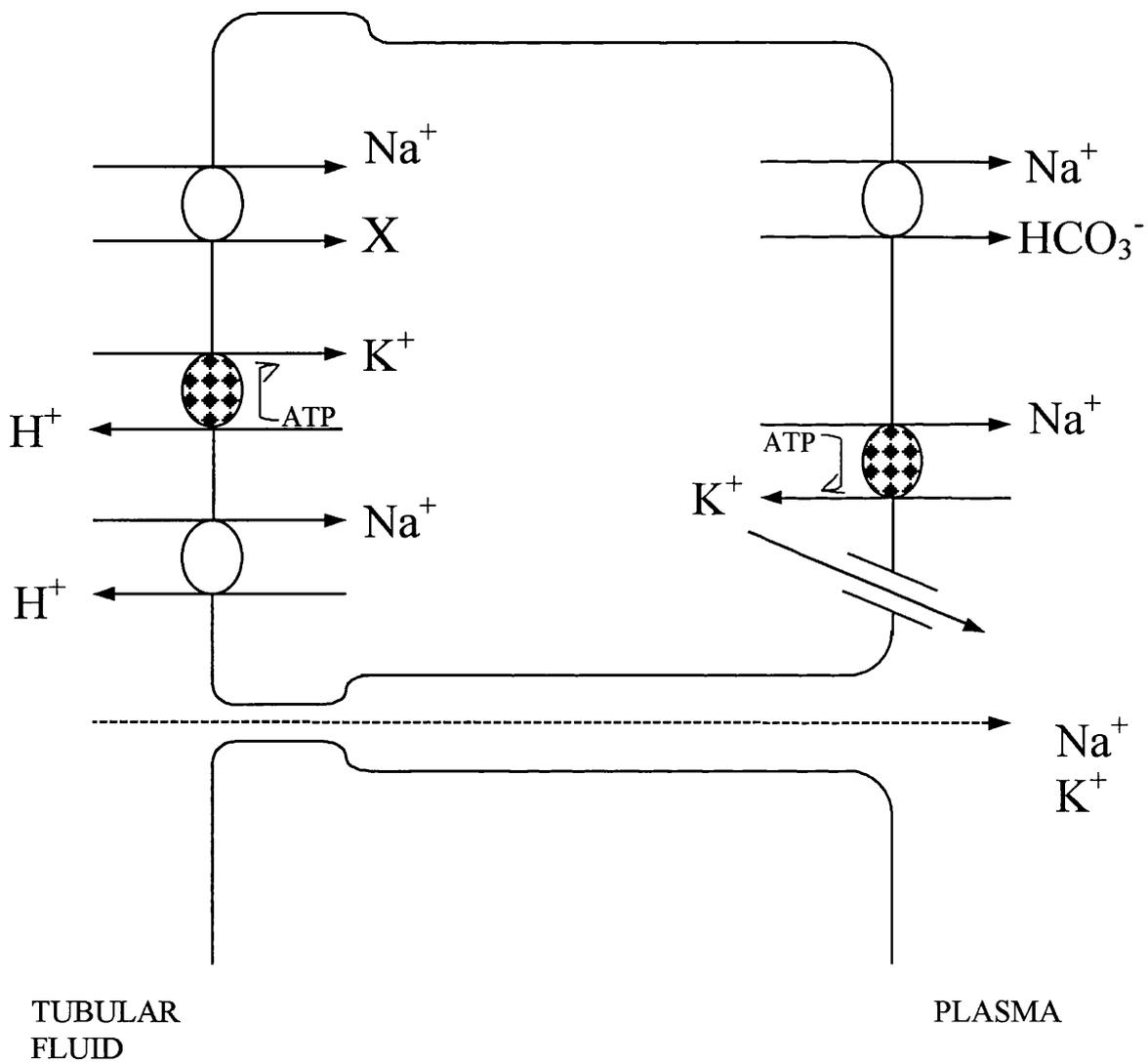
1.1. Transport mechanisms along the nephron

This thesis is predominantly concerned with sodium and potassium transport, and, to a lesser extent, acid-base balance. Hence, the transport mechanisms of these substances only, and their regulation by corticosteroids, are the focus of this introduction.

1.1.1. Proximal Tubule

The proximal tubule accounts for the reabsorption of 60-70% of the filtered loads of sodium, potassium and water. In addition, this nephron segment reabsorbs all of the filtered glucose and amino acids and in excess of 80% of the filtered bicarbonate.

Reabsorption of sodium in the proximal tubule is driven by Na^+/K^+-adenosine triphosphatase (Na^+/K^+-ATPase) in the basolateral membrane, extruding sodium and creating a favourable electrochemical gradient for coupled sodium entry across the apical membrane (Figure 1.2). This gradient is harnessed to the movement of other ionic species against their individual concentration gradients. The importance of the Na^+/K^+-ATPase has been clearly demonstrated: inhibition of this pump with ouabain reduces proximal reabsorption to zero and changes in sodium delivery are matched by almost simultaneous changes in pump activity (Morgunov and Boulpaep, 1987). Thus, the pump controls and balances the basolateral extrusion of sodium to its apical entry.

Mechanisms of sodium entry differ along the length of this segment. In the early proximal convoluted tubule (PCT), the sodium gradient is coupled to the co-transport of glucose, amino acids and sulphate although there is some contribution by sodium-hydrogen exchange. The majority of these transporters are electrogenic, and this depolarises the apical membrane with respect to the basolateral membrane, rendering the transepithelial potential

difference slightly lumen negative. Sodium is reabsorbed primarily as sodium bicarbonate in the early PCT. Proton secretion (via H^+-ATPase and the Na^+/H^+ antiporter) is utilised in the reclamation of filtered bicarbonate: secreted protons combine with bicarbonate in the tubular fluid to form H_2CO_3. This is rapidly converted to carbon dioxide and water in the presence of carbonic anhydrase at the apical membrane. The carbon dioxide diffuses into the cell, combines with the hydroxide that was generated in the production of the initial proton and, again in the presence of carbonic anhydrase, forms bicarbonate which exits the cell across the basolateral membrane. It is important to stress that the proximal tubule in no way acidifies the tubular fluid since there is no net proton secretion.

In both the late PCT and the proximal straight tubule (PST), reabsorption of glucose and other substrates is largely complete and sodium-hydrogen exchange is the predominant mechanism of sodium entry into the cells lining this segment. The preferential reabsorption of bicarbonate and the relative impermeabilty of the early PCT to chloride causes the concentration of chloride in the tubular fluid to rise. In the latter sections of the proximal tubule, this concentration gradient is sufficient to drive passive, transcellular chloride reabsorption. Movement of chloride down this gradient, and the low permeability to bicarbonate backflux cause a reversal of the transepithelial potential difference, (which has been recorded as lumen-positive by 2mV). This creates a favourable electrochemical gradient for paracellular sodium reabsorption. It is generally accepted that approximately a third of sodium reabsorption in the proximal tubule occurs in this way.

The mechanisms of potassium reabsorption in the proximal tubule are less clear. Although in recent years, an isoform of H^+/K^+-ATPase has been found to be expressed in the proximal tubule of the rat, the functional significance of these findings remains unknown: *in vivo* microperfusion of the proximal tubule with a solution containing Sch28080, an inhibitor of H^+/K^+-ATPase, was found not to affect potassium reabsorption in this segment (Wilson *et al*, 1997). Nevertheless, potassium reabsorption can be accounted for solely on the

basis of passive transport: solvent drag, by which the movement of water entrains that of electrolytes, is thought to be especially important for potassium (Figure 1.2). Moreover, the tubular fluid:plasma potassium concentration ratio for fluid harvested from the end of the PCT exceeds unity. Coupled with the lumen-positive potential difference measured in the PST, the electrochemical driving force favours potassium reabsorption.

1.1.2. The Loop of Henle

The loop of Henle is an heterogeneous nephron segment, consisting of the proximal straight tubule (pars recta), the thin descending limb (tDL), ascending thin limb (aTL; juxtamedullary nephrons only) and the thick ascending limb (TAL).

Sodium and water reabsorption in the pars recta is qualitatively, if not quantitatively, the same as that in the proximal convoluted tubule (see above). The tDL is highly permeable to water and may reabsorb as much as 20% of the filtered load. This reabsorption occurs due to the hyperosmolarity of the surrounding interstitium. Electrolyte handling in the tDL is poorly understood. Studies have indicated the presence of sodium, potassium and chloride ion channels in this nephron segment, the function of which remain elusive. However, concentration gradients favour secretion of electrolytes into the tubular fluid and no active transport has been detected. The aTL is highly permeable to both sodium and potassium and reabsorption is considered to be passive. This segment is, however, impermeable to water.

Not only does the rat TAL account for 20-25% of the reabsorption of filtered sodium but it has a very large reserve capacity, ie, sodium reabsorption increases with delivery, thus compensating for any failure in the proximal tubule. For this reason, acetazolamide, an inhibitor of carbonic anhydrase, is not very effective as a diuretic. As in most segments of the nephron, basolateral Na^+/K^+-ATPase activity maintains the electrochemical driving force for sodium entry; in this segment the sodium gradient is coupled to the

Figure 1.3:
Major electrolyte transport
mechanisms in the TAL.

Na^+
$2Cl^-$
K^+

$2Cl^-$

Na^+ ATP

K^+

Na^+
K^+
Ca^{2+}
Mg^{2+}

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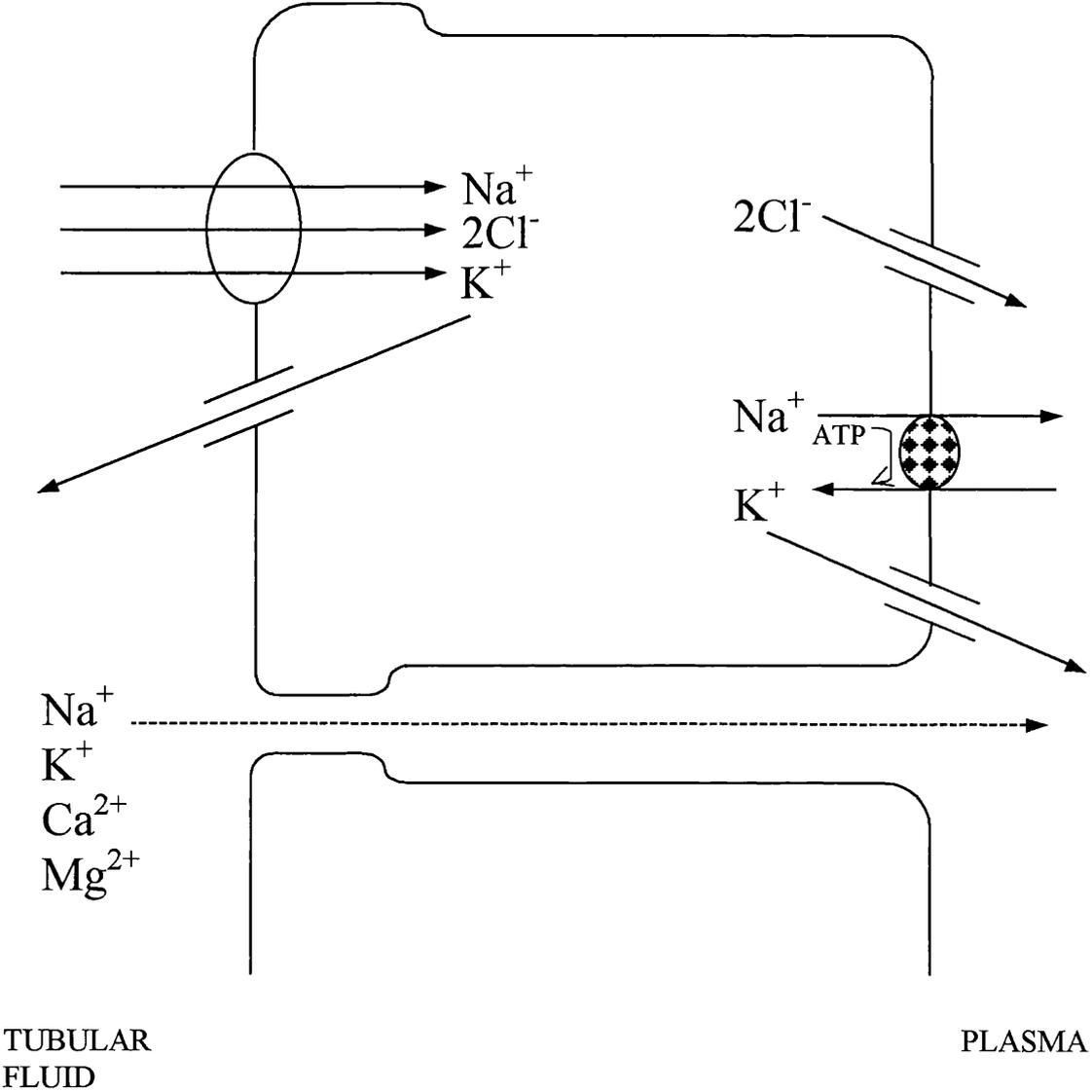
co-transport of potassium and chloride (Figure 1.3). The Na-2Cl-K triple co-transporter is the site of action of loop diuretics, such as bumetanide and furosemide. Sodium exits across the basolateral membrane in the usual way, and chloride exit is facilitated by specific ion channels in the basolateral membrane, although the presence of potassium-chloride co-transport has been suggested. The fate of potassium is less clear. The delivery of sodium chloride to the triple co-transporter is an order of magnitude greater than that of potassium. Given that the 1:2:1 ratio for sodium:chloride:potassium binding is essential for the conformational change resulting in ion translocation, then a proportion, if not all of the potassium that enters the cell by this pathway is obliged to recycle across the apical membrane by means of specific ion channels. Studies, both *in vitro* (Greger and Schlatter, 1981) and *in vivo* (Wang *et al*, 1995; Walter *et al*, 1997), in which apical potassium conductances are blocked, using barium or glibenclamide for example, clearly demonstrate an inhibition of sodium reabsorption in the thick ascending limb.

Nevertheless, the TAL is an important site for potassium reabsorption, which appears paradoxical if potassium is recycled. However, this recycling confers electrogenicity onto the intrinsically electroneutral triple co-transporter, generating a lumen-positive potential difference of approximately 10mV. Cations, notably potassium, magnesium and calcium are driven by this gradient through the paracellular shunt pathway (Figure 1.3).

The TAL, like the aTL before it, is impermeable to water. The separation of sodium and water transport in this segment is of vital importance in the fine control of water balance. By the removal of sodium chloride and consequent dilution of the tubular fluid, the medullary interstitium is rendered hypertonic. This provides the potential for subsequent concentration of the urine.

In the rat, approximately 10 to 20% of filtered bicarbonate is absorbed between the late proximal and the early distal tubule, indicating that the loop of Henle absorbs most of the bicarbonate that escapes the PCT. Studies with

isolated, perfused cortical and medullary TAL (cTAL and mTAL, respectively) have shown that these segments absorb bicarbonate at rates comparable to values measured in the cortical and medullary collecting ducts (Good, 1990) Furthermore, bicarbonate absorption by isolated cTAL and mTAL fully account for the absorption of bicarbonate by the entire loop of Henle perfused *in vivo.*

Absorption of bicarbonate by the TAL occurs against both chemical and electrical driving forces and, therefore, is an active process. As in other nephron segments, transcellular bicarbonate absorption involves secretion of protons across the apical membrane and efflux of bicarbonate across the basolateral membrane. In addition, the TAL facilitates the excretion of net acid by reabsorbing ammonium, causing the accumulation of this species in the renal medulla and thus enhancing secretion via the collecting ducts. The sequential abilities of the thin descending limb to absorb water in excess of bicarbonate and of the TAL to actively absorb bicarbonate provide an efficient mechanism that enables the loop of Henle to reclaim most of the filtered bicarbonate that escapes the proximal tubule.

1.1.3. The Distal Tubule

The distal tubule is a short nephron segment connecting the thick ascending limb of Henle to the collecting duct system. It is comprised of three segments: the early distal tubule, investigated *in vivo* micropuncture studies, consists of the distal convoluted tubule (DCT), connecting tubule and the initial collecting tubule (ICT). In the two former segments, sodium chloride reabsorption is mediated by apical Na-Cl co-transport (site of action of thiazide diuretics) coupled to basolateral exit via the Na^+/K^+-ATPase and through chloride ion channels (Costanzo, 1985; Ellison *et al*, 1987). There is little or no water transport in this segment.

1.1.4. The Collecting ducts

The late distal tubule is the ICT and is both anatomically and functionally very similar to the cortical collecting duct (CCD) which it precedes. Thus, the two segments will be considered together. It is the CCD which is thought to be the major site of mineralocorticoid action.

Although in percentage terms, the collecting duct system appears relatively unimportant with regard to whole kidney water and electrolyte balance, it is here that the final composition of the urine is determined. Consequently, it is the principal site of action of many hormones, which fine tune solute handling in response to prevailing systemic conditions. Indeed, up to 10% of the filtered sodium and water is reabsorbed here, which for the latter corresponds to 18 litres per day in humans. Moreover, the collecting ducts are important sites for the secretion of potassium and protons; 0-150% of the filtered potassium can be excreted in the final urine, and urine pH can be up to two units lower than that of the tubular fluid leaving the loop of Henle.

The CCD, outer medullary collecting duct (OMCD) and first third (initial part) of the inner medullary collecting duct ($IMCD_i$) are composed of two distinct cell types: the principal cell (accounting for around 60% of the cell population in each segment) mediates sodium, potassium and water transport, whilst the intercalated cell is involved in the acidification of tubular fluid.

1.1.4.1. Principal cells

In the current section, the actions of the principal cells are described with particular reference to the CCD, since this is the collecting duct segment in which the transport mechanisms of this cell predominates. However, the general transport mechanisms and their regulation by corticosteroids are also applicable to both the OMCD and $IMCD_i$.

Electrophysiologically, the CCD is characterised by a large, lumen-negative transepithelial potential difference that arises due to the relative

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Figure 1.4:
Major electrolyte transport
mechanisms in the Principal Cell.

Na^+

K^+

Na^+ ATP

K^+

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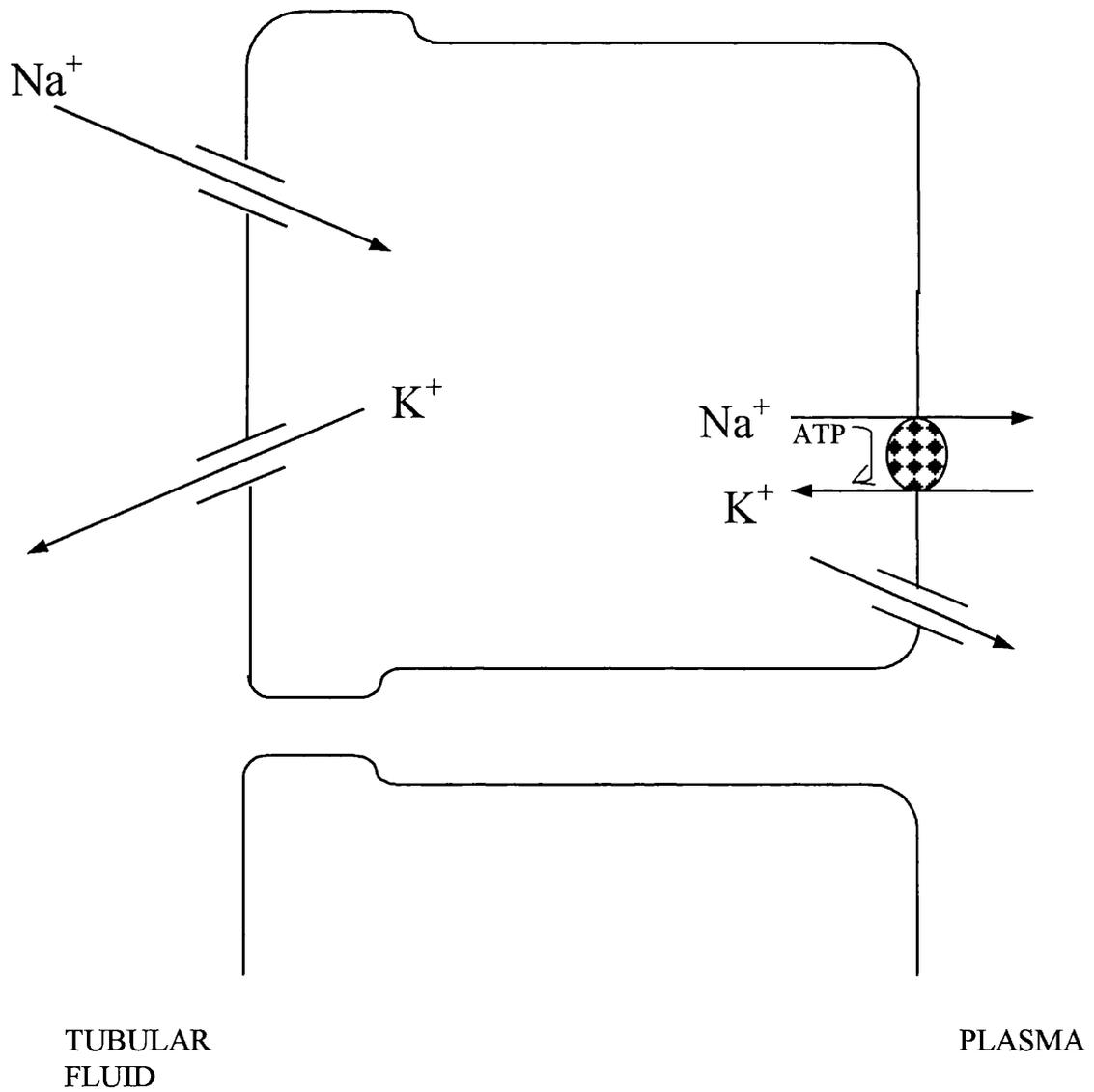
depolarisation of the apical membrane of the principal cell with respect to its basolateral membrane. The apical membrane is depolarised due to the large sodium permeability: sodium enters the cell down its electochemical gradient through specific epithelial sodium channels (ENaC). It is the sensitivity of these channels to amiloride that gives that compound diuretic activity.

Both the apical and basolateral membranes contain potassium channels (Figure 1.4), but their relative open probability favours net secretion of this cation into the tubular lumen. It is commonly believed that sodium reabsorption is linked to potassium secretion as when the former is stimulated, by increased delivery for example, so is the latter. This link is indirect and results in part from the voltage-sensitivity of apical potassium channels: increased sodium reabsorption further depolarises the apical membrane causing potassium channels to open. This increase in potassium permeability returns the membrane potential towards the potassium equilibrium potential and thus maintains the electrochemical gradient for sodium reabsorption. Nevertheless, in certain circumstances, the connection between sodium and potassium transport is broken. Dietary potassium depletion reduces secretion to very low levels, yet sodium transport is unaffected.

As previously stated, the triple co-transporter in the TAL generates a hyperosmotic medullary interstitium and renders the fluid that enters the early DCT hypotonic. The anatomical arrangement of the kidney dictates that this hypotonic fluid is redirected down through an increasingly concentrated interstitium. Thus, there is a large osmotic gradient for water transport out of the collecting duct and it is the action of vasopressin (AVP) that controls this water movement. In the absence of AVP, the collecting duct is water impermeable and thus the dilute tubular fluid passes through unaffected. If AVP is present in the circulation, however, the collecting duct is permeable and a concentrated urine is formed. AVP binds to receptors (V2-type vasopressin receptors) in the basolateral membrane of the CCD. This activates adenylate cyclase to form cyclic adenosine 3',5'-cyclic

monophosphate (cAMP). cAMP activates protein kinase A, and the phosphorylation of proteins still undefined, causes vesicles that contain pre-formed water channels to fuse with the apical membrane. The result is a 10- to 20-fold increase in water permeability of the apical membrane of the CCD. Upon removal of AVP, water channels are recycled into the cell interior by an endocytic pathway. A family of water channels called aquaporins has recently been identified and cloned. One of these aquaporins, aquaporin 2, is selectively found in the apical membranes and subapical vesicles of CCD cells and is likely to represent the AVP-stimulated water channel (Schnermann and Sayegh, 1998).

1.1.4.2 Intercalated cells

In the proximal nephron there is little net proton secretion. Movement of protons across the apical membrane provides a mechanism for the reclamation of filtered bicarbonate and relies on the presence of carbonic anhydrase. Despite such avid bicarbonate reabsorption, the generation of large amounts of non-volatile acids from the metabolism of dietary protein is sufficient to cause a metabolic alkalosis unless new bicarbonate is generated. This is performed in the collecting ducts. In the presence of carbonic anhydrase, hydroxide combines with generated carbon dioxide to form bicarbonate which is subsequently reabsorbed across the basolateral membrane. This leaves a proton, which must be removed from the intercalated cell. By one of the mechanisms described below, protons are secreted into the tubular fluid and combine with buffers such as Na_2HPO_4 or ammonia before secretion into the urine. The net result is the generation of a new bicarbonate ion for every proton thus excreted. This process occurs in the intercalated cells.

Intercalated cells can be further subdivided. For the sake of simplicity only two of these groups will be discussed, although classification on the basis of H^+-ATPase localisation suggests that there can be as many as 5 types. The α-intercalated cell displays dense arrays of H^+-ATPase in the apical membrane. These pumps are capable of generating an electromotive force

Figure 1.5:
Major electrolyte transport
mechanisms in α- and β- intercalated cells

α)

β)

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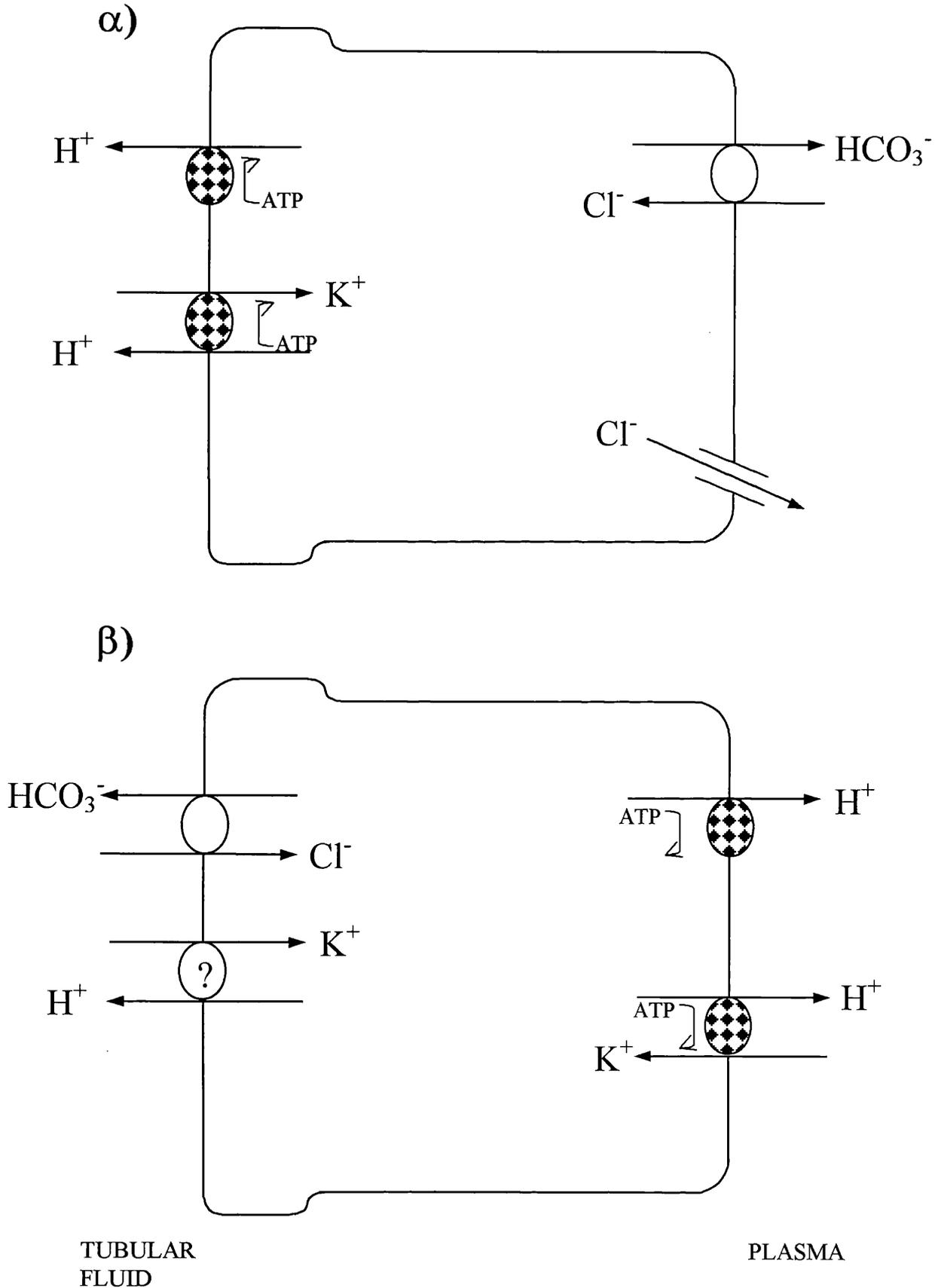
equivalent to 2 pH units and thus there is a large capacity for proton secretion in this segment. The α-intercalated cell is abundant in OMCD but also present in CCD and IMCD$_i$, in which segments it is responsible for proton secretion and bicarbonate reabsorption (Figure 1.5). The β-intercalated cell, situated in CCD only, has the reverse polarity and thus mediates bicarbonate secretion (Figure 1.5). The rat CCD is comprised of approximately equal amounts of both α- and β-intercalated cells.

Despite the importance of the OMCD in urine acidification and its regulation by mineralocorticoids, a striking feature of this nephron segment is the gradual disappearance of active transport of sodium and potassium (Stokes, 1990). In the rat, the α-intercalated cells in the OMCD have been demonstrated to secrete protons in a sodium-independent electrogenic process. Until recently it was thought that any potassium reabsorption by the OMCD occurred passively; via the paracellular pathway. However, functional and structural evidence has indicated that the collecting duct possesses at least one, and probably more than one, isoform of a proton and potassium activated ATPase (H^+/K^+-ATPase). This transporter was initially characterised in hypokalaemic animals (Wingo, 1989) and its expression thought to be limited to situations demanding potassium retention. Recent studies, however indicate that H^+/K^+-ATPase, albeit a different isoform, is expressed in the CCD and OMCD of potassium-replete animals (See Wingo and Smolka, 1995).

The IMCD consists of 2 subsegments with different cell types. The cellular composition of the initial IMCD (IMCD$_i$) is similar to that of the earlier parts of the collecting duct; both principal and α-intercalated cells are present. The terminal part of the IMCD (IMCD$_t$), appears to consist only of one cell type, the IMCD cell. The IMCD has been shown to respond to an acute metabolic acidosis by enhanced proton secretion, bicarbonate reabsorption and ammonium secretion. Conversely, chronic metabolic alkalosis suppresses the secretion of protons (although this does not induce bicarbonate secretion) along this region of the collecting duct. In contrast to the OMCD, the IMCD is
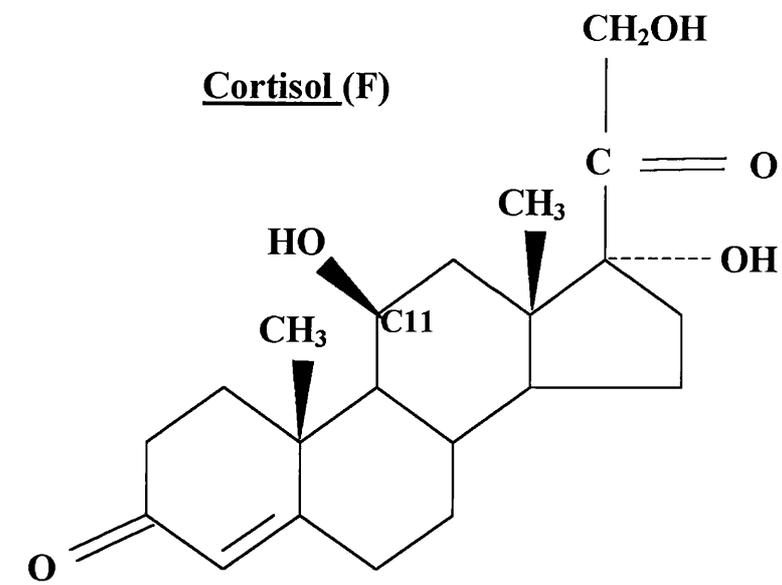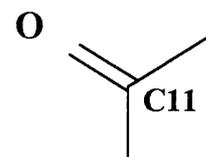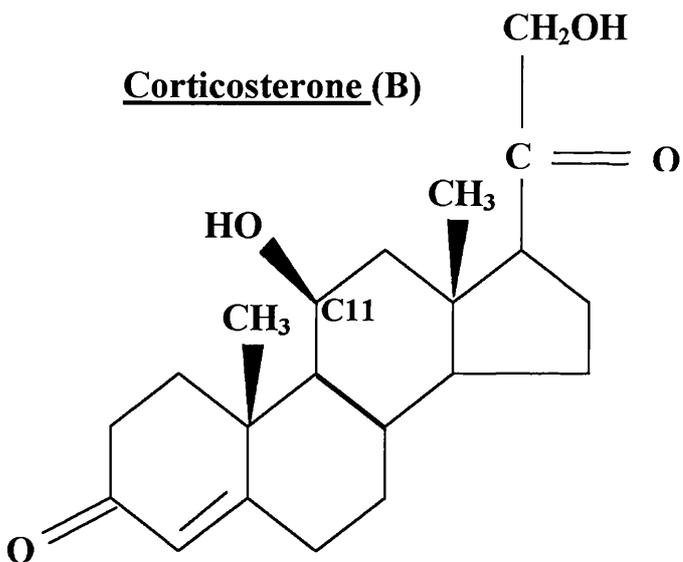
Figure 1.6: Structures of cortisol (F), corticosterone (B), 11-dehydrocorticosterone (A) and aldosterone

thought to be involved not only in acid-base balance, but in sodium and chloride transport. The majority of sodium transport appears to occur via entry across the apical membrane through an amiloride-sensitive sodium channel and exit across the basolateral membrane via the Na^+-K^+ pump. However, despite the reappearance of sodium transport in the terminal portion of the collecting duct, net potassium transport by the IMCD is not thought to occur.

1.2 Adrenal steroids

The hormones of the adrenal cortex are derivatives of cholesterol, containing the cyclopentanoperhydrophenanthrene nucleus (Figure 1.6). The adrenocortical steroids are of 2 structural types: those that have a two carbon side-chain attached at position 17 of the D ring and contain 21 carbon atoms ("C_{21} steroids"), and those that have a keto or hydoxyl group at position 17 and contain 19 carbon atoms ("C_{19} steroids"). The C_{19} steroids have androgenic activity whilst the C_{21} steroids are classified as either mineralocorticoids or glucocorticoids. All secreted C_{21} steroids, otherwise known as corticosteroids, have both mineralocorticoid and glucocorticoid activity; traditionally, mineralocorticoids are those in which effects on sodium and potassium excretion predominate, and glucocorticoids those in which effects on carbohydrate and protein metabolism predominate.

1.2.1. Corticosteroid Synthesis

In all species from amphibia to humans, the major corticosteroid hormones secreted by the adrenocortical tissue appear to be aldosterone, deoxycorticosterone (DOC), cortisol (F) and corticosterone (B), although the ratio of cortisol to corticosterone varies. Birds, mice and rats secrete corticosterone almost exclusively, dogs secrete roughly equal amounts of the two glucocorticoids, and cats, sheep and primates secrete predominantly cortisol.

Figure 1.7: Adrenal steroid synthesis pathways. Enzymes mediating each conversion are italicised.

17α-hydroxylase

C17,20-lyase

CHOLESTEROL

Cholesterol side chain cleavage
(CYP11A)

PREGNENOLONE → 17α-HYDROXY-PREGNENOLONE → DEHYDROEPIANDOSTERONE

3β-hydroxysteroid dehydrogenase/
Δ⁴, Δ⁵ isomerase
(HSD 3β2)

PROGESTERONE → 17α-HYDROXY-PROGESTERONE

21-hydroxylase
(CYP21)

21-hydroxylase

DEOXY-CORTICOSTERONE

11-DEOXYCORTISOL

11β-hydroxylase
(CYP11β1)

CORTICOSTERONE

CORTISOL

18-hydroxylase
(CYP11β2)

18-HYDROXYCORTICOSTERONE

Aldosterone synthase
(CYP11β2)

ALDOSTERONE

The adrenal gland consists of an inner medulla which synthesises catecholamines and an outer cortex, responsible for the production of steroids. The division of the cortex into three distinct zones is an important feature of the adrenal gland, since the zones produce different steroids. The outer layer of cells in the zona glomerulosa synthesises aldosterone, the zona fasciculata produces mainly cortisol (or corticosterone in myomorph rodents) and the zona reticularis secretes adrenal C_{19} steroids. Figure 1.7 summarises the pathways of steroid biosynthesis occurring in the adrenal cortex. Cholesterol, the starting point for all steroid hormone biosynthesis is obtained mostly from circulating low-density lipoprotein. As shown in Figure 1.7, cholesterol is modified by a series of hydroxylation reactions. Five of the enzymes (CYP11A, CYP17, CYP21, CYP11B1 and CYP11B2) are members of the cytochrome P450 superfamily of haemoproteins. The enzymes of the CYP11 family are located in the mitochondria and the remainder are located in the endoplasmic reticulum (ER), so the substrates have to move around the cell for the process of steroidogenesis to be complete.

The hypothalamus plays an important stimulatory role in regulating the secretion of adrenocorticotropic hormone (ACTH); which in turn stimulates the synthesis of glucocorticoids by the adrenal gland. Following secretion, corticotropin-releasing hormone (CRH) is transported via the portal hypophyseal vessels from the hypothalamus to the anterior pituitary gland where it stimulates the secretion of ACTH. ACTH interacts with G-protein-linked cell surface receptors in the zonae fasciculata and reticularis of the adrenal gland, acting via adenylate cyclase to stimulate the production of cortisol by increasing cholesterol side-chain cleavage to form pregnenolone (Figure 1.7). The end product of the action of ACTH, namely cortisol/corticosterone, has a direct inhibitory action on the pituitary. It also exerts a negative feedback action on the hypothalamus, to inhibit the release of CRH.

In the resting state, basal levels of CRH, ACTH and cortisol/corticosterone are released in a pulsatile and circadian fashion. At these baseline concentrations, the main functions of glucocorticoids are to sustain euglycaemia and to prevent arterial hypotension. Activation of the HPA axis during physical and/or emotional stress and the resulting increase in plasma glucocorticoid concentrations are required for central nervous system activation, higher blood glucose concentrations, and an elevated mean blood pressure in the stress state.

Aldosterone is produced exclusively in the zona glomerulosa and is the most potent mineralocorticoid produced by the adrenal. The renin-angiotensin system (RAS) is the predominant regulator of the secretion of aldosterone. Renin is secreted from the juxtaglomerular cells that surround the renal afferent arterioles as they enter the glomeruli. Aldosterone secretion is regulated via the RAS in a negative feedback loop. A drop in ECF volume or intra-arterial vascular volume leads to a reflex increase in renal nerve discharge and decreases renal arterial pressure. Both changes increase renin secretion. Renin, a proteolytic enzyme, cleaves angiotensin I from a circulating $\alpha2$-globulin, angiotensinogen. Angiotensin I is then converted in several tissues (most notably the lungs) to angiotensin II in the presence of angiotensin converting enzyme (ACE). Angiotensin II acts directly on the zona glomerulosa cells to stimulate aldosterone secretion, which, in turn, stimulates sodium and water reabsorption, expanding ECF volume and terminating the stimulus that initiated increased renin secretion. Potassium and ACTH can also directly stimulate the rate of synthesis of aldosterone by the glomerulosa cells. DOC is a mineralocorticoid that is normally secreted in about the same amount as aldosterone but has only 3% of the mineralocorticoid activity of aldosterone. Its synthetic acetate (desoxycorticosterone acetate, DOCA) is used clinically as a mineralocorticoid.

1.2.2. Corticosteroid Receptors

In 1973, two studies were employed to establish the binding characteristics of corticosteroid receptors in the kidneys of adrenalectomised rats. In the first study, the binding of [^{3}H]-aldosterone and [^{3}H]-dexamethasone was investigated *in vitro* (Funder *et al* 1973a); a simultaneous study in the same laboratory investigated the binding characteristics following *in vivo* infusion of the same two tracers (Funder *et al*, 1973b). Several conclusions were drawn from these studies. First, the higher affinity (Type I) aldosterone binding sites had equivalent affinity for aldosterone and DOC, but much lower affinity for corticosterone and dexamethasone, and, as such, these sites were taken to represent physiological mineralocorticoid receptors (MR). Second, the lower affinity (Type 2) aldosterone binding sites were shown to bind dexamethasone better than corticosterone, and corticosterone better than aldosterone and, on the basis of this specificity profile, were postulated to be the physiological glucocorticoid receptor (GR).

1.2.3 Molecular mechanisms of corticosteroid action

Similar to other steroid hormones, aldosterone binds to its cytosolic receptor of target cells and induces a change in the oligomeric structure of the receptor which involves dissociation from the heat shock protein (hsp) 90 (Rafestin-Oblin *et al*, 1989). This modification, which is termed receptor transformation, allows the activated hormone-receptor complex to translocate to the nucleus, bind to specific DNA sequences [hormone response elements (HRE)], and modulate the transcriptional activity of specific genes (Evans, 1988, Beato, 1989). The synthesis of several proteins, collectively known as the aldosterone-induced protein, is then induced, and these modulate the transport of various ions, including sodium, potassium and protons. GR activation is similar in many ways: ligand binding causes dissociation of the GR/hsp90 complex and nuclear translocation of the ligand-receptor complex. Within the nucleus, the activated GR binds to glucocorticoid response elements (GRE) and can either induce or suppress the transcription of target

genes or affect the stability of mRNA.

Steroid nuclear receptors are composed of different domains, including the ligand binding domain and the DNA binding domain, which display both amino acid and functional homology. In particular, the GR and MR share more than 90% amino acid homology in the central cysteine region of the protein. Hormone binding to the MR is followed by receptor activation, which is a result of conformational changes and phoshorylation events. Once in the nucleus, MR modulates transcription by binding as homodimer to DNA sequences. Recent data demonstrate that MR is also able to heterodimerise with GR, leading to either synergy or inhibition of transactivation depending on the promoter and cellular context. This is particularly important when considering that MR and GR are coexpressed in several tissues.

1.3. Regulation of renal electrolyte transport by corticosteroids

The effects of corticosteroids on renal electrolyte transport, which are nephron specific, are summarised in Table 1.1, and described in detail in specific nephron segments below.

Table 1.1. A summary of the effects of adrenal steroid on electrolyte transport in the proximal convoluted tubule (PCT), loop of Henle (LOH), distal convoluted tubule (DCT), cortical collecting duct (CCD) and outer and inner medullary collecting ducts (OMCD and IMCD, respectively). ↑ denotes simulation; 0 denotes no effect; ? denotes controversial or unstudied to date. See text for references.

Nephron segment & Transport Mech	Mineralocorticoids	Glucocorticoids
PCT		
Na^+/K^+-ATPase	0	↑
Na/HCO_3^- exchange	0	↑
Na/H exchange	?	↑
LOH		
Na^+/K^+-ATPase	?	↑
Na^+ reabsorption	↑	?
K^+ reabsorption	↑	↑
Na/H exchange	↑	?
HCO_3^- reabsorption	?	↑
DCT		
Na^+ reabsorption	↑	?
CCD		
Na^+/K^+-ATPase	↑	↑
ENaC	↑	?
K^+ secretion	↑	↑
OMCD		
H^+-ATPase	↑	?
H^+ secretion	↑	?
Cl^-/HCO_3^- exchange	↑	?
H^+/K^+ATPase	?	?
IMCD		
Na^+ reabsorption	↑	↑
H+ secretion	↑	?
HC03- reabsorption	↑	?

1.3.1. Proximal convoluted tubule

Initial micropuncture studies indicated that the proximal tubule was responsive to aldosterone (Hierholzer, 1975). Although a direct effect of aldosterone on the proximal tubule cannot be ruled out entirely, subsequent studies suggest that sodium and potassium reabsorption in this segment (which amount to 60-70% of the filtered load), are not regulated by aldosterone. This is supported by two lines of evidence. First, there are very few, if any, MR in the proximal tubule (Doucet *et al*, 1981, Vandewalle *et al*, 1981, Farman *et al*, 1982). Second, adrenalectomy and dietary modulation of aldosterone levels have no effect on sodium reabsorption in the isolated perfused proximal tubule of the rabbit (Knepper, *et al*, 1981). It is possible that the effects of pharmacological doses of mineralocorticoids on sodium transport in early studies resulted from hormone binding to GR in the proximal tubule (Marver *et al*, 1984).

In contrast to the failure of any of the above techniques to localise MR to the proximal tubule, it is generally accepted that GR are present in the proximal tubule (Doucet *et al*, 1981; Mishina *et al*, 1981; see Marver *et al*, 1984). Despite several studies reporting the absence of immunostaining with GR antibody in the proximal tubule (See Farman *et al*, 1992), functional studies performed in this nephron segment support the view that GR are indeed present in the proximal tubule.

Both acute and chronic dexamethasone administration have been shown to restore Na^+/K^+-ATPase activity in the PCT of adrenalectomised rats and rabbits (Charney *et al*, 1974); an effect which was not blocked by spironolactone (an MR antagonist). Studies of sodium-dependent phosphate transport in isolated brush border membrane from the renal proximal tubule have demonstrated that dexamethasone, when administered at physiological doses, reduces sodium-dependent phosphate transport by about 30% (Freiberg *et al*, 1982). At a higher dose of dexamethasone, administered for

the same period, there was greater than a 50% reduction in transport activity (Freiberg *et al*, 1982). In both these studies (Charney *et al*, 1974; Freiberg *et al*, 1982), the administration of either acute or chronic glucocorticoid hormones to adrenal-intact animals did not alter 'base-line' Na^+-K^+-ATPase activity, indicating that the effects mediated via GR may only be permissive.

In terms of acid-base balance, glucocorticoids appear to play a role in acid-base homeostasis, by increasing acid production and net ammonia and acid excretion. The proximal tubule appears to be largely involved in the 2 former processes. In a recent study utilising microperfusion techniques, the administration of physiological doses of dexamethasone to rats increased bicarbonate reabsorption in the proximal tubule by 54% compared with control animals (Ruiz *et al*, 1995). Consistent with this, glucocorticoid hormones have been shown to stimulate both Na^+/H^+ antiporter and Na^+-HCO_3^- cotransporter activity in cultured proximal tubules (Damasco and Malnic, 1987, Cardoso 1996). Furthermore, in the same cells, the presence of cortisol increased Na^+-HCO_3^- cotransport activity, whereas aldosterone was without effect (Cardoso *et al*, 1995).

Thus, although mineralocorticoids do not appear to have any regulatory function in the proximal tubule, the presence of GR and GR-specific mediated transport processes suggests a role for glucocorticoids in the proximal tubule, although the importance of this regulation under normal physiological conditions is unknown.

1.3.2. The Loop of Henle

Patients with Addison's disease (a syndrome of adrenal insufficiency) and animals deficient in adrenal corticosteroid hormones have a decreased ability to generate a maximally concentrated urine (See Dietl *et al*, 1990). Free-flow micropucture studies (Cortney, 1969, Welch *et al*, 1985) and *in vivo* microperfusion techniques in rats (Murayama *et al*, 1968, Stanton, 1986) have shown that adrenalectomy reduces net sodium chloride reabsorption by

the loop of Henle, thereby increasing the sodium concentration of fluid entering the early distal tubule. Since absorption of sodium along the water-impermeable TAL is responsible for reducing the sodium concentration in the early distal tubule below that in plasma, it can be inferred from these studies that the defect in loop NaCl absorption was located, at least in part, in the TAL. Likewise, continuous microperfusion studies of the rat loop of Henle have demonstrated that both potassium (Stanton, 1986) and bicarbonate reabsorption (Unwin et al, 1995) were reduced following adrenalectomy. It is likely that the reported effects of adrenalectomy on electrolyte transport in the loop of Henle are a result of the loss of both mineralocorticoids and glucocorticoids as discussed below.

MR have been identified in cortical and medullary segments of rabbit TAL by autoradiographic detection of [^{3}H]-aldosterone-receptor complexes in tubular segments isolated by microdissection in the rabbit (Vandewalle et al, 1981, Farman et al, 1982) and in the rat (Farman et al, 1983). Although binding studies did not detect aldosterone-binding sites in rabbit TAL (Doucet and Katz, 1981), the obtention of an antiidiotypic antibody that recognises the steroid binding domain of MR (Lombes et al, 1989 and 1990) enabled the examination of the intrarenal localisation of MR, regardless of the presence or absence of endogenous ligand. Immunocytochemical studies performed by Rundle et al (1989), not only confirmed the presence of MR in the TAL of rats and humans, but showed new target cells for aldosterone: thin limbs of the loop.

In two studies using free-flow micropuncture techniques (Murayama et al, 1968, Hierholzer and Lange, 1974) the acute administration of a large dose of aldosterone to adrenalectomised rats decreased early distal tubule sodium concentration, consistent with a stimulation of sodium absorption in the loop of Henle. Conclusions drawn from both studies, however, were limited by the use of large doses of aldosterone, since changes in sodium concentration could have been mediated via MR or GR (or both). These complications were eliminated in a subsequent study in which adrenalectomised rats received

physiological doses of aldosterone for 10 days after which time sodium transport rates were measured in loop segments by continuous microperfusion *in situ* (Stanton, 1986). Stanton demonstrated that this physiological dose of mineralocorticoid reduced early distal sodium delivery (presumably by increasing sodium reabsorption by the loop of Henle) to levels observed in sham-operated animals. Consistent with this finding, chronic physiological replacement with aldosterone in adrenalectomised rats also restored to control levels the NaCl absorptive capacity of the mTAL isolated and perfused *in vitro* (Work and Jamison, 1987). Although there is a clear association between plasma aldosterone and NaCl reabsorption in the TAL, none of above studies show *direct* stimulation of TAL by aldosterone (ie, stimulation mediated directly by hormone-receptor binding). To date, acute *in vitro* studies have not been performed to clarify this uncertainty.

Several studies have examined the effects of mineralocorticoids on potassium transport by the loop of Henle. Acute infusion of aldosterone into adrenalectomised rats did not significantly alter potassium reabsorption by the loop of Henle as measured by free-flow micropuncture (Hierholzer and Lange, 1975, Higashihara and Kokko, 1985). In contrast, in experiments in which rats were given physiological doses of aldosterone for 10 days and in which *in vivo* microperfusion was used to study transport, aldosterone increased potassium absorption to levels observed in control animals (Stanton, 1986). The differing results are presumably related to the length of hormone treatment and/or to the fact that small differences in the flow rate and composition of fluid entering the loop were eliminated in the microperfusion experiments. Both factors are known to influence potassium reabsorption by the loop of Henle (Hierholzer and Lange, 1974, Stanton, 1986).

The effects of aldosterone on acid transport have been studied directly in the amphibian diluting segment (similar in structure and function to the mammalian TAL) and in the rat mTAL. In the diluting segment, direct exposure of tubule cells to aldosterone *in vitro* increased cellular acid

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extrusion and stimulated luminal fluid acidification; the increase in acid secretion resulted from stimulation by aldosterone of apical membrane Na^+/H^+ exchange (Oberleithner *et al*, 1987, Weigt *et al*, 1987). In isolated, perfused mTAL from rats, the acute addition of a very high concentration (10μmol/l) of aldosterone to the basolateral solution increased bicarbonate reabsorption by 30% over a period of 1-2 hours (Good, 1990). A subsequent study has shown however, that only high (1μg/100g body weight.24hr) and not physiological (0.5μg/100g body weight.24hr) doses of aldosterone administered to adrenalectomised rats stimulated bicarbonate absorption along the loop of Henle (Unwin *et al*, 1995). The fact that only high doses of aldosterone have been shown to stimulate bicarbonate reabsorption along the Loop of Henle may be related to the relatively low numbers of MR compared to that of GR in this segment (see below). Hence, it is possible that the apparent aldosterone effects at the high doses were mediated by occupancy of GR. Additional studies *in vitro* with physiological concentrations of specific MR and GR agonists and antagonists are needed to resolve these issues.

The same approaches (autoradiography on isolated tubules and immunohistochemistry) have been used to examine GR expression along the nephron. High levels of [^{3}H]-dexamethasone binding sites, as detected by autoradiography, have been reported in the rat and rabbit thin and thick ascending limbs of the loop, at a level approximately 5 times that of [^{3}H]-aldosterone (Farman *et al*, 1983). Immunolocalisation studies of GR in TAL performed nearly a decade later by the same group, demonstrating high GR immunostaining in mTAL, supported the earlier findings obtained from their binding studies.

The role of glucocorticoids in the regulation of sodium absorption by the TAL is less well defined than that of the mineralocorticoids. Physiological levels of glucocorticoids increase Na^+/K^+-ATPase activity in TAL *in vitro* (Doucet, 1988). Both acute and chronic dexamethasone administration restored Na^+/K^+-ATPase of adrenalectomised animals in rabbit (El Mernissi and

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Doucet, 1984, Garg et al, 1985) and rat (Doucet *et al*, 1986) mTAL, and this effect was not blocked by spironolactone (an MR antagonist) (El Mernissi and Doucet, 1983). The acute stimulation of the enzyme in the mTAL was not accompanied by an increment in the number of pump units and was therefore attributed to increased specific activity (turnover rate) of pre-existing pump units rather than *de novo* synthesis in these segments. Furthermore, the stimulation was independent of an increase in glomerular filtration rate (GFR) and sodium delivery as dexamethasone also increases Na^+/K^+-ATPase activity in isolated mTAL. Both dexamethasone (Rodriguez *et al*, 1981) and methylprednisolone (Fisher et al, 1975) can stimulate Na^+/K^+-ATPase independent of alterations in sodium reabsorption.

One study has examined the effects of selective glucocorticoid replacement on potassium transport by the loop of Henle (Stanton, 1986). In that study, although the chronic (10 day) administration of physiological doses of dexamethasone to adrenalectomised rats partly restored potassium absorption, this increase was less than that observed with physiological doses of aldosterone (see above), and was not sufficient to return potassium absorption to control (adrenal intact) levels (Stanton, 1986). Conversely, *in vivo* microperfusion studies in rats indicate that, at physiological doses, glucocorticoids are more effective than mineralocorticoids in restoring bicarbonate reabsorption in the loop of Henle in the adrenal-deficient state (Unwin *et al*, 1995). Whether this effect is due to direct stimulation of Na^+/H^+ exchanger, as is the case in the proximal tubule, is unknown. Likewise it is possible that the dexamethasone enhanced bicarbonate reabsorption observed by Unwin *et al* (1995), was caused by increased sodium delivery to the loop as a result of a glucocorticoid-induced increase in GFR.

1.3.3. The distal convoluted tubule

In addition to the localisation of both MR and GR to the DCT (Farman, 1992), functional evidence supports a role for aldosterone in the regulation of thiazide-sensitive Na-Cl cotransport activity. Adrenalectomy reduces Na^+/K^+-

ATPase activity along the DCT, as well as other segments of the distal nephron (El Mernissi and Doucet, 1984). Furthermore, aldosterone has also been reported to stimulate sodium reabsorption by the *in vivo* perfused superficial distal tubule of rats (Hierholzer, *et al*, 1965; Stanton, 1986). Although this effect was attributed to a stimulation of electrogenic sodium reabsorption by cells of the ICT, a recent *in vivo* study has reported that aldosterone infusion into adrenalectomised rats increased the thiazide-sensitive component of sodium transport by perfused distal tubules, up to 20-fold (Valazquez *et al*, 1996). This functional capacity was accompanied by large increases in the number of high-affinity receptors for the thiazide-like drug [^{3}H]-metolazone (Chen *et al*, 1994, Valazquez *et al*, 1996). Since thiazide-sensitive Na-Cl cotransport is expressed primarily by DCT cells (Obermuller *et al*, 1995; Plotkin *et al*, 1996), these functional data raise the possibility that DCT cells are targets of aldosterone action. The role of glucocorticoids in this nephron segment has yet to be investigated.

1.3.4. Principal cells of the collecting ducts

It has been shown by numerous laboratories that the CCD is the major site of mineralocorticoid action. Elevation of plasma mineralocorticoid levels results in a stimulation in the rate of sodium reabsorption and potassium secretion in this segment (Stanton *et al*, 1981; Garg *et al*, 1981; Field *et al* 1984; Field and Giebisch, 1985, Stanton *et al*, 1985; Kaissling, 1985). Moreover, it is now generally agreed that it is the principal cell of the CCD which is the major site of mineralocorticoid action. These cells not only have cytoplasmic MR (see Farman, 1992 and Todd-Turla, 1993), but aldosterone has been reported to elicit numerous actions in the principal cell ranging from ultrastructural changes and stimulation of protein synthesis to activation of transport processes for sodium, potassium and hydrogen ions.

The response of the CCD to mineralocorticoids can, in general, be divided into two stages; early and late phases. Enhancement of sodium reabsorption and potassium secretion by mineralocorticoids occurs by a sequence of

events that has, as an absolute requirement, an increase in sodium permeability of the apical membrane (Kleyman *et al*, 1992). The early phase (within 1-2hr) of aldosterone action results in a relatively rapid increase in the number of sodium channels located at the apical membrane of the urinary bladder of the toad (Garty, 1986). This increase appears to be caused by the activation of pre-existing sodium channels at the apical surface, and not by increased synthesis of new channels. This hypothesis is consistent with studies demonstrating that the total cellular pool of sodium channels in A6 cells does not change after aldosterone treatment, despite the stimulation of sodium transport (Kleyman *et al*, 1992). Time-course studies on mineralocorticoid treatment have also been studied in mammalian CCDs. Sansom and O'Neil (1985), using electrophysiological techniques, showed that a primary effect of DOCA treatment in adrenal-intact rabbits was an increase in the apical membrane sodium conductance of the CCD within 24hr.

An early aldosterone-induced increase in the number of functional Na^+/K^+-ATPase units has been described in the CCD of adrenalectomised rats. For instance, several studies showed that the decrease in functional sodium pumps (Na^+/K^+-ATPase) of the rat CCD produced by adrenalectomy was compensated within 1-3 hr when aldosterone was given *in vivo* or *in vitro* (El Mernissi and Doucet, 1983; Verrey *et al*, 1996). In accordance with the hypothesis that this transcription-dependent effect corresponds to the activation of pre-existing pumps, CCD Na^+/K^+-ATPase mRNA was only marginally decreased by adrenalectomy (Farman *et al*, 1992). With aldosterone depletion, the Na^+/K^+-ATPase can be decreased to 20% of normal, whereas with aldosterone excess it can be increased to 200-300% of basal, reflecting a range of control of more than an order of magnitude.

In the late phase of mineralocorticoid action, sodium transport continues to increase for 6-12hr, although morphological and functional changes continue to occur over a much longer period in experimental animals that are chronically exposed to mineralocorticoid (Sansom and O'Neil, 1985, 1986;

Figure 1.8:
late and early phases of aldosterone (aldo) action
in the principal cell. AIP; aldo-induced proteins
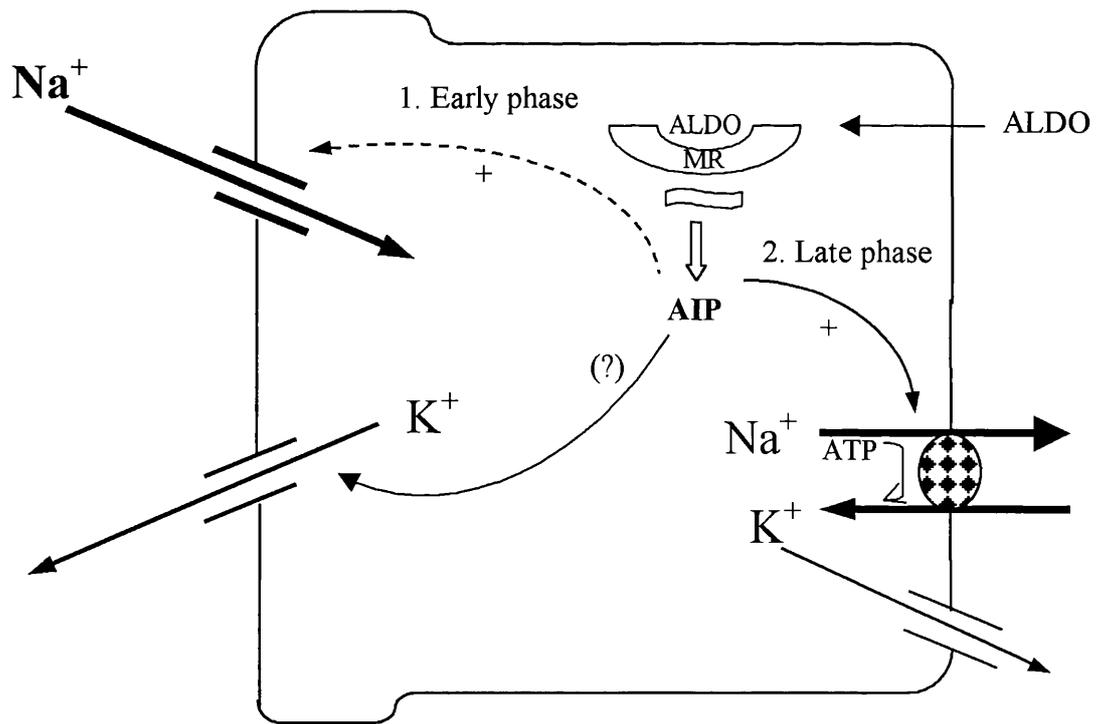
Wade *et al*, 1990). In this late phase, the transcription of the gene encoding Na^+/K^+-ATPase is stimulated. Aldosterone (at a concentration which also activates the GR) increases the transcription rate of Na^+/K^+-ATPase genes within 15 minutes. This effect is followed by an accumulation of the corresponding mRNAs and an increase in the biosynthesis of subunits that can be measured after 3 hr (Verrey, 1990). The cellular pool of Na^+/K^+-ATPase subunits, as well as the expression of subunits at the basolateral surface, is then significantly increased after 5 hr of aldosterone.

The stimulated sodium influx causes a modest depolarisation of the apical membrane during the early phase. When this is combined with the increased potassium uptake via the basolateral Na^+/K^+-ATPase pump, and a modest elevation in the intracellular potassium concentration, the result is a more favourable gradient for potassium secretion into the tubule lumen.

Together therefore, the early and late phases of aldosterone action on the principal cell results in a sustained increase in the CCD capacity to reabsorb sodium and to secrete potassium. This series of events is summarised in Figure 1.8.

The GR is expressed all along the distal and collecting tubule, as assessed by quantification of mRNA abundance (Escoubet *et al*, 1996) and immunodetection of the protein (Farman *et al*, 1991).

Despite the localisation of GR along the CCD, the question of whether glucocorticoids exert direct tubular effects is unanswered. Early studies which reported the influence of glucocorticoids on water, potassium and sodium excretion were generally ascribed to cross-reactivity of glucocorticoids to MR (Campden *et al*, 1983, Adam *et al*, 1984). However, studies by Bia *et al* (1982) suggested that the relatively selective glucocorticoid agonist, dexamethasone, directly affects distal tubular transport. Furthermore, dexamethasone treatment *in vivo* has been shown to increase the basolateral membrane area of principal cells (Wade *et al*, 1979). *In vivo* studies in the

microperfused distal tubule of the rat, demonstrated that dexamethasone increased sodium reabsorption under conditions when flow rate did not change, although to a lesser extent than aldosterone (Stanton, 1986). *In vitro* methods have been employed in an attempt to elucidate the underlying mechanisms of glucocorticoid-mediated electrolyte transport in the CCD. Naray-Fejes-Toth and Fejes-Toth (1990) utilised the highly specific GR agonist RU28362 (Quirk and Funder, 1988; Sutanto and de Kloet, 1987 and 1988) to investigate the specific effects of glucocorticoids on sodium transport and potassium secretion by monolayers of cultured cells isolated from the CCD of rabbit kidney. They reported that RU28362-GR complexes were capable of mediating mineralocorticoid-like effects, even in the presence of the specific MR antagonist ZK91587.

1.3.5. Intercalated cells of the collecting ducts

Since the action of mineralocorticoids is less well elucidated in the intercalated cells compared to principal cells, many studies have been carried out on nephron segments, with little discrimination between individual cells. Hence, in the current section, the effects of corticosteroids on intercalated cells are discussed with respect to each nephron segment of the collecting duct rather than in individual cell types.

In addition to its well known effects on sodium reabsorption and potassium secretion, aldosterone has been shown to influence proton and bicarbonate transport in the collecting ducts. In the CCD, a general consensus suggests that the effects of aldosterone on sodium and potassium transport are direct but it is not known whether this is the case regarding aldosterone's actions on the intercalated cell in the same segment. Due to the mixture of $\alpha-$ and $\beta-$ intercalated cells in the CCD, the interpretation of the effects of aldosterone on this segment has thus far been impeded. Furthermore, the presence of MR in the intercalated cell is controversial. Whereas some investigators have demonstrated the presence of MR in the β-intercalated cell (Todd-Turla *et al*, 1993), others have not (Farman *et al*, 1991).

The role of mineralocorticoids in the medullary section of the collecting duct is better elucidated than in the cortical regions. Firstly, MR and its mRNA have been localised to the OMCD (Farman *et al*, 1991; Todd-Turla *et al*, 1993). Secondly, there is substantial evidence that proton secretion by OMCD α-intercalated cells is stimulated by aldosterone. Administered at physiological doses to adrenalectomised rabbits or rats, aldosterone increases the enzymatic activity of N-ethylmaleimide (NEM)-sensitive H^+-ATPase in the OMCD (Garg and Narang, 1988; Khadouri *et al*, 1989; Eiam-Ong *et al*, 1993). It has been reported that the rate of proton secretion by perfused inner (Stone *et al*, 1983) and outer (McKinney and Davidson, 1987) OMCD segments dissected from control and adrenalectomised rabbits is decreased following adrenalectomy and was restored to control levels by *in vivo* (Stone *et al*, 1983) or *in vitro* (McKinney and Davidson, 1987) aldosterone treatment. Aldosterone-induced enhancement of proton secretion appears to be a result of a stimulation of both apical H^+-pump activity and basolateral Cl^-/HCO_3^- exchange activity in the α-intercalated cell (Figure 1.5) (Kuwahara *et al*, 1992; Hays *et al*, 1992).

The role of aldosterone in the regulation of the Sch28080-sensitive H^+/K^+-ATPases is uncertain. Whilst aldosterone-induced stimulation of this ATPase in Madin-Darby canine kidney (MDCK) cells (taken to represent intercalated cells) has previously been reported (Oberleithner, *et al*, 1990) a later study performed by Eiam-Ong and colleagues failed to demonstrate a role for this mineralocorticoid in the regulation of H^+/K^+-ATPase in the rat medullary collecting duct (Eiam-Ong *et al*, 1993).

Receptors for a variety of hormones have been demonstrated in the IMCD. Both MR and GR have been demonstrated by autoradiography in the rat papilla, albeit at lower levels than in the CCD (Farman and Bonvalet, 1983). Aldosterone has been shown to stimulate sodium transport by the IMCD *in vivo* (Ullrich and Papavassiliou, 1979). Likewise, *in vivo* data indicates that mineralocorticoids have an effect on proton secretion in the IMCD. In

mineralocorticoid-deficient rats given an acid infusion, a fall in pH along the IMCD was diminished, relative to that in control rats given a similar infusion. The fall in pH was partially restored with the acute administration of aldosterone (Higashihara *et al*, 1984). Dubrose and Caflish (1988) found that with mineralocorticoid deficiency, the ammonia concentration gradient between the interstitium and the IMCD lumen was decreased, accounting for a decrease in ammmonia secretion. *Ex vivo* experiments with isolated, perfused IMCD tubules have demonstrated enhanced bicarbonate absorption following chronic deoxycorticosterone (DOC) administration to the rats as compared with controls (Wall *et al*, 1990). Similarly, Husted *et al*, (1990), have shown that, in addition to aldosterone-MR complexes, dexamethasone-GR complexes also stimulate electrogenic sodium transport across monolayers of rat IMCD cells in primary culture.

In summary, many studies have been undertaken to investigate the mammalian collecting duct system; the principal site of adrenal corticosteroid action. The fact that this system can finely regulate the urinary composition of major electrolytes is made possible by its remarkable heterogeneity. Although the three major subsegments of the collecting duct, CCD, OMCD and $IMCD_i$ all contain at least two of the three main cell types, their role in electrolyte balance appears to be very different. The distal nephron and the CCD are predominantly involved in the regulation of sodium and potassium excretion, primarily under the control of mineralocorticoids. Both sodium and potassium transport in the OMCD is, in general, passive; active transport in this segment has thus far only been observed under extreme conditions of potassium deprivation. The primary role of the IMCD is the regulation of acid-base balance, in which both mineralocorticoids and glucocorticoids appear to play a role. Despite many studies however, it is apparent that the role of corticosteroids, particularly glucocorticoids, in the regulation of electrolyte balance in certain areas of the nephron (namely the TAL and medullary regions of the collecting duct) remain incompletely understood.

Although the localisation of MR and GR determines in which segment of the nephron adrenal corticosteroids regulate renal function, the magnitude of their actions is ultimately regulated by their rate of synthesis and metabolism, both of which determine the concentrations of these steroids at their site of action. The following section discusses the role of glucocorticoid metabolism in the regulation of renal function.

1.4. Glucocorticoid metabolism

1.4.1. The inherent non-selectivity of MR

Until the early 1980s, it had been widely assumed that MR had a 25 to 50 times higher affinity for aldosterone than for corticosterone as had been demonstrated in the original kidney slice studies carried out by Funder *et al* (1973). However, subsequent *in vitro* experiments with the MR, either as cytosolic preparations or as cloned receptor expressed in transfected cells, showed that its affinity was similar for aldosterone, cortisol, corticosterone and DOC (Krozowski and Funder, 1983; Funder, 1985; Arriza *et al*, 1987).

High levels of extravascular corticosteroid-binding globulin ('tissue transcortin') present in the kidney were originally hypothesised to be responsible for conferring aldosterone selectivity on renal MR (Stephenson *et al*, 1984). The mechanism proposed was one of a counter-current, whereby renewable sequestration of free corticosterone by extravascular transcortin served to lower the levels of glucocorticoid competing for MR occupancy. However, this hypothesis was rendered untenable by subsequent *in vivo* studies in 10-day old rats (Sheppard and Funder, 1987a; Sheppard and Funder, 1987b) which demonstrated that transcortin is not required for the aldosterone selectivity of renal MR. In these studies, 1-day adrenalectomised, 10 day old rats, which have low or absent transcortin levels, were injected with either [^{3}H]-aldosterone or [^{3}H]-corticosterone, and the uptake and retention of tracer compared in the hippocampus, kidney, parotid and colon.

In the hippocampus, both tracers bound equally well. However, in the classic mineralocorticoid target tissues (kidney, parotid and colon), binding of [^{3}H]-aldosterone was up to 20 times higher than that of [^{3}H]-corticosterone. In contrast, *in vitro* cytosol binding of the two tracers was the same for each tissue. One possible explanation for these discrepancies was the existence of an *in vivo* mechanism to exclude glucocorticoids from 'classical' (not hippocampal) MR, which was absent from *in vitro* cytosol preparations.

1.4.2. Glucocorticoid metabolism pathways

The metabolism of steroids has long been recognised as an important mechanism for regulating steroid hormone action. In both humans and rats, despite high concentrations of total plasma glucocorticoids (between 150-600nmol/l), there is very little free glucocorticoid in the plasma, due to the fact that around 85% of glucocorticoids are bound to an α-globulin called transcortin or corticosteroid-binding globulin (CBG). Hence, circulating levels of free (ie, active) glucocorticoids vary between 0.5 and 100nmol/l (Munck, 1992). The normal plasma half-life of cortisol is between 60 and 80 min, in contrast to aldosterone which has a half-life of less than 20 min due, in large part, to the lower affinity of aldosterone for plasma proteins. In normal humans and rats, aldosterone circulates at around 200pmol/l.

The liver and kidney are the prevalent organs involved in metabolising glucocorticoids and clearing them from circulation. Metabolism decreases the biological activity of these hormones and increases their water solubility by converting them to hydrophilic compounds that can be excreted in urine. Free cortisol is also present in urine but normally comprises only 0.1% of the total cortisol metabolites.

Figure 1.9: Pathways of glucocorticoid metabolism. The major reactions involved in the metabolism of cortisol include reduction of the C-4,5 double bond and reduction of the C-3 ketone group to yield tetrahydrocortisol and tetrahydrocortisone. Enzymes mediating each conversion are italicised.

Cortisol (F)

CH_2OH

$C == O$

HO

CH_3 C11

OH

CH_3

O

Cortisone (E)

O

C11

11β-dehydrogenase

11-ketoreductase

5β-reductase

5α-reductase

CH_3

O

H

5ß-dihydrocortisol (5ßDHF)

CH_3

O

H

5α-dihydrocortisol (5αDHF)

3α-dehydrogenase

CH_3

HO

H

5ß-tetrahydrocortisol (THF)

CH_3

HO

H

5α-tetrahydrocortisol
(alloTHF)

tetrahydrocortisone
(THE)

52
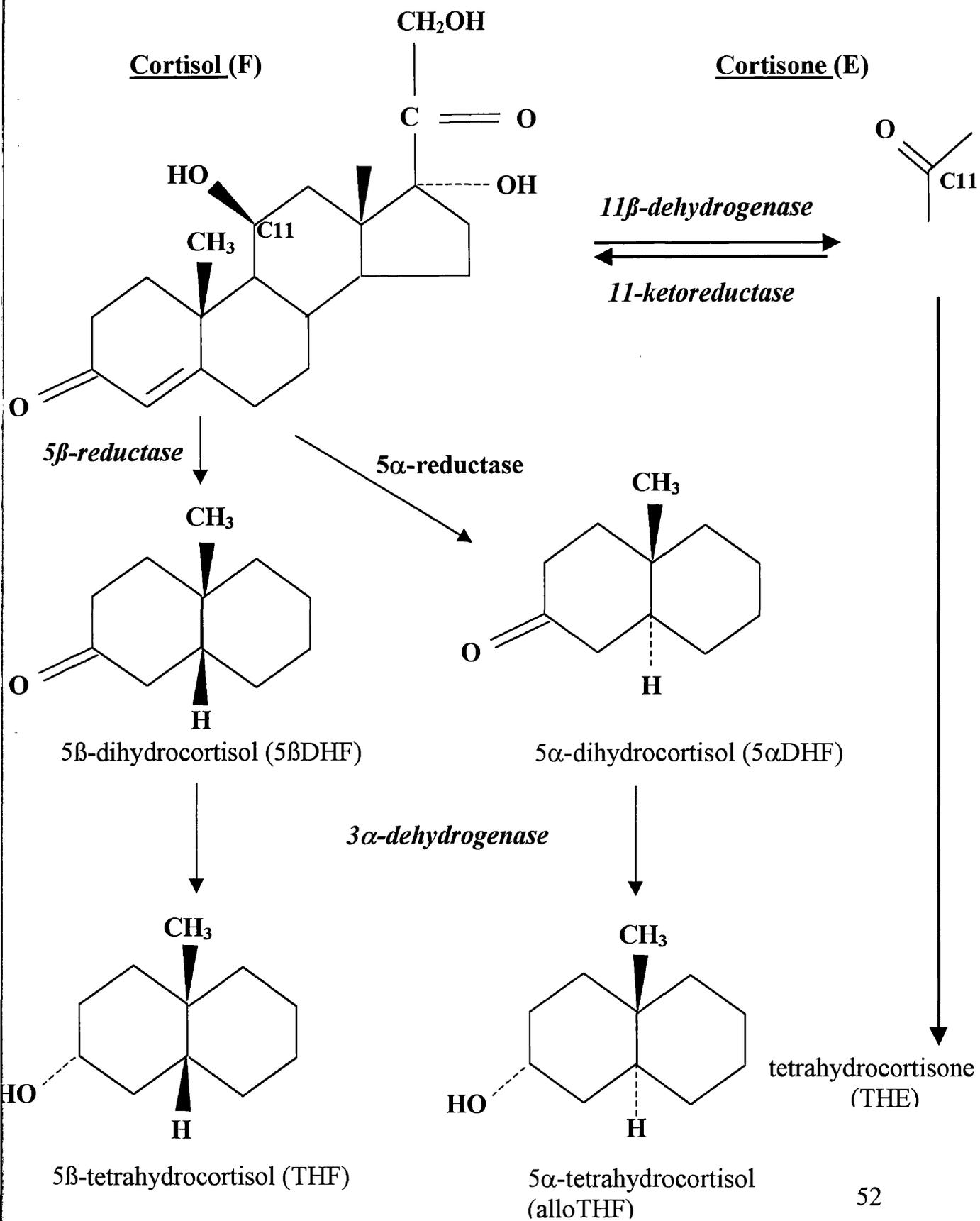
Cortisol and corticosterone are metabolised similarly as shown in Figure 1.9. The major reactions involved in the metabolism of cortisol include reduction of the C-4,5 double bond and reduction of the C3 ketone group. The products of these 'A ring' reductions are tetrahydrocortisol (if 5ß-reduced) and allo-tetrahydrocortisol (if 5α-reduced). Cortisol may be oxidised at the 11-hydroxy position to cortisone, dihydrocortisone or tetrahydrocortisone. Tetrahydrocortisol (THF) and tetrahydrocortisone (THE) can also undergo reduction at the C-20 position to yield cortol and cortolone, respectively.

1.4.3. Evidence for glucocorticoid metabolism

As early as the 1950's, studies of patients with Cushing's disease demonstrated that cortisol was the predominant secretory product of the human adrenal cortex, and thus presumed to be the glucocorticoid hormone of most importance (Mason, 1950). At the same time, it was noted that cortisone when administered systemmically was effective in treating inflammatory conditions (Hench et al, 1950), but had little therapeutic effect when injected directly into the joint (Hollander et al, 1951). Moreover, since systemically administered cortisone was partially excreted as cortisol, it was postulated that cortisone only became active when converted to cortisol and that this biological activation would occur in some parts of the body but not in others. The enzymatic activity catalysing the conversion of inactive C_{21} steroids (cortisone and 11-dehydrocorticosterone) to active glucocorticoids (cortisol and corticosterone) by reduction of the ketone group situated on C-11 (Figure 1.9) was identified in rat liver (Amelung et al, 1953, Hubener et al, 1956) and consequently termed 11-ketosteroid reductase (11KSR). The enzyme responsible for the inactivation of glucocorticoids by oxidation of the hydroxyl group on C-11 was named 11ß-hydroxysteroid dehydrogenase (11ßHSD).

1.4.4. Clinical relevance of glucocorticoid interconversion: Apparent Mineralocorticoid Excess

Although the interconversion of cortisol and cortisone was subsequently found in many tissues, the full physiological consequences of this metabolism, with regard to renal function, were not appreciated until the late 1970s when Ulick and New began detailed investigations into the syndrome of apparent mineralocorticoid excess (Ulick *et al*, 1979, New *et al*, 1982). This very rare disorder is most common in young children who present with severe hypertension, caused by excess sodium retention, and neurological symptoms associated with hypokalaemia. Since ACTH exacerbated the hypertension, and a low salt diet or blockade of MR ameliorated the rise in blood pressure, (Werder *et al*, 1974, New *et al*, 1977, Ulick *et al* 1977), it was initially suspected that these patients had elevated levels of an unidentified ACTH-inducible mineralocorticoid. However, levels of all known mineralocorticoids were low and, consequently, the condition was named 'apparent mineralocorticoid excess' (AME) (Ulick *et al*, 1979).

Subsequent studies have shown that AME patients exhibit profound alterations in their patterns of urinary cortisol metabolites. As well as preferential 5α reduction of the A ring of cortisol, markedly elevated urinary ratios of cortisol to cortisone metabolites (THF:THE) were also observed in these patients (Ulick *et al*, 1979; Wilson *et al*, 1995; Milford *et al*, 1995). These urinary metabolites are normally in the order of 1:1. In patients with AME however, the ratio is in excess of 10:1 (See Mune *et al*, 1995 and White *et al*, 1997).

Attempts to isolate a novel mineralocorticoid from the plasma or urine of AME patients proved futile and, as such, it was proposed that these patients had either MR abnormalities, or that cortisol itself was somehow acting as a mineralocorticoid. The latter proposal was investigated by Stewart *et al* (1985) who, having studied the oldest surviving AME patient, proposed a role

Figure 1.10: Structures of carbenoxolone (CBX), Glycyrrhetinic acid (GE) and Glycyrrhizic acid (GA).
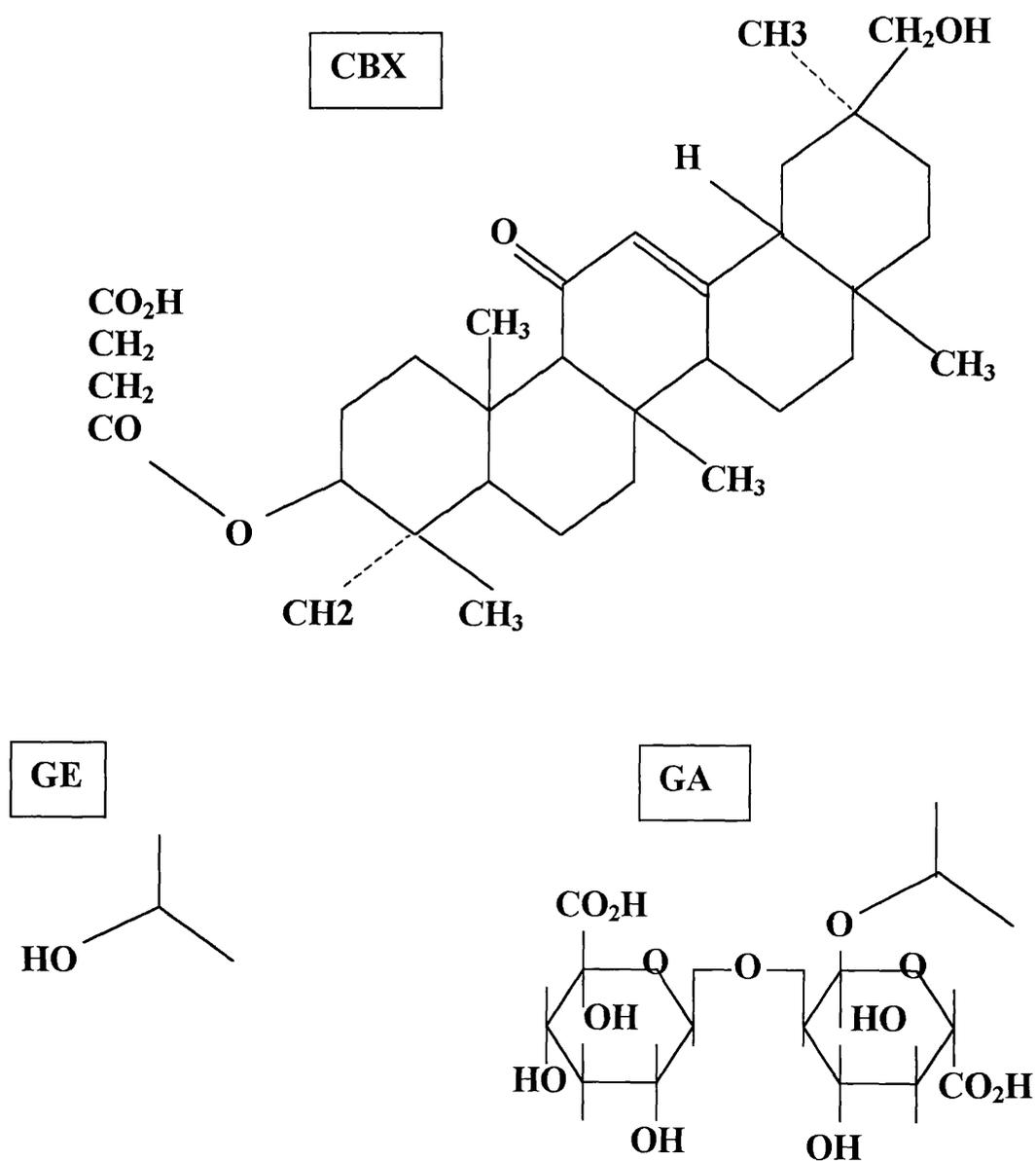
for the cortisol-cortisone shuttle. They proposed that in the liver, for example, cortisone was favourably converted to cortisol (by the 11-KSR activity of 11ßHSD), whilst in mineralocorticoid target tissue, such as the kidney, cortisol was predominantly converted to cortisone by the oxidative activity of 11ßHSD. Although, the interconversion of cortisol and cortisone by various tissues was already recognised, Stewart *et al* were the first to emphasise the importance of the renal component of the shuttle as a possible contributor to the pathogenesis of AME. In further studies, healthy volunteers who had consumed 250g liquorice for 10 days exhibited a doubling of their urinary ratio of cortisol:cortisone metabolites, increased sodium retention and kaliuresis (Stewart *et al*, 1987). Moreover, infused [^{3}H]-cortisol in these subjects had a half life more than twice as long as usual. The results demonstrated that the predominant mineralocorticoid action of liquorice was due to its inhibition of glucocorticoid metabolising enzymes, resulting in abnormally high intrarenal cortisol levels and enhanced occupancy of MR. Stewart *et al* (1987) proposed that, whereas AME represented a congenital deficiency of this enzyme, liquorice intoxication resulted from pharmacological inhibition of 11ßHSD.

1.4.5. Pharmacological inhibition of 11ßHSD

Liquorice is an extract of the roots of *Glycyrrhiza glabra* and clinically it has been used in the treatment of peptic ulcers. However, for more than 40 years it has been known that liquorice induces undesirable mineralocorticoid-like side effects including hypertension, oedema, and hypokalaemia (Card *et al*, 1953). The active component of liquorice is glycyrrhizic acid (GE), the aglycone of which is glycyrrhetinic acid (GA). Carbenoxolone (CBX) is the water-soluble hemi-succinate ester of GA. The structures of CBX, GE and GA are shown in Figure 1.10. The biological effects of these compounds are not identical; it has been reported that CBX inhibits both 11ßHSD and 11KSR activities, whereas GA appears to inhibit only the 11ßHSD activity (Stewart *et al*, 1990).

Two studies (Edwards *et al*, 1988 and Funder *et al*, 1988) provided fundamental evidence for 11ßHSD activity as the specificity-conferring mechanism in 'classical' mineralocorticoid target tissues. Results from both laboratories demonstrated that 11ßHSD activity, as determined by percent conversion of [^{3}H]-corticosterone to [^{3}H]-11-dehydrocorticosterone (Edwards *et al*, 1988) or of [^{3}H]-cortisol to [^{3}H]-cortisone (Funder *et al*, 1988), in tissue homogenates taken from 10 day old rats, was highest in kidney with lower levels in the parotid gland. Little or no enzyme activity was found in the hippocampus or heart. In addition, Edwards and colleagues (1988) reported that both proximal and distal enriched fractions of rat kidney were capable of converting cortisol to cortisone, although distal enriched fractions had higher enzyme activity (30% compared to 20% conversion in proximal tubules). Furthermore, autoradiography with [^{3}H]-aldosterone (Edwards *et al*, 1988) showed expected binding patterns in the cortex and outer medulla, whilst in contrast, very little uptake of [^{3}H]-corticosterone was observed in these sites. However, after inhibition of 11ßHSD (GE administration 60 min prior to [^{3}H]-corticosterone administration), the pattern of [^{3}H]-corticosterone binding was similar to that observed with [^{3}H]-aldosterone. Similarly, Funder *et al* (1988), demonstrated that *in vivo* injection of [^{3}H]-aldosterone to 1-day adrenalectomised, 10 day old rats showed characteristic high levels of uptake and retention in kidney, parotid gland, colon, hippocampus and heart. [^{3}H]-corticosterone, in contrast, whilst displaying equivalent binding to hippocampus and heart, bound at much lower levels in kidney, parotid and colon. In rats pre-treated with CBX, this aldosterone-selectivity in classic mineralocorticoid target tissues was lost, so that binding of injected [^{3}H]-corticosterone equalled that of [^{3}H]-aldosterone in the kidney and parotid.

Together the two studies revealed the inherent non-selectivity of MR *in vivo*, and thus confirmed the significance of the cortisol to cortisone shuttle in conferring aldosterone specificity within mineralocorticoid target tissues.

1.4.6. 11ßHSD1: Isolation and localisation

1.4.6.1. Molecular biology of 11ßHSD1

Rat liver expresses high levels of 11ß-dehydrogenase and 11KSR activities in the presence of $NADP^+$ and NADPH, respectively. The 11ß-dehydrogenase activity was purified to homogeneity and identified as a glycoprotein with a molecular mass of 34kDa. A cDNA clone encoding this enzyme was isolated using an antiserum to the purified rat protein (Agarwal *et al*, 1989). The full-length cDNA was 1.4kb long including an open reading frame of 876bp, predicting a protein of 292 amino acids. The corresponding human cDNA was then isolated (Tannin *et al*, 1991). The amino acid sequences predicted from the cloned rat and human cDNA sequences are 77% identical, although the human enzyme is slightly larger.

A search of sequence databases revealed that this isoform of 11ßHSD (termed 11ßHSD1) is a member of the "short-chain alcohol dehydrogenase" (SCAD) enzyme family. Such enzymes all have a molecular mass of around 30kDa and share a conserved consensus sequence of Tyrosine (Y), X, X, Ser (S), Lysine (K). Highly conserved amino acids in these enzymes are situated near the amino terminus in a region proposed to constitute part of the binding site for the cofactors which, in the case of 11ßHSD1, is NADP(H). Absolutely conserved Y and K residues are located toward the carboxyl terminus (Persson *et al*, 1991). In rat 11ßHSD1 these are Y179 and K183. Even conservative substitutions of either of these residues destroy enzymatic activity (Obeid and White, 1992); observations which confirm that these residues represent the substrate-binding site.

11ßHSD1 was subsequently isolated in several more mammalian species: sheep (Yang *et al*, 1992), squirrel monkey (Moore *et al*, 1993) and mouse (Rajan *et al*, 1995). Several observations however, concerning the enzymatic properties and localisation of 11ßHSD1, suggested that this isoform of the

enzyme was not responsible for conferring MR specificity.

1.4.6.2. Enzymatic properties of 11ßHSD1

The recombinant enzyme expressed from the cloned cDNA of 11ßHSD1 exhibited both 11ß-dehydrogenase and oxoreductase activity when expressed in mammalian cells (Agarwal et al, 1989). At physiological pH in cell lysates, the kinetic constants for 11ß-dehydrogenation and 11 ketosteroid reduction (K_m of 1.1 and 1.4 µmol/l, respectively) were similar (Agarwal et al, 1990). Hence, since the affinity of this form of 11ßHSD for endogenous glucocorticoids is relatively low, and circulating levels of free glucocorticoids vary between 0.5 and 100nmol/l (Munck and Naray-Fejes-Toth, 1992), it is unlikely that an enzyme with a K_m orders of magnitude higher could decrease glucocorticoid concentrations to the very low levels needed to ensure aldosterone specificity.

Moreover, a prerequisite of an 11ßHSD isoform which is able to protect MR from inappropriate glucocorticoid occupation must be that predominantly dehydrogenase activity occurs, such that the majority of glucocorticoids are inactivated. However, although the enzyme purified from rat liver functioned only as a dehydrogenase, the recombinant enzyme expressed from cloned cDNA exhibited both 11ß-dehydrogenase and 11-ketoreductase activity when expressed in mammalian cells (Agarwal et al, 1989). The recombinant enzyme expressed using vaccina virus also exhibits both 11ß-dehydrogenase and 11keto-reductase activities (Agarwal et al, 1990).

1.4.6.3. Localisation of 11ßHSD1

In the rat, 11ßHSD1 cDNA hybridised to RNA from a wide range of tissues including liver, kidney, testis, lung, heart and colon, with strength of hybridisation in approximately that order (Agarwal, 1989, Krozoswki, 1990). Immunoreactivity was similarly distributed (Monder and Lakshmi, 1990). In general, the distribution of expression of 11ßHSD1 more closely paralleled that of the GR than that of the MR, suggesting that this enzyme might, at least in some tissues, modulate levels of glucocorticoids reaching the GR

(Whorwood et al, 1992). In the rat brain, hybridisation was strongest in the hippocampus and cortex but also found in the pituitary, hypothalamus, brain stem, and cerebellar cortex (Moisan et al, 1990). In humans, a 1.5-kb RNA band was observed in samples from liver, testis, lung, ovary, colon and kidney (Tannin et al, 1991). Of the human tissues tested, by far the highest level of expression was in the liver, whereas (in contrast to the rat) expression was much lower in the kidney. Moreover, it was found that, surprisingly, 11ßHSD1 was expressed in the proximal tubule (Edwards et al, 1988; Rundle et al, 1989) not, as would be expected, in renal mineralocorticoid target segments, namely the distal tubule. Thus, it was hypothesised that 11ßHSD acted within the proximal tubules to prevent active glucocorticoids from reaching the distal nephron.

Taken together, the results implied that 11ßHSD1, cloned from rat liver and ubiquitously expressed, was unlikely to be the mechanism responsible for conferring aldosterone selectivity on the MR in mineralocorticoid target cells within the kidney. Indeed, four years later it was demonstrated that mice produced with targeted disruption of the 11ßHSD1 gene were unable to convert inert 11-dehydocorticosterone to corticosterone in vivo but did not display any phenotypic characteristics of the syndrome of AME (Kotelevtsev et al, 1997). It was thus assumed that such renal cells possessed an enzyme with characteristics distinct from that of the hepatic isoform. Such a hypothesis led to the isolation of a second isoform of 11ßHSD.

1.4.7. 11ßHSD2: Isolation and localisation

Indirect evidence for an additional isoform of 11ßHSD active in mineralocorticoid target tissue came when Mercer and Krozowski (1992) reported that 11ß-hydroxyandrostenedione, a non-physiological substrate, was oxidised in rat renal DCT and medullary collecting ducts, in the presence of NAD^+. The histochemical technique used in this study utilises the NADH produced by 11ßHSD oxidative activity to reduce diformazan dye, giving a blue colouration.

More direct evidence for a distinct 11ßHSD isoform was obtained from biochemical studies of isolated CCD from rabbit kidney (Naray-Fejes-Toth *et al*, 1991; Rusvai and Naray-Fejes-Toth, 1993). In these studies, the existence of an exclusively NAD^+-dependent 11ßHSD enzyme was detected in intact collecting duct cells, with an apparent K_m for corticosterone of approximately 30nmol/l; a value more than 100 times lower than the K_m of rat liver 11ßHSD (~2μmol/l). Moreover, when grown in monolayer (Naray-Fejes-Toth *et al*, 1991) these cells were able to metabolise 98% of corticosterone as it passed from the basolateral to the apical compartment; an activity sufficient to decrease intracellular corticosterone concentrations and confer aldosterone specificity on the MR.

1.4.7.1. Molecular biology of 11ßHSD2

Cloning of cDNA encoding 11ßHSD2 occurred by expression-screening strategies in which pools of cDNA clones from sheep (Agarwal *et al*, 1994; Yang and Yu, 1994) and human (Albiston *et al*, 1994) kidney were assayed for their ability to confer NAD^+-dependent 11ßHSD activity on *Xenopus* oocytes or cultured mammalian cells. Positive pools were divided into smaller pools and rescreened until a single positive clone was identified. Subsequently, rat (Zhou *et al*, 1995), rabbit (Naray-Fejes-Toth and Fejes-Toth, 1995) and mouse (Cole, 1995) cDNAs were isolated.

The protein is predicted to contain 404 (sheep) or 405 (human) amino acid residues with a total molecular mass of 41kDa. A search of sequence databases revealed sequence similarity to members of the SCAD superfamily, although 11ßHSD2 is only 20-26% identical to 11ßHSD1 (Wu *et al*, 1993). Regions of similarity between the isoforms include part of the putative binding site for the nucleotide cofactor and the absolutely conserved tyrosine and lysine residues (Y232 and K236 in this enzyme). The fact that these residues are well conserved in both isoforms is consistent with their postulated role in binding the substrate.

1.4.7.2. Enzymatic properties of 11ßHSD2

Recombinant 11ßHSD2 has properties that are virtually identical to the activity found in mineralocorticoid target tissues. The recombinant enzyme appears to act exclusively as a 11ß-dehydrogenase; no reductase activity is detectable with either NADH or NADPH as a cofactor (Agarwal *et al*, 1994; Albiston *et al*, 1994; Zhou *et al*, 1995). Moreover, in contrast to the liver isoform, 11ßHSD2 has been reported to be inhibited by its end-product: in collecting duct cells incubated with increasing concentrations of 11-dehydrocorticosterone, oxidation of [^{3}H]-corticosterone was significantly inhibited in a concentration-dependent manner (Naray-Fejes-Toth and Fejes-Toth, 1994). The 11ß-dehydrogenase activity of the human, rat and rabbit 11ßHSD2 has an almost exclusive preference for NAD$^+$ as a cofactor. The sheep 11ßHSD2 is able to utilise NADP$^+$ to oxidise corticosterone (but not cortisol) approximately 25% as efficiently as it utilises NAD$^+$. The enzyme has a very high affinity for glucocorticoids, but corticosterone is the preferred substrate, with first order rate constants 10-fold higher than those for cortisol, even in mammalian species in which cortisol is the predominant glucocorticoid. Reported K_m values for corticosterone are between 1 and 13nmol/l, and for cortisol, 14-60nmol/l (Naray-Fejes-Toth *et al*, 1991; Brown *et al*, 1993; Kenouch *et al*, 1994; Stewart *et al*, 1994; Yang and Yu, 1994).

It should be noted that several groups have recently reported that NADH-dependent reduction of cortisone occurs in human kidney microsomes (Diederich *et al*, 1997; Li *et al*, 1997). Since the properties of the enzyme reported in their study were consistent with those of the cloned type 2 11ßHSD isoform, the findings suggest that 11ßHSD2 is capable of reductase activity; an observation at odds with the generally held theory that 11ßHSD2 activity is unidirectional. Although reductase activity is unlikely to occur in the kidney *in vivo* (predominantly due to limited NADH availability), the findings are important in demonstrating that, as for type 1 11ßHSD, the environment in which 11ßHSD2 resides could influence the activity (dehydrogenase *vs* reductase) of this enzyme.

1.4.7.3. Localisation of 11ßHSD2

The tissue distribution of 11ßHSD2 expression has been examined by RNA blot hybridisation in human adults (Albiston *et al*, 1994; Stewart *et al*, 1995) and foetuses (Stewart *et al*, 1995) and in sheep (Agarwal *et al*, 1994), rats (Zhou *et al*, 1995) and mice (Cole, 1995). In all species, this isoform is expressed in placenta and mineralocorticoid target tissues, particularly in the kidney, whereas it has not, to date, been detected in the liver or heart. In salivary glands, it is expressed in tubular elements with minimal expression in acini (Roland and Funder, 1996). In the colon it is found in the mucosa but not in the submucosa (Kyossev, 1996; Roland and Funder, 1996).

Expression of 11ßHSD2 within mineralocorticoid target tissues has been further localised by immunohistochemistry and *in situ* hybridisation. In the kidney, 11ßHSD2 is expressed in DCT and CCD and as such is colocalised with MR (Naray-Fejes-Toth and Fejes-Toth, 1995; Cole, 1995; Roland and Funder, 1996; Whorwood *et al*, 1995; Krozowski *et al*, 1995; Kyossev *et al*, 1996). Glucocorticoid oxidation has also been detected in rat and human cTAL and OMCD (Kenouch *et al*, 1994). Although Kenouch and colleagues did not specify which isoform of 11ßHSD was responsible for the observed metabolism, the recent findings of Smith *et al* (1997), that low levels of 11ßHSD2 are present in tubules thought to represent TAL, would suggest that 11ßHSD2 activity is present in this nephron segment.

1.4.8. Evidence for further isoforms of 11ßHSD

At present, the question remains as to whether more 11ßHSD isoforms exist in addition to the cloned type 1 and type 2 isoforms. In terms of the kidney, there have been several studies performed over the past decade which have provided indirect evidence for the presence of two isoforms of 11ßHSD in the CCD. Stewart *et al* (1991) performed *in situ* hybridisation of liver 11ßHSD mRNA in rat renal tissue and found 11ßHSD mRNA to be expressed in both proximal and cortical collecting tubules. Paradoxically, in the same study,

Stewart *et al* reported that an immunohistochemical study (in which the same antibody was employed to isolate the cDNA sequence encoding hepatic 11ßHSD) localised 11ßHSD1 immunoreactivity to the proximal tubule but not to the distal tubules or CCD. It was suggested by the authors that either post translational modification of the enzyme occurred which may prevent detection of the functional protein by hepatic 11ßHSD antisera in the distal tubule, or that another isoform of 11ßHSD was present in the more distal regions of the kidney. Evidence for the latter proposal has been provided following the isolation of the high affinity NAD^+-dependent type 2 11ßHSD isoform. However, the question remains as to whether the initial observation by Stewart *et al* (1991) was indeed the result of either type 1 11ßHSD in the distal tubule that could not be detected, or is in fact another isoform of the enzyme. On the basis of cofactor preference, there is further evidence to suggest that another isoform (besides type 1 and type 2) may exist in the kidney. Walker *et al* (1992) reported that in isolated tubules enriched for proximal or distal segments, levels of 11ßHSD activity were equal in the presence of $NADP^+$ and NAD^+. In their study, Walker and colleagues (1992) attributed the lack of a demonstrable difference between cofactor utilisation in proximal versus distal renal tubules to inadequate purity of the two fractions. However, a subsequent study performed by Alfaidy *et al* (1995) using individually microdissected nephron segments suggested that two isoforms of 11ßHSD may be present in the CCD on the basis that kinetic analyses revealed the coexistence of two isoforms which differed in affinity for corticosterone by an order of magnitude (K_m, 65 *vs* 550nmol/l).

Evidence for a third high affinity $NADP^+$-dependent isoform of 11ßHSD has been described for non-renal tissues including a human choriocarcinoma cell line, rat Leydig cells and human ovarian cells (Gomez-Sanchez *et al*, 1996; Ge *et al*, 1997; Michael *et al*, 1997). In these studies, the K_m for this enzyme is significantly lower than that for 11ßHSD1 and actively predominates in the mitochondrial and large microsomal fraction, not in the nuclei, where NAD^+-dependent activity is found. Together, these studies provide evidence that in both renal and extrarenal tissues another isoform of 11ßHSD may exist.

1.4.9. 11ßHSD isoform mutations: Correlations with AME

The clinical studies involving AME patients, which had initiated the hunt for the enzyme responsible for endowing mineralocorticoid target cells with aldosterone selectivity, were the 'final' evidence confirming the role of 11ßHSD2. With the structure of the genes of both 11ßHSD1 and 11ßHSD2 elucidated, it has been possible to analyse the corresponding genes from patients with AME to determine whether mutations in this gene are responsible for the disease.

To date, no mutations have been identified in the gene encoding the type 1 isoform of 11ßHSD (HSD11B1) in any patients suffering from AME (see White *et al*, 1997). In contrast to the findings with HSD11B1, mutations in the gene encoding the type 2 isoform (HSD11B2) have been detected in all AME patients studied to date. In 1997, 11 different mutations had been detected in 20 patients from 15 kindreds (White *et al*, 1997). A wide range of urinary cortisol:cortisone metabolite ratios is seen in patients who carry mutations that apparently completely destroy 11ßHSD2 enzyme activity. This observation is not surprising since small variations in the very low levels of cortisone metabolites (ie, THE) excreted by these patients will lead to marked differences in ratios for which this value is the denominator. Efforts to correlate biochemical severity with measures of clinical severity are likewise impeded by the fact that the number of patients is small and the possibility that prior antihypertensive therapy may confound effects. Anecdotal reports, however, do suggest that mutations which do not completely destroy activity (eg, R337C, which has at least 50% of normal 11ßHSD activity) may be associated with milder disease (Wilson *et al*, 1995; Mune *et al*, 1995).

Despite such hinderences, the findings that mutations in HSD11B2, but not in HSD11B1, are associated with renal dysfunction, has been widely accepted as the confirmation that it is indeed the type 2 isoform responsible for protecting the MR from physiological concentrations of cortisol (Wilson *et al*, 1995, Mune *et al*, 1995, Wilson *et al*, 1995, Stewart *et al*, 1996).

1.4.10. Decreased 11ßHSD2 activity: consequences for renal function

In the renal collecting duct of an AME patient devoid of 11ßHSD2 activity, inappropriate glucocorticoid occupation of MR would be expected to result in increased activity of sodium channels and Na^+/K^+-ATPase, and the consequent antinatriuresis and kaliuresis (see section 1.3.4) Despite convincing clinical evidence linking genetic defects of the type 2 isoform of 11ßHSD with the genesis of AME, studies in which attempts have been made to inhibit 11ßHSD pharmacologically have yielded far less consistent results regarding the physiological roles of this enzyme.

The first major study to attempt to relate pharmacological inhibition of 11ßHSD to renal function was carried out by Stewart *et al* (1990). In this study, it was shown that the administration of CBX to healthy human volunteers resulted in a decrease in sodium excretion, consistent with the proposed guardian role for 11ßHSD in protecting the MR. However, no increase in potassium excretion was demonstrated. Another human study (Baron *et al*, 1969) had previously reported a fall in urinary sodium excretion without a concomitant change in potassium excretion following the administration of CBX. Furthermore, in the study performed by Stewart *et al*, it was impossible to determine directly whether or not renal 11ßHSD activities were affected, because although CBX administration was associated with both increased plasma half-life of [11α-^{3}H]-cortisol, and elevated urinary excretion of free cortisol, no effect on the urinary ratio of THF:THE was observed.

Souness and Morris (1989) had likewise observed a dissociation between the effects of CBX on 11ßHSD activity and changes in renal electrolyte transport. In their study, Souness and Morris administered CBX (2.5mg/rat; subcutaneous (s.c)) to adrenalectomised rats receiving high dose corticosterone replacement. Although antinatriuresis and kaliuresis were both

observed in these animals, the pronounced 'mineralocorticoid-like' sodium-retaining properties conferred on corticosterone by CBX were disproportionately larger than the observed increases in the kaliuretic response. A further discrepancy was revealed when subsequent studies by the same authors showed that while hepatic 11ßHSD activity was significantly inhibited with acute (up to 2 hours) administration of 2.5 and 10mg CBX, neither of these doses had any impact on renal corticosterone oxidation in adrenalectomised rats (Latif *et al*, 1992), despite changes in urinary electrolytes. Furthermore, in studies performed in the same laboratory, the administration of CBX to adrenalectomised rats by the same subcutaneous route was also found to potentiate the antinatriuretic effects of low dosages of the mineralocorticoids, aldosterone and DOC (Morris and Souness, 1990). Since neither of these compounds serve as substrates for 11ßHSD, it is questionable whether the effects of CBX on urinary electrolyte excretion are mediated exclusively by inhibition of renal 11ßHSD activities.

1.5. Aims of the experiments described in this thesis

It is evident from both human and animal studies performed to date, that a direct correlation between inhibition of renal 11ßHSD activities and altered renal electrolyte excretion has yet to be determined. The experiments described in chapters 2 and 3 were designed to provide further evidence relating the inhibition of renal 11ßHSD activities *in vivo* to alterations in urinary sodium and potassium excretion both at the whole kidney and single nephron level. The experiments described in the subsequent three chapters were designed to investigate the possible regulation of renal 11ßHSD in both the whole kidney and individual nephron segments. The overall aim of the experiments reported in this thesis was to improve the understanding of the physiological roles for isoforms of 11ßHSD and of glucocorticoids in the rat kidney.

Chapter 2:

The effect of *in vivo* inhibition of 11ß-hydroxysteroid dehydrogenase with carbenoxolone on renal sodium and potassium transport

2.1. Introduction

As stated in Chapter 1, there is unequivocal clinical evidence linking genetic defects of the type 2 isoform of 11ßHSD with the genesis of AME. However, studies in which attempts have been made to inhibit 11ßHSD pharmacologically have yielded far less consistent results regarding the physiological roles of this enzyme. Two previous studies have both reported that rats administered CBX (Morris and Souness, 1989) or humans administered liquorice (Stewart *et al*, 1990) exhibit antinatriuresis but not kaliuresis. Moreover, it was uncertain whether renal 11ßHSD activities had actually been inhibited in either study.

A direct correlation between inhibition of renal 11ßHSD activities and altered renal electrolyte excretion has yet to be determined. The aim, therefore, of this first series of experiments was to determine whether acute, graded inhibition of renal 11ßHSD activities *in vivo* results in predicted graded changes in urinary sodium and potassium excretion in adult rats.

2.2. Materials and Methods

2.2.1. Optimisation of radiometric conversion assay of renal 11ßHSD activities

Before any 11ßHSD assays were performed on tissue obtained from experimental animals, the assay was initially optimised with regard to i) homogenate preparation and ii) the incubation period to ensure that all measurements of enzyme activity were made under first order kinetics. The optimum homogenate concentration (25mg wet weight/ml) had previously been determined in this laboratory. The substrate concentration selected for all 'whole kidney' enzyme assays was 500nmol/l, regardless of whether cortisol (F) or corticosterone (B) was used, as this concentration was within the physiological range of glucocorticoids in the rat. Likewise, the cofactor concentration was pre-selected; 400µmol/l was used for both $NADP^+$ and NAD^+ to ensure that sufficient (excess) concentrations of these cofactors were present in the incubate. Renal tissue was obtained from normal male Sprague-Dawley rats (weight 150-220g) which had been allowed free access to water and standard rat diet (140 mmol/l sodium and 180 mmol/l potassium per kg dry weight). Animals were anaesthetised with pentobarbitone sodium (60mg/kg, i.p.; Sagatal, Rhone Merieux, Harlow, UK) and their kidneys rapidly excised and snap-frozen in liquid nitrogen. Kidneys were then stored at -20°C until required.

Renal tissue was homogenised in hypotonic lysis buffer (5mmol/l Tris, 1.5mmol/l $MgCl_2$, 1.5mmol/l Ethylenediaminetetra-acid; EDTA) (Rusvai and Naray-Fejes-Toth, 1993) using a glass dounce homogeniser. Isotonicity was restored by the addition of 1.5M KCl (final KCL concentration 150mmol/l; tissue concentration 25mg wet weight/ml). Dulbeccos modified phosphate-buffered saline (PBS) was then added (7:1 (v/v) PBS:homogenate).

2.2.1.1. Homogenate preparation

In preliminary studies to determine optimal homogenate preparation, an aliquot of homogenate (prepared as described above) was centrifuged at 1000 xg for 15 min at 4°C. The supernatant was then aspirated and labelled as S1; the pellet was resuspended in PBS to an equal volume of the original homogenate and labelled as P1. An aliquot of S1 was then recentrifuged at 1000 xg for a further 15 min at 4°C, the supernatant aspirated and labelled as S2 and the pellet resuspended in PBS to the volume of S1, and labelled as P2. 11ßHSD activities were subsequently determined in all homogenate preparations using the radiometric conversion assay described below except that samples were incubated for 60min instead of 30min. On the basis of the results obtained from these preliminary studies (enzyme activity was highest in the S1 supernatant compared to other preparations), in all subsequent experiments, the supernatant of homogenates which had been centrifuged at 1000x g for 30 min at 4°C, were used in all incubations to derive a preparation which has high enzyme activity with the minimum amount of solid residue.

800µl of each supernatant was transferred to glass assay tubes and pre-incubated at 37°C for 30 min. 100µl 4mmol/l NADP$^+$ or NAD$^+$ (final concentration 400µmol/l; Sigma Chemical Company, Poole, UK) and 100µl 5000nmol/l [1,2,6,7-^{3}H]-cortisol (final concentration 500nmol/l; 1µCi/ml; Amersham International) were then added to each tube. Optimum incubation assays were then established.

2.2.1.2. Incubation period

In these preliminary experiments, homogenates were incubated over a time course of 10, 20, 30, 60 and 120 min at 37°C. The results of these preliminary studies (Figure 2.1) demonstrate that the rate of F oxidation was linear in the first 30 minutes of incubation time. Thus, all subsequent experiments using 'whole' renal tissue were incubated for 30 min.

Figure 2.1: 11ßHSD activities (pmol F oxidised/mg protein.unit time) in rat renal homogenates, measured *ex vivo* in the presence of 400μmol/l $NADP^+$ or NAD^+ and 500nmol/l F over 10 to 120 min time period.
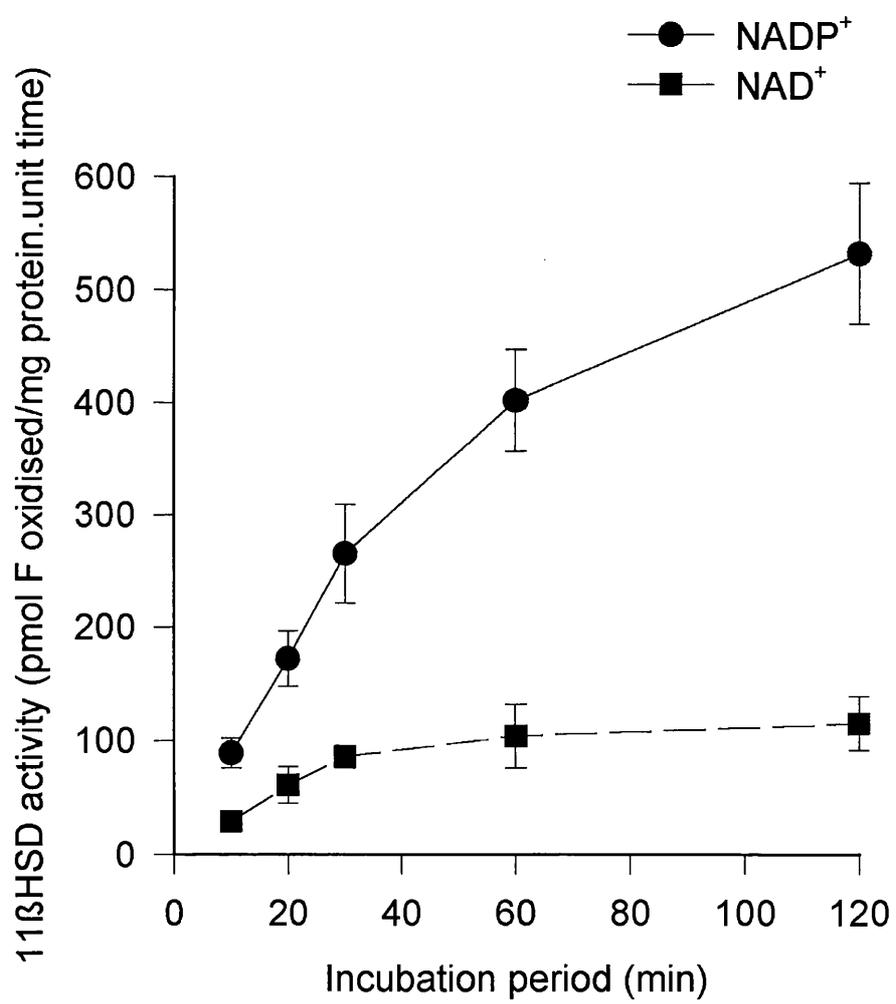
After incubation, the [³H]-steroids were extracted with two volumes of ice-cold chloroform and the tubes vortexed and centrifuged at 1000x *g* for 30min at 4°C. The aqueous phase was then aspirated and the organic extract evaporated to dryness under nitrogen at 40°C. [³H]-steroid residues were resuspended in ethyl acetate containing 1mmol/l F and 1mmol/l E, and the samples transferred onto aluminium-backed Silica 60 TLC plates. These were then resolved in 92:8 (v/v) chloroform:95% (v/v) ethanol (Edwards *et al*, 1988). The bands containing the F and E were identified by absorption of short-wave (254nm) UV light and conversion of [³H]-F to [³H]-E was quantified using a Bioscan 200 TLC radiochromatogramme scanner (Lablogic, Sheffield, UK). 11ßHSD activities were expressed in pmol F oxidised/mg protein.30min. The protein concentration of homogenates was assayed by the protein-binding dye assay of Bradford (Bradford, 1976).

2.2.1.3. Routine 11ßHSD assay conditions for renal homogenates

Unless stated otherwise, in all subsequent enzyme assays using 'whole' (ie, biopsies of whole organs) tissue homogenates (tissue concentration 25mg wet weight/ml) to determine *ex vivo* 11ßHSD activities, incubations were carried out for 30min in the presence of 400μmol/l cofactor and 500nmol/l substrate. All conditions in each experiment were performed in triplicate.

2.2.1.4. CBX concentration

Preliminary studies were required to determine which CBX concentrations to use for the establishment of CBX dose-response curves *in vivo*. Six CBX concentrations (6μg, 0.06mg, 0.6mg, 3mg, 6mg and 15mg/hr) were initially infused i.v. to anaesthetised male Sprague-Dawley rats (as described in section 2.2.2). 11ßHSD assays of kidneys obtained from these animals were assayed using the optimal conditions described above and the results are presented in table 2.1. On the basis of these results, 3 doses of CBX (0.06mg, 0.6mg and 6mg/hr) were used in the subsequent clearance studies.

Table 2.1. *Ex vivo* 11ßHSD activities in renal tissue taken from CBX-treated rats incubated with 400µmol/l NADP$^+$ or NAD$^+$ and 500nmol/l F. 11ßHSD activities are expressed pmol F oxidised/mg protein.30min. All values are mean (± sem) n=4 rats for each CBX dose.

	[CBX]/hr						
	0	6µg	0.06mg	0.6mg	3mg	6mg	15mg
NADP$^+$	332 ± 29	325 ± 15	189 ± 28	107 ± 11	112 ± 33	5 ± 1	2 ± 0
NAD$^+$	72 ± 6	79 ± 11	37 ± 6	42 ± 6	38 ± 9	3 ± 1	0 ± 0

2.2.2. Clearance studies: The effect of CBX administration on renal sodium and potassium transport

(clearance studies were performed by K.J. Sewell and D.G Shirley in the renal physiology laboratory at Charing Cross and Westminster Medical School, London, UK)

2.2.2.1. Surgical protocol

Experiments were performed on male Sprague-Dawley rats (weight range 200-250g). Prior to experimentation, animals were allowed free access to water and standard rat diet (140 mmol/l sodium and 180 mmol/l potassium per kg dry weight). All procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986.

Animals were anaesthetised with Intraval (110mg/kg body weight, i.p; May and Baker Ltd., Dagenham, UK) and prepared surgically for renal clearance studies as follows. Core body temperature (measured via a rectal thermometer) was maintained at 37°C, using a thermostatically controlled heating blanket (Braun Medical, Aylesbury, UK). Two polythene cannulae (PP50 tubing; Jencons (Scientific) Ltd., Leighton Buzzard, UK) were placed in the right jugular vein, the bladder was catherterised (PP100), a tracheotomy was performed, and the right femoral artery was cannulated with polyethylene tubing (PP50), containing heparinised saline to allow blood sample collection and blood pressure measurement. This cannula was connected to an electronic pressure transducer (Lectromed, Letchworth, UK). Arterial blood pressure was monitored continuously throughout the period of renal clearance measurement on a Macintosh LCII (Rothwell Group, Farnborough, UK) using MacLab peripheral and Chart v3.3.5 software (AD instruments, Hastings, UK).

2.2.2.2. Infusion protocol

Isotonic saline was infused i.v. at 2ml/hr throughout the experiments. One hour after completion of surgery, [^{3}H]-inulin (2µCi primer, 2µCi/hour; Amersham International, Aylesbury, UK) was included in the i.v. saline infusion. Following a one hour equilibration period, all rats underwent a one hour control period during which clearance measurements were made. The animals were then divided into four treatment groups:

Control (vehicle-only) animals (n=10) continued to receive isotonic saline at 2ml/hr throughout the remainder of the experiment (3.5 hours). During the final 1.5 hours (designated the experimental period), urine was continuously collected for analysis. The two hour interval between the control and experimental periods allowed sufficient time for any mineralocorticoid-like effect to develop following inhibition of 11ßHSD with CBX (Morris and Brem, 1987).

In CBX-treated animals, at the end of the control period, CBX was included in the saline infusion at one of three doses: 0.06 mg/hr (n=8 rats), 0.6 mg/hr (n=8 rats) or 6 mg/hr (n=8 rats) for the remainder of the experiment (3.5 hours). Clearance measurements were made in the final 1.5 hours as above.

In all four groups of rats, at the end of each experiment, the left kidney and a biopsy of the liver were removed and snap frozen in liquid nitrogen. A final arterial blood sample was taken for the measurement of haematocrit and of plasma sodium and potassium concentrations.

2.2.3. Radiometric conversion assay of renal 11ßHSD activities

11ßHSD activities in the snap-frozen renal tissue removed from rats at the end of the clearance experiments were measured using the radiometric conversion assays described in section 2.2.1.3. For each assay, a quarter of each kidney was prepared by sequentially bisecting the kidney in the sagittal and transverse planes.

2.2.4. Radiometric conversion assay of hepatic 11ßHSD activities

Hepatic 11ßHSD activities were assayed in the presence of 400μmol/l $NADP^+$ using a homogenate of rat liver (25mg wt weight/ml) prepared as for the renal homogenates. For the liver assays, 11KSR activities (expressed as pmol/l cortisone (E) reduced/mg protein.30min) were measured as for the renal homogenate assays except the liver homogenates were incubated in the presence of 400μmol/l NADPH using 100nmol/l $[1,2,6,7-^3H]$-cortisone (0.1μCi/ml) as substrate.

The $[^3H]$-cortisone was synthesised in house by overnight incubation of renal homogenate at 37°C with $[^3H]$-cortisol and $NADP^+$. After resolution of the $[^3H]$-steroids by TLC, the $[^3H]$-cortisone was eluted from the relevant area of the TLC plate using dichloromethane. This organic extract was then evaporated under nitrogen and the $[^3H]$-cortisone was resuspended in ethanol to a final specific activity of 0.1μCi/5μl.

2.2.5. Analyses

Sodium and plasma concentrations in plasma and urine were measured by flame photometry (Model 543, Instrumentation Laboratory, Warrington, UK). [^{3}H]-inulin activities in 5μl samples of urine and plasma, dispensed in 8ml Aquasol 2 scintillation cocktail (Packard, Pangbourne, UK), were measured by ß-emmission spectroscopy (model 2000 CA, Packard, Pangbourne, UK).

2.2.6 Calculations

Glomerular filtration rate (GFR) was determined as the clearance (C) of [^{3}H]-inulin and calculated using the standard formula:

$$C_X = (U_X/P_X)V,$$

where U_X and P_X are the concentrations of x in urine and plasma respectively and V is urine flow rate. The urinary excretion rates of sodium and potassium were calculated using the following expression: $U_X.V$.

2.2.7 Statistics

Data are presented as means ± standard errors of the mean (sem). Statistical comparisons were made using one-way analysis of variance (ANOVA) to assess differences between groups within the control and experimental period. Point to point comparisons were made using *post-hoc* Tukey-Kramer multiple comparisons tests and in both tests statistical significance was taken as *P*<0.05. In all 4 groups, n=8-10 animals.

2.3. Results

2.3.1. Animal parameters

There were no significant differences in body weights and plasma sodium and potassium concentrations between the 4 groups of rats (Table 2.2).

Table 2.2. Body weight and plasma data from rats infused i.v with CBX (0mg, 0.06mg, 0.6mg and 6mg/hr). P_K and P_{Na} are the plasma concentrations of potassium and sodium, respectively.

[CBX]	0mg/hr	0.06mg/hr	0.6mg/hr	6mg/hr
Body wt (g)	262 ± 3	262 ± 3	260 ± 2	263 ± 3
P_K (mmol/l)	3.9 ± 0.1	4.4 ± 0.2	4.1 ± 0.1	4.0 ± 0.1
P_{Na} (mmol/l)	140 ± 1	139 ± 1	140 ± 2	138 ± 1

There was no change in mean arterial blood pressure (MABP) between the control and experimental period and there were no differences between the 4 groups of animals. Blood pressure fell slightly during the course of the experiment, but the administration of CBX had no effect (Figure 2.2). No changes in GFR were observed either between control and experimental periods or after CBX infusion (Figure 2.2).

Figure 2.2: Mean arterial blood pressure (MABP) and glomerular filtration rate (GFR) during the control and experimental periods in rats infused i.v. with CBX (0.06mg, 0.6mg, and 6mg/hr).
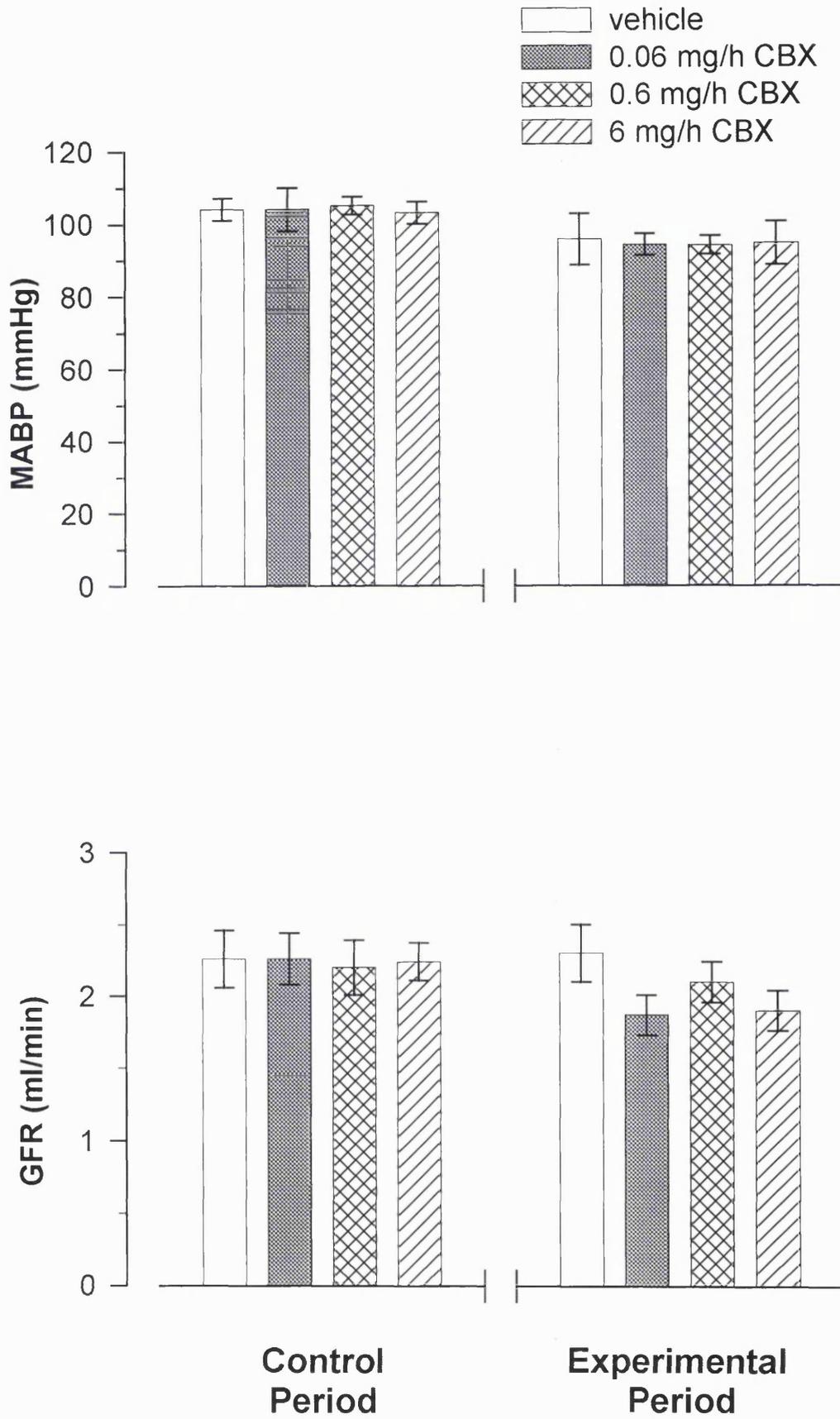
2.3.2. Urinary sodium and potassium data

Urinary fractional excretion of sodium (FE_{Na}) was the same in all 4 treatment groups during the control period (Figure 2.3). During the experimental period, when compared to vehicle-infused rats, FE_{Na} was significantly lower only in animals who had received the highest dose of CBX (6mg/hr) ($P<0.05$; Figure 2.3). Similar to the sodium excretion observations, there were no differences in the fractional excretion of potassium (FE_K) between the 4 groups of animals during the control period (Figure 2.3). There was no change in FE_K with the administration of any of the 3 doses of CBX when compared to vehicle-infused rats (Figure 2.3).

No significant difference in urinary sodium/potassium ratio (U Na/K ratio) was observed between the 4 groups of animals during the control period (Figure 2.4). During the experimental period, the U Na/K ratio was significantly lower in the high dose (6mg/hr) CBX-infused rats compared to those which received vehicle only (Figure 2.4; $P<0.01$). At doses of 0.6 and 0.06mg/hr, however, CBX had no effect on the U Na/K ratio.

Figure 2.3: Fractional sodium excretion (FE_{Na}) and fractional potassium excretion (FE_K) during the control and experimental periods in rats infused i.v. with CBX (0.06, 0.6, 6mg/hr). *$P<0.05$ compared with vehicle-infused rats during the experimental period.
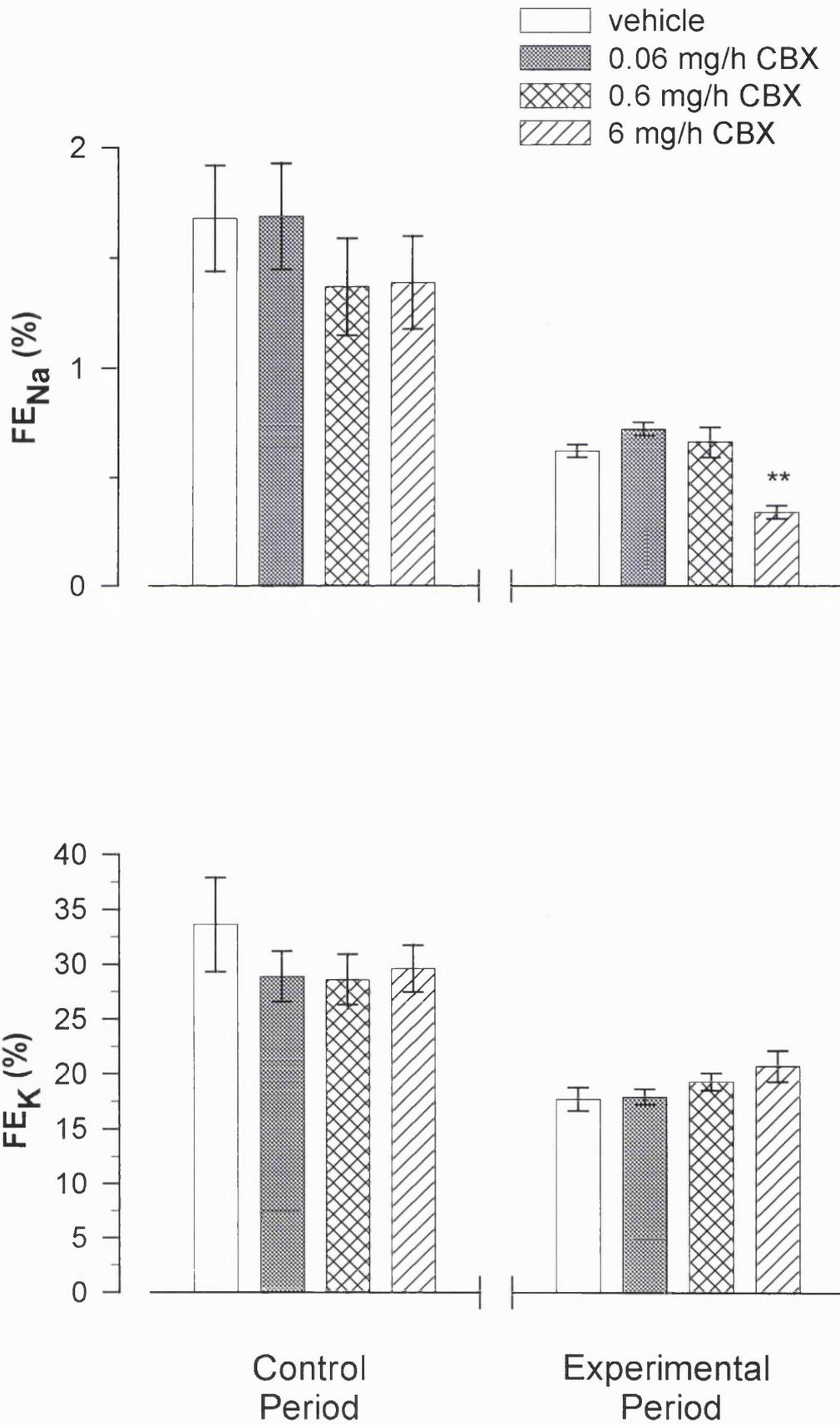
Figure 2.4: Urinary Sodium/potassium ratio (U Na/K ratio) during the control and experimental periods in rats infused i.v. with CBX (0.06, 0.6, 6mg/hr). **$P<0.01$ compared with vehicle-infused rats during the experimental period.
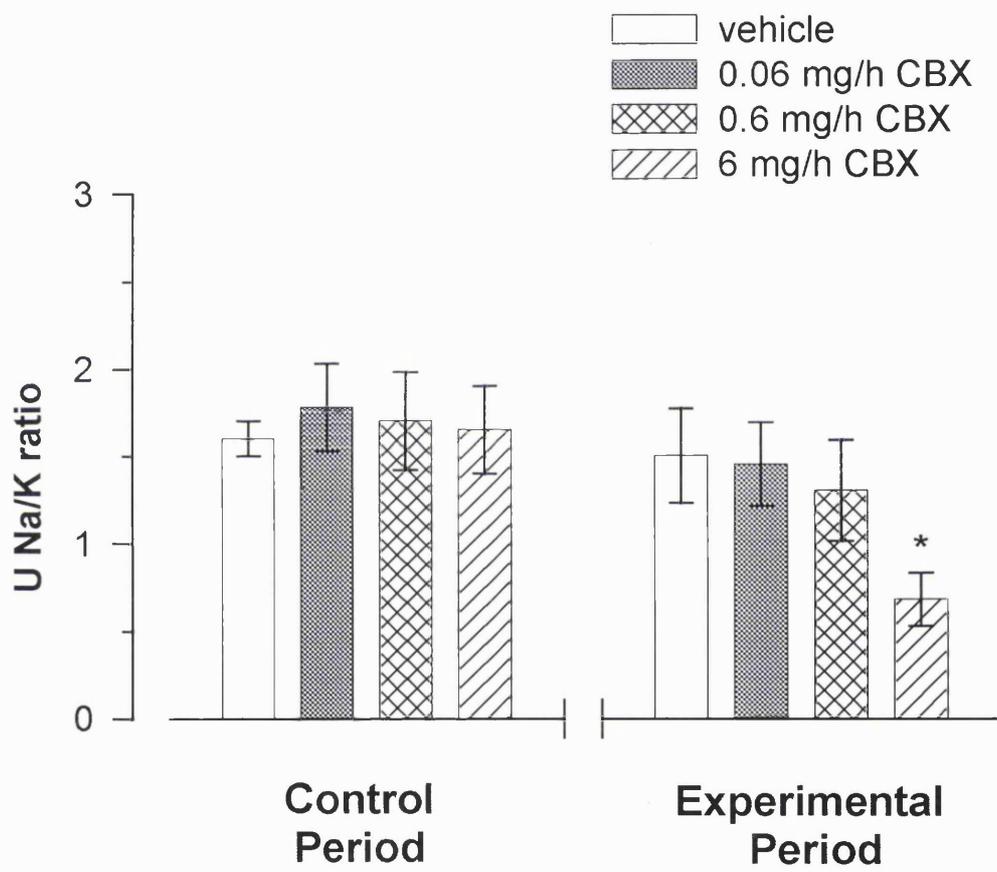
2.3.3. 11ßHSD activities.

CBX caused a dose-dependent inhibition of renal 11ßHSD activity regardless of which cofactor was used (Table 2.3; Figure 2.5a). While $NADP^+$-dependent cortisol oxidation was inhibited only at the intermediate (0.6mg/hr) and high (6mg/hr) doses of CBX ($P<0.001$ for both), NAD^+-dependent 11ßHSD activity was significantly inhibited at all 3 tested CBX doses ($P<0.001$ for 0.6 and 6mg/hr; $P<0.05$ for 0.06mg/hr).

Table 2.3. Ex vivo 11ßHSD activities in renal tissue taken from CBX-treated (0.06mg, 0.6mg, 6mg/hr)rats and incubated with 400µmol/l $NADP^+$ or NAD^+ and 500nmol/l F. 11ßHSD activities are expressed as pmol F oxidised/mg protein.30min. All values are mean (± sem), n=8-10 rats. *$P<0.05$, ***$P<0.001$ compared with control value.

	[CBX]			
	0mg/hr	0.06mg/hr	0.6mg/hr	6mg/hr
$NADP^+$	292 ±72	182 ± 28	66 ± 27***	4 ± 1***
NAD^+	86 ± 22	44 ± 8*	20 ± 5***	4 ± 2***

Hepatic $NADP^+$-dependent 11ßHSD activity was significantly inhibited in rats infused with both the intermediate and high dose CBX (Table 2.4; Figure 2.5b, $P<0.001$ for both). In contrast, inhibition of NADPH-dependent 11KSR reduction of cortisone was less pronounced and only achieved statistical significance at the highest dose of 6mg/hr (Table 2.4; Figure 2.5b, $P<0.05$).

Table 2.4: *Ex vivo* activities of 11ßHSD and 11KSR in hepatic tissue taken from CBX-treated rats and incubated with 400µmol/l $NADP^+$ and 500nmol/l F or 400 NADPH and 100nmol/l E, respectively. 11ßHSD activity is expressed as pmol F oxidised/mg protein.30min. 11KSR activity is expressed as pmol E reduced/mg protein.30min. All values are mean (± sem), n=8-10 rats. $*P<0.05$, $***P<0.001$ compared with control value.

	[CBX]			
	0mg/hr	0.06mg/hr	0.6mg/hr	6mg/hr
11ßHSD ($NADP^+$)	626 ± 149	537 ± 93	94 ± 21***	11 ± 3***
11-KSR (NADPH)	74 ± 15	50 ± 12	39 ± 15	12 ± 4*

Figure 2.5: a) Renal 11ßHSD activities and b) Hepatic 11ßHSD and 11-KSR activities (% of control) in rats administered CBX (0.06, 0.6, 6mg/hr). Each value is calculated as the comparison of the mean 11ßHSD or 11-KSR activity in CBX-infused rats with that of the vehicle-infused rats (see Tables 2.3 and 2.4).

KIDNEY

Legend:
- ● NAD$^+$ (11ßDH)
- ■ NADP$^+$ (11ßDH)

% control vs CBX (mg/hr)

LIVER

Legend:
- ■ NADP$^+$ (11ßDH)
- ▲ NADPH (11KSR)
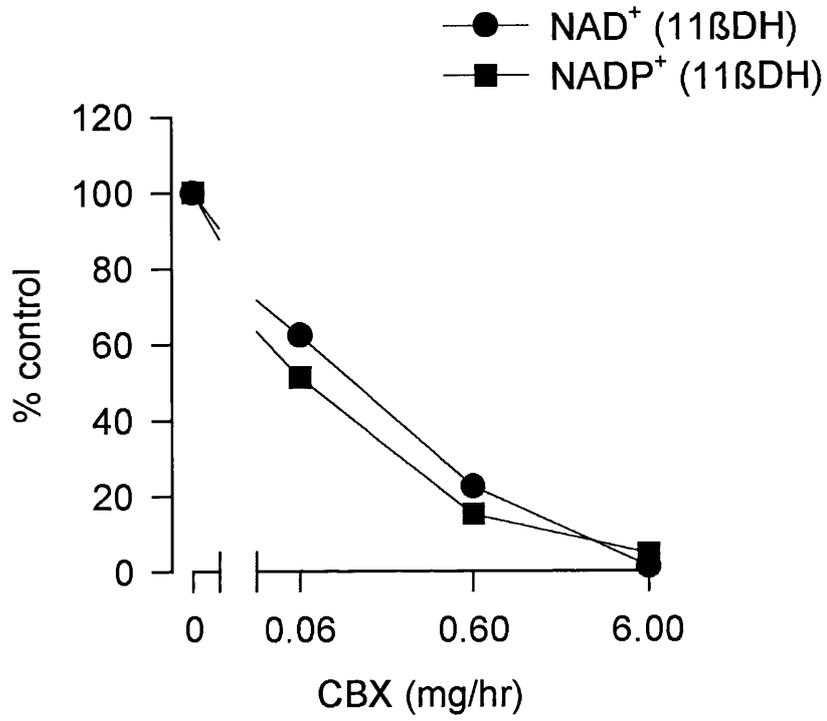
% control vs CBX (mg/hr)
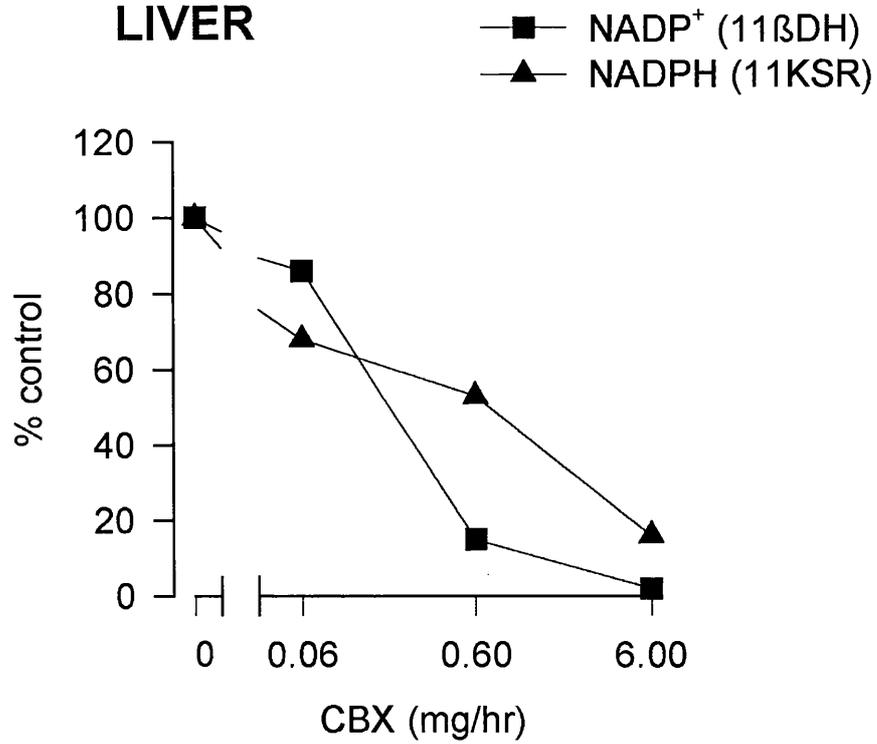
2.4. Discussion

The putative physiological role of 11ßHSD within the kidney is based on the effects of mutations in the HSD11B2 gene, or pharmacological inhibition, which results in an antinatriuresis and a decrease in urinary sodium/potassium ratio (New et al, 1977, Ulick et al, 1997, Souness and Morris, 1989, Stewart et al, 1990).

In the series of experiments described in the current chapter, the substantial decrease in renal glucocorticoid oxidation following the infusion of 6mg/hr CBX was associated with a decrease in urinary fractional sodium excretion and a concomitant fall in the urinary sodium/potassium ratio. This apparent increase in mineralocorticoid effect is consistent with increased glucocorticoid access to the MR following inhibition of 11ßHSD activities and as such supports the hypothesis of a 'guardian' role for the enzyme.

It is recognised that the use of different cofactors ($NADP^+$ and NAD^+) does not allow absolute discrimination between the activities of specific isoforms of 11ßHSD, since 11ßHSD1, though preferentially utilising $NADP^+$ as an oxidant cofactor, can also employ NAD^+, while 11ßHSD2 has an absolute requirement for NAD^+ (Agarwal et al, 1994; Albiston et al, 1994). Consequently, glucocorticoid oxidation in the presence of $NADP^+$ can be attributed solely to 11ßHSD1 whereas oxidation in the presence of NAD^+ will reflect the activities of both 11ßHSD isoforms. Therefore, substantial decreases in NAD^+-dependent cortisol oxidation could reflect inhibition of 11ßHSD1 which is not necessarily accompanied by inhibition of the type 2 isoform in the distal nephron. In the study described in the current chapter however, the ability of 0.06mg/hr CBX to significantly inhibit NAD^+-dependent 11ßHSD activity without affecting $NADP^+$-dependent cortisol oxidation does indicate inhibition of 11ßHSD2 in vivo at this lowest tested dose. Irrespective of specificity of action on particular renal 11ßHSD isoforms, the fact remains that at the intermediate CBX dose of 0.6mg/hr, both $NADP^+$-dependent and NAD^+-dependent 11ßHSD activities were

inhibited by at least 75% without significantly affecting overall urinary sodium or potassium excretion.

Since high dose CBX administration was associated with substantial inhibition of renal 11ßHSD activities, it is reasonable to assume that the concomitant antinatriuresis observed in these animals reflects enhanced renal mineralocorticoid effects elicited by the higher intrarenal concentrations of glucocorticoids. It might be expected therefore, that an increase in urinary potassium excretion as a result of MR-stimulated potassium secretion may have occurred in these CBX treated animals. However, despite a tendency for potassium excretion to increase during the experimental period following infusion of high dose CBX (whilst the converse was true during the control period) this change was not statistically significant. The absence of kaliuresis, despite antinatriuresis following CBX or liquorice administration is consistent with previous studies in both rats (Souness and Morris, 1989) and humans (Stewart *et al*, 1990). Since potassium secretion by the distal nephron only accounts for around 2% of renal potassium transport, it is likely that the secretion of this cation within this nephron segment is 'masked' by potassium transport changes elsewhere in the kidney, such that no overall change in urinary potassium excretion is observed. Indeed, Field *et al* (1984) have demonstrated that in adrenalectomised rats, the administration of aldosterone resulted in the expected increase in potassium secretion in perfused distal tubules but *overall* potassium excretion was unaffected despite a fall in urinary sodium excretion. Thus, the absence of a demonstrable kaliuresis, even in the highest dose of CBX-infused rats in the study described in this chapter, may simply reflect the fact that overall urinary potassium excretion is an insensitive marker of an MR-mediated response.

The substantial inhibition of glucocorticoid metabolism, even at the lowest dose of CBX, observed in the current study, is in contrast to the findings of Latif *et al* (1992) who failed to demonstrate inhibition of 11ßHSD activities in kidneys obtained from CBX-treated adrenalectomised rats. This difference may be attributable to the differences in the route of CBX administration. Since, in the current study, the drug was administered intravenously, it is likely that the uncertainty of drug delivery, so implicit in the subcutaneous route of administration used in the prior study, was avoided. It is also possible that the difference in adrenal status between the rats used in the two studies may somehow account for the contrasting effects of CBX on renal 11ßHSD activities, although why such a phenomenon should occur is unknown. It should be emphasised, however, that despite the discrepancy between the two studies, regarding the extent of 11ßHSD inhibition, they both reveal a dissociation between the ability to inhibit renal 11ßHSD by CBX and its 'expected' effects on urinary electrolyte excretion. Thus, while Latif *et al* demonstrated increased mineralocorticoid effects of glucocorticoids without inhibition of renal 11ßHSD activities, this study has demonstrated that substantial inhibition of both $NADP^+$-dependent and NAD^+-dependent cortisol oxidation can occur without effects on urinary electrolyte excretion. This dissociation again accords with the study by Stewart *et al* (1990) in which it was found that CBX administration to humans did not result in an obvious correlation between urinary indices of 11ßHSD activities and sodium excretion.

The observed inhibition of hepatic 11KSR activity in the CBX-treated rats in the current study are in agreement with the conclusions reached by Stewart *et al* (1990), which were based on the attenuation of the rise in plasma cortisol following administration of cortisone acetate in human subjects who had ingested CBX. The most significant finding of the hepatic enzyme assays was the demonstration that the lower doses of CBX, which inhibited $NADP^+$-dependent 11ßHSD activity in both hepatic and renal tissue by at least 50%, failed to inhibit NADPH-dependent hepatic 11KSR activity. This

demonstration of differential sensitivities of hepatic reductase versus dehydrogenase activities is consistent with previous *in vitro* studies for recombinant 11ßHSD1 (Agarwal *et al*, 1990). The differential sensitivities of dehydrogenase and reductase activities suggest that administration of low-dose CBX may inhibit the oxidation of glucocorticoids preferentially in the kidney with less of an impact on hepatic glucocorticoid metabolism. Hence, in subjects treated with low-dose CBX, it is plausible that the urinary ratio of F:E (or THF:THE) preferentially reflects changes in *renal* 11ßHSD activities.

The findings of this first series of experiments suggest that the commonly held paradigm of the role of renal 11ßHSD may not be as straightforward as once perceived. There is no questioning the significance of HSD11B2 mutations in the genesis of AME, but the discrepant observations of inhibiting 11ßHSD activities with pharmacological agents continues to raise questions as to whether this enzyme-mediated protection of MR is impenetrable or, indeed, the only major factor in the protection of the promiscuous MR. One option is that 11ßHSD-mediated protection of renal MR is extremely efficient and has a high functional capacity, such that the enzyme can be 80% inhibited but still fulfil its role as 'guardian' of MR. Indeed, as stated in chapter 1, there is evidence to suggest that partial inactivation of the enzyme results in milder symptoms of AME (Wilson *et al*, 1995; Mune *et al*, 1995).

Alternatively, it is possible that the antinatriuretic effect of high-dose CBX may result from a direct interaction between CBX and MR. Indeed, not only have both CBX and GA, at high concentrations, been shown to displace the binding of [^{3}H]-aldosterone from MR (Arminini *et al*, 1982) but CBX has also been reported to alter renal sodium and potassium excretion in the absence of adrenal steroids (Arminini *et al*, 1982). Although this option cannot be ruled out, a direct effect of CBX on MR-mediated electrolyte transport is unlikely since it has been shown that the affinity of CBX for MR is only approximately 1/15 000 that of aldosterone (Arminini *et al*, 1982).

It is also possible that an alternative biochemical mechanism is accountable for at least some of the renal effects observed at the high dose of CBX. CBX is a non-specific inhibitor of short chain alcohol dehydrogenases (SCADs) (Baker, 1991; Ghosh *et al*, 1995) and as such, it is possible that the acute effects of high-dose CBX on renal sodium excretion may be attributable to the inhibition of enzymes other than 11ßHSD. 15-Hydroxyprostaglandin dehydrogenase (PGDH), a member of the SCAD family, is responsible for the metabolism, and hence, rapid inactivation of prostaglandins. PGDH has been shown to be inhibited by CBX, as evidenced by the increased half life of prostaglandins (Peskar *et al*, 1976; Baker, 1991) Since renal prostaglandins are known to affect renal function (See Bonvalet *et al*, 1987), it was possible that elevated intrarenal prostaglandins, in addition to the higher intrarenal glucocorticoid concentrations, may have been responsible for the changes in electrolyte excretion observed in this series of experiments. To test this hypothesis, preliminary assays of [^{3}H]-PGF2α metabolism in the renal tissue obtained in this study were performed to establish any differences in PGDH activities in control rats compared to CBX-treated animals. It was found that at the highest dose of 6mg/hr, CBX inhibited renal NAD$^+$-dependent PGDH activity by 40%. At the lower doses, which had no effect on urinary electrolyte excretion, CBX had no effect on the oxidation of [^{3}H]-PGF2α. This association between the inhibition of renal PGDH activity and urinary electrolyte excretion is not necessarily causal but supports the view that acute effects of CBX on renal sodium transport cannot be attributed solely to the inhibition of renal 11ßHSD activities.

In summary, the major finding of this series of experiments was that substantial inhibition (>75%) of renal 11ßHSD activities occurred at the intermediate dose of CBX with no effect on either sodium or potassium urinary excretion. Furthermore, almost complete inhibition (>95%) of both isoforms of renal 11ßHSD was observed, with no evidence of kaliuresis, despite decreased urinary sodium excretion. These findings suggest that 11ßHSD may not be the only mechanism for conferring aldosterone specificity on renal MR.

The main limitations of this study were the possibility of changes in potassium secretion in the distal nephron being 'masked' by overall tubular potassium transport and the inability to discriminate clearly between the two isoforms of 11ßHSD, simply by incubating with $NADP^+$ or NAD^+. The next series of experiments were designed to overcome these constraints and are described in the following chapter.

Chapter 3:

Inhibition of 11ß-hydroxysteroid dehydrogenase activities in proximal and distal nephron segments: effects on sodium and potassium transport

3.1. Introduction

From the experiments described in Chapter 2, it is apparent that renal 11ßHSD activities can be substantially inhibited with no detectable alteration in sodium or potassium excretion. In addition, following almost complete inhibition of renal cortisol oxidation at 6mg CBX/hr, whilst overall urinary sodium excretion was reduced, the anticipated kaliuresis was not observed. Previous studies have shown that changes in distal tubular potassium secretion can occur which are undetectable in the final urine (Field *et al*, 1984). Moreover, the use of kidney homogenates containing different segments of the nephron does not clearly discriminate between the type 1 and type 2 isoforms of 11ßHSD and, like the urinary clearance data described in Chapter 2, can only provide indirect information on events which may be occurring at the level of the single nephron. Furthermore, the use of adrenal intact rats in the previous studies did not allow the control of endogenous corticosteroid concentrations in animals, fluctuations in which could affect the response of the tubule to CBX infusion.

In order to obtain more detailed information relating to the effects of 11ßHSD inhibition on renal tubular function, a series of free-flow micropuncture experiments was performed on adrenalectomised rats (receiving replacement physiological concentrations of aldosterone and dexamethasone) infused with high dose (6mg/hr) CBX. In this type of experiment, native tubular fluid is collected from the early and late regions of the superficial distal tubule. Thus, the technique allows aspects of single nephron function to be investigated. In parallel, 11ßHSD activities were determined in microdissected nephron segments of PCT, cTAL, mTAL, CCD and OMCD. Although these studies have previously been performed by Kenouch *et al* (1994), 11ßHSD activities in their study were investigated in female Wistar rats, and, moreover, with very low (10nmol/l) substrate concentrations, such that 11ßHSD activities were established under non-linear conditions with 70-100% conversion of substrate to product.

The aims of the studies described in this chapter were 1) to characterise $NADP^+$-dependent and NAD^+-dependent 11ßHSD activities along segments of the nephron in the male Sprague-Dawley rat using first order kinetic conditions and 2) to investigate the effects of CBX infusion i.v. on sodium and potassium transport in the distal nephron and on renal corticosterone oxidation in PCT and CCD segments.

3.2. Materials and Methods

3.2.1. Investigation into 11ßHSD distribution along the rat nephron: establishment of the 11ßHSD assay for microdissected segments

Optimisation of the enzyme assay was necessary for the measurement of 11ßHSD activities in microdissected nephron segments so that approximately 10% conversion of substrate to product was observed in CCD (the nephron segment reputed to have the highest 11ßHSD activity in the rat) using the minimal amount of tissue possible.

3.2.1.1. Microdissection study: surgical protocol

Experiments were performed on male Sprague-Dawley rats (weight range 139-150g) which had been allowed free access to water and a standard rat diet (see section 2.2.2.1).

Animals were anaesthetised with pentobarbital sodium (60mg/kg,i.p; Sagatal, Rhone Merieux, Harlow, UK) the aorta cannulated with polyethylene tubing (PP50), and the left kidney perfused with 4ml ice cold perfusion solution (containing in mmol/l concentration: NaCl, 137; KCl, 5; $MgSO_4$, 0.8; Na_2HPO_4, 0.33; KH_2PO_4, 0.44; $MgCl_2$, 1; $CaCl_2$, 1; D-glucose, 5; Tris-HCl, 10, 20% (w/v) BSA; pH 7.4) followed by perfusion of 5ml collagenase solution (same solution containing 0.1% (w/v) collagenase (186 U/mg; Worthington Biochemical Corporation, New Jersey, USA)). At the end of perfusion, both kidneys were removed; the right kidney was rapidly-frozen in liquid nitrogen.

3.2.1.2. Isolation of tubular segments

Thin pyramid pieces of the left kidney (including cortex and medulla) were incubated in 0.1% (w/v) collagenase solution gassed continually with 95% (v/v) O_2 and 5% (v/v) CO_2, at 30°C for 18 min. The pyramids were then rinsed 3 times in ice cold collagenase-free perfusion solution and

microdissection performed under a stereomicroscope in collagenase-free perfusion solution at 4°C. PCT and CCD were then isolated and 3 or 5mm segments stored in 20 μl hypotonic lysis buffer (see 2.2.1 for composition) at -20°C until the day of experiment.

3.2.1.3. 11ßHSD enzyme assay: Preliminary method 1

Since enzyme assays using renal homogenates were incubated in a final volume of 1ml, an attempt was made to optimise the enzyme assay for microdissected nephron segments in similar conditions, ie, in a final incubation volume of 1ml using the same concentrations of cofactors (400μmol/l) and substrate (500nmol/l). 3 or 5 mm nephron segments were thawed at room temperature before a further 70μl hypotonic lysis buffer was added to each micro test-tube and the samples homogenised for 2 min. 10μl 1.5mol/l KCl was then added to the sample before its transferal (all 100μl) to a glass incubate tube containing 700μl PBS. The homogenate was then pre-incubated at 37°C for 30 min, before the addition of 100μl 167, 1000 or 5000nmol/l [^{3}H]-F (final concentration, 16.7, 100 and 500nmol/l, respectively) and 100μl 4mmol/l NADP$^+$ or NAD$^+$ (final concentration, 400μmol/l). The incubation was then continued at 37°C for 1hr. Each condition was performed in triplicate.

Table 3.1: 11ßHSD activity (% F oxidised/mm.1hr) in individual rat PCT and CCD incubated with 400μmol/l NADP$^+$ or NAD$^+$. All values are mean (± sem) n=4 animals.

	PCT		CCD	
[F] nmol/l	NADP$^+$	NAD$^+$	NADP$^+$	NAD$^+$
16.7	11 ± 2	3 ± 1	1 ± 0	1 ± 0
100	7 ± 2	2 ± 1	0 ± 0	1 ± 0
500	6 ± 1	4 ± 2	0 ± 0	0 ± 0

Despite detectable 11ßHSD activity observed in the PCT at all substrate concentrations, very little conversion was observed in the CCD under these conditions. Moreover, increasing the sample size from 3mm to 7mm and 13mm/tube with either 16.7 or 100nmol/l F did not increase 11ßHSD activity (0.6 ± 0.1% and 0.8 ± 0.3% F oxidised/mm.1hr), respectively, n=3 animals) in the CCD even in the presence of both cofactors.

Neither the presence of $NADP^+$ nor NAD^+ in the incubations increased 11ßHSD activities in CCD to any detectable level. Given the fact that high levels of glucocorticoid oxidation could be measured in PCT there was concern that the presence of collagenase may have been decreasing NAD^+-dependent 11ßHSD activity. This possibility was investigated by incubating renal homogenate, taken from 3 untreated male Sprague-Dawley rats, as described in section 2.2.1, in 4 concentrations of collagenase (0, 0.05, 0.1, 0.2%; (w/v)), commonly used for *in vitro* perfusion of the kidney. The results (Figure 3.1) show that although both $NADP^+$- and NAD^+-dependent 11ßHSD activities are significantly decreased by collagenase *in vitro*, they are decreased to the same degree. The lack of any substantial difference between inhibition of the two isoforms did not explain why sufficient $NADP^+$-dependent 11ßHSD activity could be observed in PCT whilst no activity could be detected with either $NADP^+$ or NAD^+ in CCD. The use of 1ml final incubation volume was not adequate to allow measurement of 11ßHSD activities in rat CCD, despite various lengths of nephron segments and substrate concentrations.

Figure 3.1: NADP$^+$- and NAD$^+$-dependent 11ßHSD activities (pmol F oxidised/mg protein.30min) in rat renal homogenates incubated in the presence of collagenase *in vitro.*
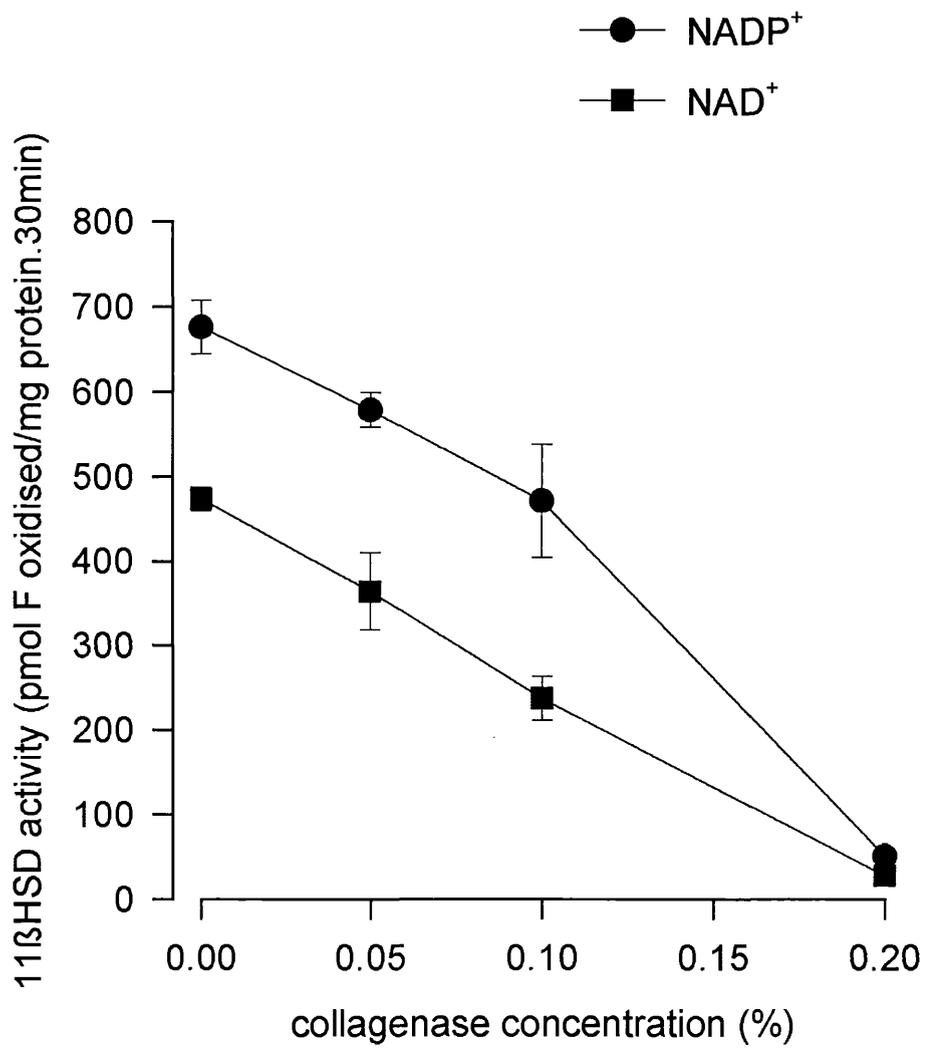
3.2.1.4. 11ßHSD enzyme assay: Preliminary method 2

Three previous studies, in which 11ßHSD activity was quantified in microdissected nephron segments, had been performed with incubation volumes of only 5µl (as opposed to a final incubation volume of 1000µl) using either 10nmol/l (Kenouch *et al*, 1992; Kenouch *et al*, 1994) or 1µmol/l [^{3}H]-B (Alfaidy *et al*, 1995). 11ßHSD activities ranged from 30 to 775 fmol [^{3}H]-A formed/3mm.10 min in PCT and CCD, respectively. All three studies reported at least 1.5-fold higher 11ßHSD activities in CCD compared to PCT. These observations are in contrast to the findings reported above, where no 11ßHSD activity could be detected in CCD.

One possible explanation for these discrepancies was the use in the aforementioned published studies of female Wistar rats and/or the use of B as a substrate. Since it was possible that gender-related differences in 11ßHSD activities may occur, incubations of both PCT and CCD from a female Sprague Dawley rat were performed using method 1 in which 500nmol/l B was as substrate instead of 500nmol/l F. The very low percentage of B oxidised (0.6% and 0.2% in PCT and in CCD segments, respectively) contrasted with the ~16% conversion of B to A reported in the Alfaidy study (1995) using 1µmol/l B.

Since the substrate concentrations (10nmol/l and 1µmol/l) used in the published studies described above encompassed the concentration employed in the current study (500nmol/l), it was considered unlikely that differences in substrate concentrations were the cause of the discrepancies. The other major difference between *Preliminary Method (1)* and the published studies was the incubation volumes. In order to test this, a series of incubations was performed using PCT or CCD (6mm sample/tube) in final volumes of 10, 100, 500 and 1000µl PBS (except 10µl which was carried out in microdissection buffer) containing 1mmol/l NADP$^+$ and 1mmol/l NAD$^+$ and 1µmol/l [^{3}H]-B for 4hr.

Table 3.2: 11ßHSD activities (% B oxidised/mm.4hr) in rat PCT and CCD, in final volumes of 10, 100, 500 and 1000μl, incubated with 1mmol/l $NADP^+$ plus 1mmol/l NAD^+ and 1μmol/l B. Sample size= 6mm/tube. All values are mean (± sem) n=3 animals.

Volume (μl)	PCT	CCD
10	16 ± 2	1 ± 1
100	0 ± 0	0 ± 0
500	1 ± 0	0 ± 0
1000	1 ± 1	0 ± 0

Smaller incubation volumes appear to be necessary to measure 11ßHSD activity in PCT. 11ßHSD activity in CCDs was still undetectable and contradicts the results of Kenouch *et al*, (1994) and Alfaidy *et al*, (1995), who reported ~2-fold higher 11ßHSD activities in CCD compared to PCT.

3.2.1.5. 11ßHSD enzyme assay: Final method 3

The final modification to the enzyme assay is described below and was the method subsequently used in all enzyme assays for microdissected nephron segments described within this thesis. The most important difference between this and *Preliminary Method (2)* was that the enzyme assay was performed *directly* after the tubules were isolated, not after snap-freezing and storage. 3μl 2mmol/l $NADP^+$ or NAD^+, or microdissection buffer (no cofactor condition) and 2 μl $[^3H]$-B (final concentration 1μmol/l; 0.025μCi/μl) were added to each micro test-tube. 1mm PCT or CCD in 1μl microdissection buffer were then added to the appropriate micro test-tube. Tubes were then snap-frozen in liquid N_2 and thawed at room temperature three times to ensure that the nephron segments were permeabilised (Alfaidy *et al*, 1995). Incubation was then carried out for 1 hour at 37°C, after which time the reaction was terminated by the addition of 14μl ice-cold ethyl acetate (containing 1mmol/l A and 1mmol/l B) to each tube. Samples were then stored at -20°C overnight before all 20μl of each sample were transferred to TLC plates and resolved in an atmosphere of 92:8 (v/v)

chloroform:95% (v/v) ethanol (see section 2.2.1). 11ßHSD activities (% [^{3}H]-B oxidised to [^{3}H]-A) were quantified using the Bioscan 200 TLC scanner (see section 2.2.1) and expressed as fmol B oxidised/mm.hr.

The above enzyme assay was repeated using 3mm samples and an incubation time of 30 min to establish which method would provide the least variable data.

Table 3.3: 11ßHSD activities (fmol B oxidised/mm. unit time) in rat PCT and CCD, in the presence of 1mmol/l NADP$^+$ or NAD$^+$ and 1μmol/l B over 60min (1mm/tube) or 30 min (3mm/tube). All values are mean (± sem) n=3 animals for each condition.

	PCT			CCD		
	None	NADP$^+$	NAD$^+$	None	NADP$^+$	NAD$^+$
1mm/60 min	1 ± 1	6 ± 1	6 ± 2	4 ± 2	6 ± 2	10 ± 2
3mm/30 min	1 ± 1	4 ± 2	7 ± 2	3 ± 2	5 ± 2	10 ± 4

Performing the enzyme assay in a small (6μl) volume and on the same day as tubular isolation enables the detection of both NADP$^+$- and NAD$^+$-dependent 11ßHSD activities in PCT and CCD. The optimum amount of tissue and duration of incubation employed in the assay was 1mm incubated for 60 min since this method allowed detection of 11ßHSD activities with less variability than that seen in 3mm samples incubated for 30 min.

3.2.1.6. Routine assay conditions for measurement of 11ßHSD activities in microdissected segments

For the remainder of all assays for measurement of 11ßHSD activities in microdissected nephron segments described within this thesis, the following assay conditions applied: 1mm nephron segments, incubated at 37°C for 60 min with 1μmol/l B (containing [^{3}H]-B; 0.025μCi/μl) and 1mmol/l NADP$^+$ or 1mmol/l NAD$^+$ (final volume; 6μl).

3.2.2. Determination of 11ßHSD activities along the rat nephron.

11ßHSD activities in rat PCT, mTAL, cTAL, CCD and OMCD were measured using the assay conditions described in section 3.2.1.6. Each nephron segment was incubated with either NADP$^+$ or NAD$^+$. Nephron segments were not incubated in the absence of cofactors due to the lack of consistent measurable 11ßHSD activity (except in the CCD) observed in preliminary studies.

3.2.3. Assessment of sodium and potassium transport in the distal tubule of the CBX-treated rat.

The micropuncture studies were performed by K.J. Sewell and D.G Shirley in the renal physiology laboratory at Charing Cross and Westminster Medical School, London, UK.

3.2.3.1. Surgical Protocols

Experiments were performed on male Sprague Dawley rats which had been allowed free access to water and a standard rat diet (see section 2.2.2.1) prior to adrenalectomy. Rats were bilaterally adrenalectomised under short-term halothane anaesthesia. An osmotic minipump supplying base-line levels of aldosterone (0.5μg/100g body weight.24hr) and dexamethasone (1.2μg/100g body weight.24hr) was implanted subcutaneously. The

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replacement dose of dexamethasone was chosen since it has been shown that this is the lowest dose that increases GFR and plasma levels of insulin and glucose in adrenalectomised animals when compared to those in which sham-operations have been performed (Stanton *et al*, 1985). The replacement dose of aldosterone was chosen because it resembled the daily secretory rate in rats and resulted in plasma levels of aldosterone similar to those measured in awake unstressed rats (Stanton *et al*, 1985). All ADX animals were allowed free access to both normal drinking water and 2.7% (w/v) saline.

7-9 days after adrenalectomy, animals (body weight range 230-260g) were anaesthetised with Intraval (110 mg/kg body weight, i.p.) and prepared surgically for micropuncture experiments. Following the surgery described in section 2.2.2.1, the rat was turned onto its right side and the left kidney exposed via a flank incision. The kidney was then cleared of perirenal fat and once isolated, the kidney was immobilized in a Perspex cup which was firmly clamped to the operating table. Throughout the duration of the experiment the kidney surface was bathed with paraffin oil heated to 37°C. Finally, the left ureter was cannulated close to the pelvis with narrowed polyethylene tubing (PP50).

3.2.3.2. Infusion protocol

Initially, each rat was infused i.v. with isotonic saline containing B (100µg/100g body weight.hr; Sigma) at a rate of 2 ml/hour. During the final hour of surgery an extra volume of saline, equivalent to 0.5% body weight (ie, 1 to 1.25ml) was infused in order to replace surgical losses. Following the completion of surgery, one hour was allowed before a bolus dose of 60µCi [^{3}H]-inulin in isotonic saline was administered; the same dose of [^{3}H]-inulin was then continued at 60 µCi/hr throughout the duration of the experiment. The animals were then split into two groups (n=12 in each group). The first group continued to receive B alone, infused at 5ml/hr, whilst

the second group received saline containing both B and CBX (6mg/hr), infused at 5ml/hr. These infusions were maintained for the next 5 hours.

3.2.3.3. Micropuncture

One hour after surgery had been completed, individual nephron sites were punctured using a glass pipette and free-flow micropuncture collections of tubular fluid were made during the final 4 hours from the early [ED] and late [LD] segments of accessible distal tubules. ED approximates to fluid arriving from the ascending limb of Henle's loop; LD to the beginning of the CCD. Typically 3-4 collections were obtained from each nephron segment per experiment. Following each collection, a silicone rubber solution (Microfil, Flowtex Inc., Boulder, Co, USA) was injected into the nephron to allow subsequent confirmation of the puncture site by microdissection (Cortell, 1969).

3.2.3.4. Renal clearance protocol

Urine from the micropunctured kidney and from the contralateral kidney was collected into pre-weighed pots during periods that encompassed each micropuncture collection. Samples of arterial blood (~40µl) were taken at regular intervals throughout the period of micropuncture for the measurement of plasma [^{3}H]-inulin activity. At the end of each experiment, following the final period of clearance measurements, a 2-3ml sample of arterial blood was taken (from the femoral artery) for the measurement of haematocrit, plasma aldosterone and electrolyte concentrations and plasma osmolality.

3.2.3.5. Analyses

3.2.3.5.1. Urine and plasma samples

Sodium and potassium concentrations in urine and plasma were measured by flame photometry; plasma aldosterone concentrations were measured by immunoassay (Coated-tube Aldosterone, DPC Ltd, Caenarfon, UK); urine and plasma osmolalities by freezing point depression (Automatic osmometer, Roebling, Camlab, Cambridge, UK). Haematocrit was measured using Hawksley microhaematocrit tubes. [^{3}H]-inulin activity in 5µl samples of urine and plasma, dispersed in 8ml Aquasol 2 scintillation cocktail (Dupont, Stevenage, UK), was measured by β-emission spectroscopy (model 2000 CA, Canberra, Packard, Pangbourne, UK).

3.2.3.5.2. Tubular fluid samples

Micropuncture collections were deposited under water-saturated oil. Sample volume was determined using calibrated constriction pipettes and duplicate samples taken for the measurement of [^{3}H]-inulin activity and sodium and potassium concentration. All 3 parameters were measured as described above (3.2.3.5.1).

3.2.3.6. Calculations and statistics

Plasma [^{3}H]-inulin activities for clearance measurements and for tubular fluid:plasma concentration ratios were interpolated from measured values. GFR was calculated using the expression cited in section 2.2.3.5. Single-nephron glomerular filtration rate (SNGFR) was calculated as:

$$(TF_{IN}/P_{IN}) \cdot V_{TF},$$

where TF_{IN} and P_{IN} are the activities of inulin in the tubular fluid and plasma samples, respectively, and V_{TF} is the rate at which fluid was collected. TF_{Na}/P_{Na} and TF_K/P_K were calculated using the plasma concentrations of sodium and potassium, respectively, measured in the terminal blood sample.

All data are presented as means ± sem. The ED and LD collections were averaged to give a mean value for each site in each rat, and this value was used in calculating the mean ± sem per group. Statistical comparisons were made using Student's *t*-test for unpaired samples (for comparisons between groups). A difference was considered to be statistically significant if $P<0.05$ (one-tailed, for between groups comparisons of sodium and potassium; two-tailed for other comparisons).

3.2.4. Measurement of 11ßHSD activities in PCT and CCD of the rat nephron following *in vivo* administration of CBX

3.2.4.1. Surgical protocol

Experiments were performed on male Sprague-Dawley rats (weight range 140-150g on day of experimentation). Animals were adrenalectomised and received replacement corticosteroids as described in section 3.2.3.1. On day 7-9 following adrenalectomy animals were anaesthetised with thiopental-sodium (Inactin; Byk Gulden, Konstanz, Germany; 110mg/Kg body weight; i.p.), and prepared surgically as described in section 2.2.2.1, except that only one catheter was inserted in to the jugular vein and the artery was not cannulated; blood pressure monitoring was not required due to the relatively short duration of experiments.

3.2.4.2. Infusion protocol

The animals were divided into two groups (n=7 per group). Both groups received saline and B (100μg/100g body weight.hr) for the first 30 min infused i.v. at a rate of 4ml/hr. Thereafter, the first group of animals continued to receive saline and B, whilst the infusion vehicle in the second group of animals was changed to saline containing both B (same dose) and CBX (6mg/hour). Infusion was continued for a further hour, after which time microdissection surgery was performed (see section 3.2.1), the contralateral (right) kidney and the liver were removed and rapidly snap-frozen in liquid N_2

before sacrificing the animal with an intracardiac injection of pentobarbitone sodium (200mg/Kg; expiral Sanofi Animal Health Ltd, Watford, UK). PCT and CCD were then obtained from the left kidney as detailed in section 3.2.1.

3.2.4.3. Measurement of 11ßHSD activities in PCT and CCD

11ßHSD activities were measured using the assay conditions described in section 3.2.1.6; PCT were incubated with no cofactors, or 1mmol/l $NADP^+$, and CCD with no cofactors or 1mmol/l NAD^+.

3.2.4.4. Measurement of 11ßHSD activities in contralateral kidney and liver

Biopsies of all samples were assayed as described in section 2.2.1.3. except that 500nmol/l [^{3}H]-B was used as substrate instead of 500nmol/l [^{3}H]-F.

3.2.4.5. Statistics; 11ßHSD activities in microdissected nephron segments

All data are presented as means ± sem. Statistical comparisons were made using Student's t-test for paired samples (for comparison between cofactor preferences in microdissected nephron segments) and unpaired samples (for comparisons between corticosterone-only and CBX-infused groups). A difference was considered to be statistically significant if $P<0.05$

3.3. Results

3.3.1. Determination of 11ßHSD activities along the rat nephron.

The highest levels of both $NADP^+$- and NAD^+-dependent 11ßHSD activity were observed in the CCD. Although corticosterone oxidation was higher in the presence of NAD^+, the differences observed between the two cofactor conditions did not achieve statistical significance (Figure 3.2). Corticosterone oxidation in the mTAL was undetectable with either cofactor whilst in the cTAL, 11ßHSD activity was approximately 2-fold higher in the presence of NAD^+ than with $NADP^+$; these cofactor preferences, however, were not statistically different. Intermediate levels of corticosterone oxidation were measured in both PCT and OMCD; there was no clear cofactor preference in either of the two nephron segments (Figure 3.2).

Figure 3.2: $NADP^+$-dependent and NAD^+-dependent 11ßHSD activities (fmol B oxidised/mm.60min) in rat PCT, mTAL, cTAL, CCD and OMCD, measured *ex vivo* in the presence of 1mmol/l $NADP^+$ or NAD^+ and 1µmol/l B. UD denotes undetectable 11ßHSD activities.
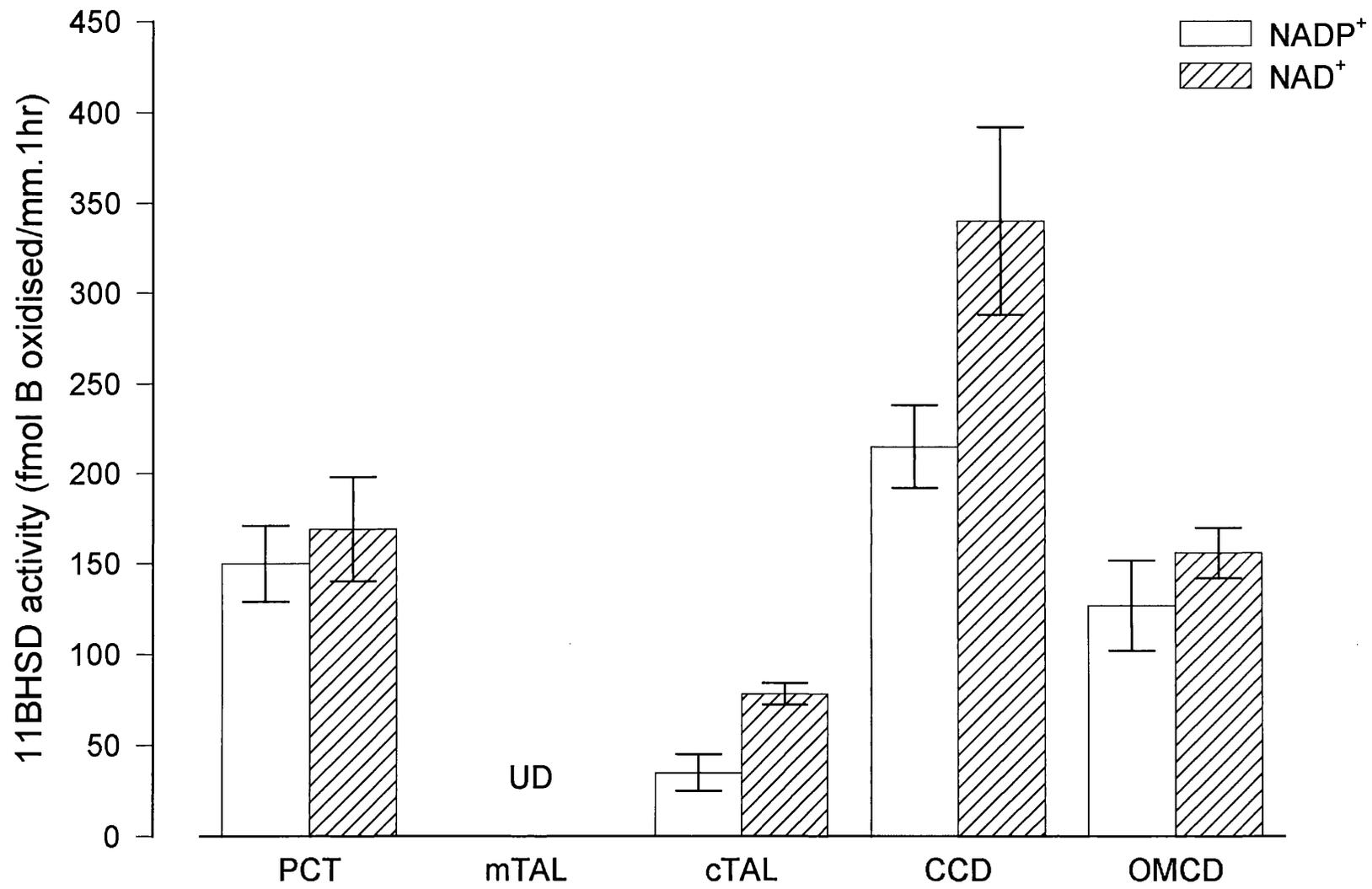
3.3.2. Micropuncture data

There were no differences in plasma sodium or potassium concentrations in rats during either the control or experimental period of CBX-treated rats *vs* control animals (infused with corticosterone only) (Table 3.4).

Table 3.4. Plasma data from control or CBX (6mg/hr) treated (5 hr duration) rats. P_{Na} and P_K are the plasma sodium and potassium concentrations, respectively. All values are mean ($\pm$ sem) n=10-12 animals for both groups.

	Control	CBX
P_{Na} (mmol/l)	136 $\pm$ 1	138 $\pm$ 2
P_K (mmol/l)	4.1 $\pm$ 0.2	4.1 $\pm$ 0.1

No differences in mean arterial blood pressure (MABP) or glomerular filtration rate (GFR) were detected between CBX or control animals (Figure 3.3). In addition, no change in SNGFR was observed in CBX treated compared to control rats (36.0 $\pm$ 2.4 *vs* 36.5 $\pm$ 2.5 nl/min, respectively).

Fractional urinary sodium excretion was lower (although not significantly) in rats administered CBX than in control animals, whereas urinary potassium excretion was unaffected by CBX infusion (Figures 3.4a and 3.4b).

Figure 3.3: Mean arterial blood pressure (MABP) and glomerular filtration rate (GFR) in rats infused i.v. with CBX (6mg/hr for 5hr) during the experimental period.
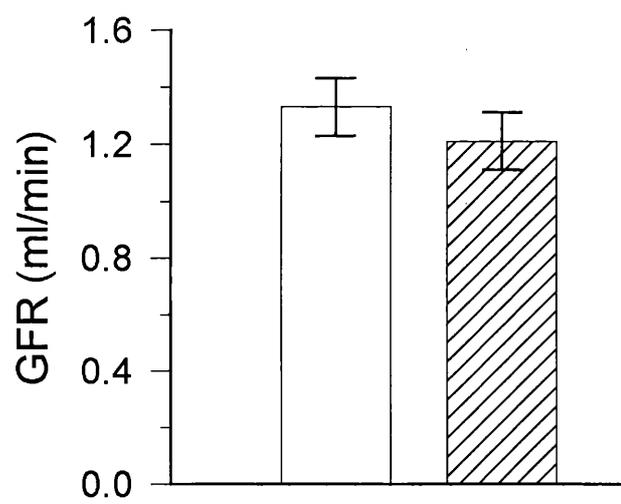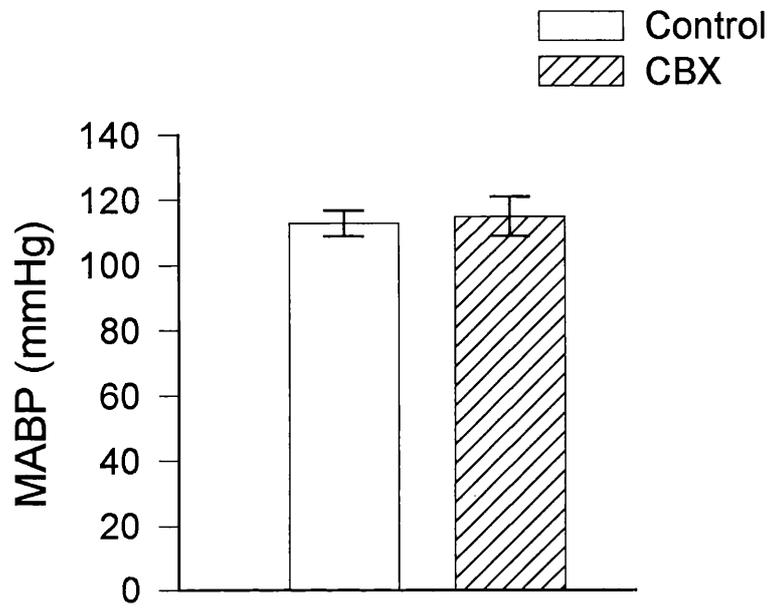
Figure 3.4: a) Fractional sodium excretion (FE_{Na}) and b) fractional potassium excretion (FE_K) in rats infused i.v. with CBX (6mg/hr for 5hr) during the experimental period. ***P<0.01 compared with control rats.
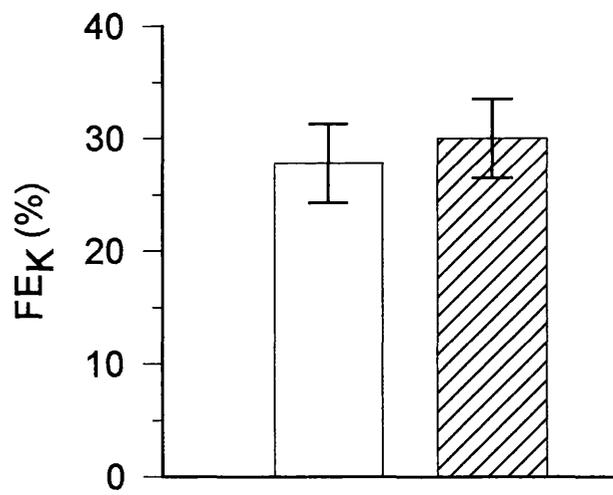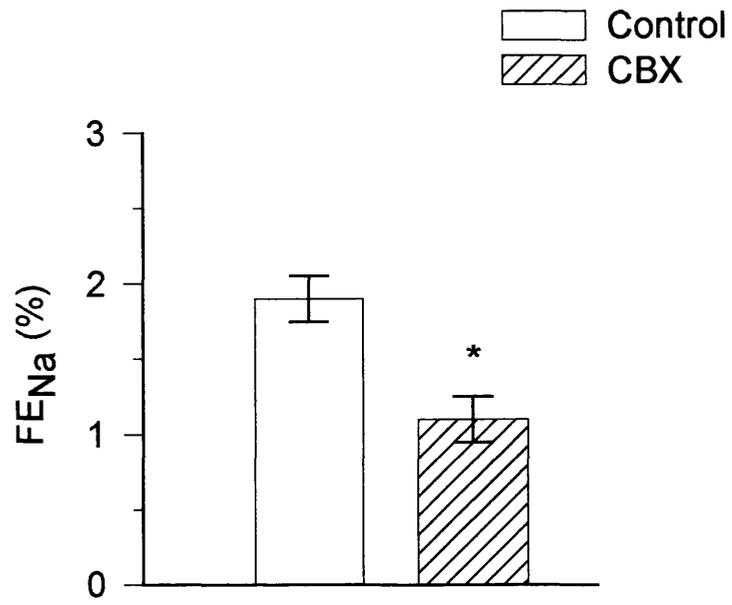
There were no differences in absolute deliveries (pmol/min) of sodium to either the early (ED) or late (LD) distal tubular sites in adrenalectomised CBX-treated rats compared to control animals (Table 3.5). In contrast, absolute delivery of potassium to the ED was significantly decreased and delivery to the LD significantly increased in CBX-infused rats compared to those receiving vehicle only (Table 3.5; $P<0.05$ for both). Tubular fluid flow rates were similar between the control and CBX animals at the ED (9.8 ± 0.6 vs 9.3 ± 0.4 nl/min), but lower (although not significantly) at the LD of CBX treated animals (5.0 ± 0.6 vs 3.8 ± 0.5 nl/min).

Table 3.5: Absolute deliveries (pmol/min) of sodium and potassium to ED and LD tubular sites in control or CBX-treated (6mg/hr for 5hr) rats. Mean ± sem. n=12 animals. *$P<0.05$ CBX compared to control rats.

	Control	CBX
Na$^+$		
ED	515 ± 30	459 ± 33
LD	208 ± 16	176 ± 24
LD-ED	$- 307 \pm 30$	$- 284 \pm 35$
K$^+$		
ED	20 ± 1	15 ± 1*
LD	26 ± 4	32 ± 4
LD-ED	$+ 6 \pm 3$	$+ 17 \pm 4$*

3.3.3 11ßHSD activities

3.3.3.1. Micropuncture study (CBX infusion for 5hr)

In the contralateral kidney obtained from rats used in the micropuncture study, the infusion i.v. of CBX (6mg/hr) for the duration of 5 hours resulted in 86% and 71% inhibition of $NADP^+$-and NAD^+-dependent 11ßHSD activities, respectively (Table 3.6 and Figure 3.5; $P<0.01$ for both cofactors).

Table 3.6: 11ßHSD activities (pmol F oxidised/mg protein.30 min) in renal tissue taken from control or CBX (6mg/hr for 5hr) treated rats, measured *ex vivo* in the presence of 400μmol/l $NADP^+$ or NAD^+ and 500nmol/l F. Values are means ± sem (n=12 per group). **$P<0.01$ compared to control animals.

	Control	CBX
$NADP^+$-dependent	28 ± 6	8 ± 1**
NAD^+-dependent	33 ± 4	5 ± 1**

Figure 3.5: 11ßHSD activities (% of control) in renal tissue obtained from adrenalectomised rats infused with CBX (6mg/hr for 5hr), measured *ex vivo* in the presence of 400µmol/l $NADP^+$ or NAD^+ and 500nmol/l F. All statistical analyses were performed on the non-standardised data presented in Table 3.6. **P*<0.01 (CBX *vs* control rats).
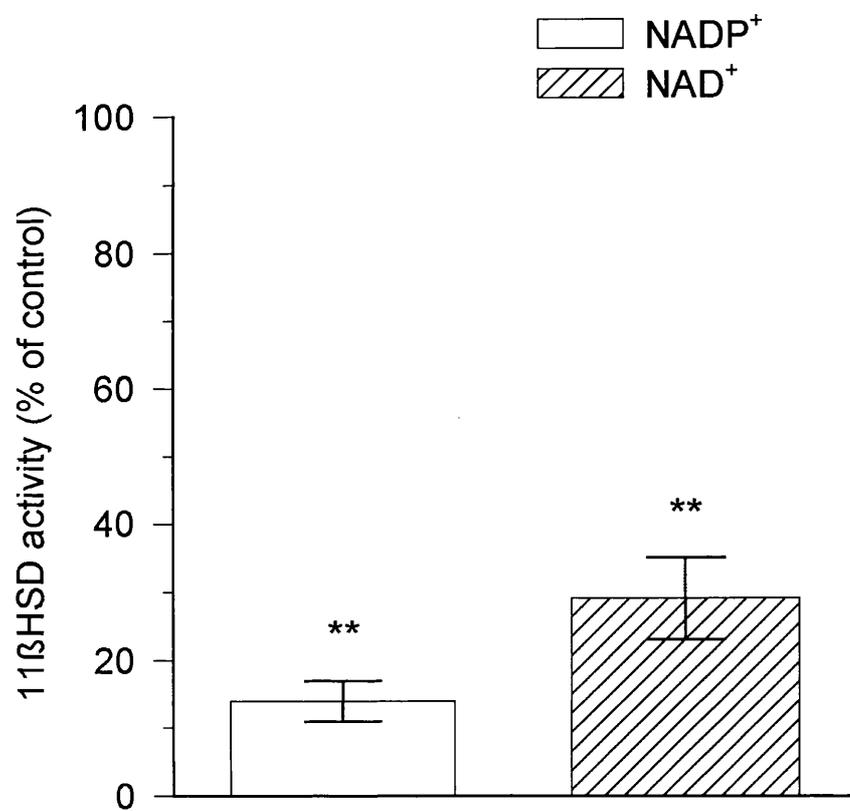
3.3.3.2. "Microdissection" data (CBX infusion for 1hr)

In the PCT and CCD of rats infused with CBX (6mg/hr) for 1hr, $NADP^+$- and NAD^+-dependent 11ßHSD activities were decreased by 43 ± 14% and 33 ± 21%, respectively, compared to corticosterone oxidation measured in the control rats; inhibition did not reach statistical significance in either nephron segment (Table 3.7 and Figure 3.6a).

Table 3.7: 11ßHSD activities (fmol B oxidised/mm.60 min) in PCT and CCD taken from control or CBX (6mg/hr for 1hr) treated rats, measured *ex vivo* in the presence of 1mmol/l $NADP^+$ (PCT) or NAD^+ (CCD). Values are means ± sem (n= 7 animals per group).

	Control	CBX
PCT ($NADP^+$)	320 ± 70	180 ± 40
CCD (NAD^+)	560 ± 160	370 ± 130

In the contralateral kidney obtained from rats in microdissection experiments, the infusion i.v. of CBX (6mg/hr) for the duration of 1 hour resulted in 78 ± 3% inhibition of $NADP^+$-dependent 11ßHSD activities (Table 3.8, Figure 3.6b; *P*<0.01). In contrast, no inhibition of NAD^+-dependent renal corticosterone oxidation was observed in rats infused with CBX under this protocol (Table 3.8 and Figure 3.6b).

Table 3.8: Microdissection study. 11ßHSD activities (pmol B oxidised/mg protein.30 min) in whole kidney tissue taken from control or CBX (6mg/hr for 1hr) treated rats, measured *ex vivo* in the presence of 400μmol/l $NADP^+$ or NAD^+ and 500nmol/l B. Values are means (± sem) n=7 animals per group. **P<0.01 compared to control animals.

	Control	CBX
$NADP^+$-dependent	170 ± 48	32 ± 14**
NAD^+-dependent	73 ± 11	97 ± 20

NADP$^+$-dependent hepatic enzyme activity in rats infused with CBX for 1hr was inhibited by 97± 3% (627 ± 139 *vs* 23 ± 10 pmol/mg protein.30 min in control and CBX-treated rats, respectively; *P*<0.001).

Figure 3.6: 11ßHSD activities (% of control) in a) PCT and CCD and b) contralateral (whole) kidney obtained from rats infused with CBX (6mg/hr for 1hr), measured *ex vivo* in the presence of a) 1mmol/l NADP$^+$ or NAD$^+$ and 1µmol/l B or b) 400µmol/l NADP$^+$ or NAD$^+$ and 500nmol/l B. All statistical analyses were performed on the non-standardised data presented in Tables 3.7 and 3.8. *******P*<0.01 (CBX *vs* control rats).
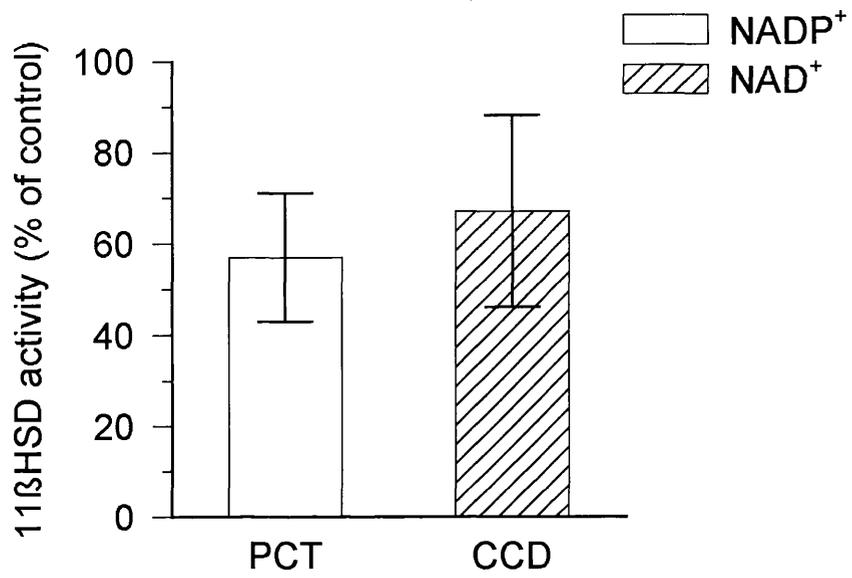
CONTRALATERAL KIDNEY
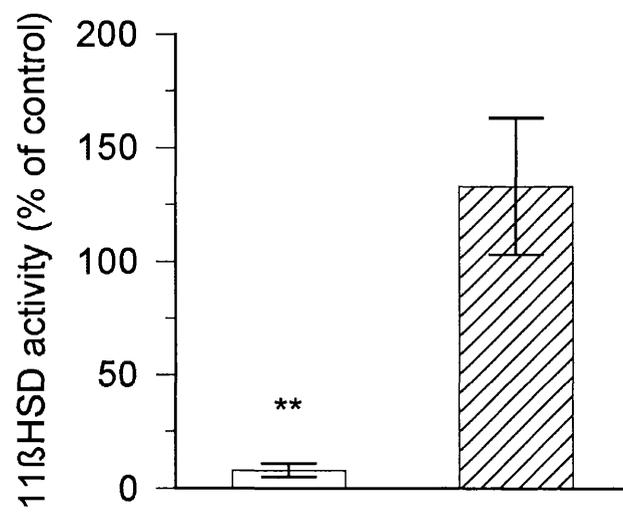
3.4. Discussion

In the studies described in chapter 2, it was demonstrated that at a high dose (6mg/hr) of CBX, inhibition of both $NADP^+$-and NAD^+-dependent renal 11ßHSD activities was associated with antinatriuresis but not kaliuresis. At the intermediate and low doses of CBX, which caused substantial inhibition (80 and 50%, respectively) of renal glucocorticoid oxidation, no effects were observed in the urinary excretion of either sodium or potassium. It was suspected that the effects of CBX on sodium and potassium transport at the level of the single nephron may be undetectable in the overall urinary excretion of these cations. The aims of the experiments described in this chapter were to establish 11ßHSD activities along the rat nephron and to investigate the effect of inhibition of this enzyme on sodium and potassium transport in the distal tubule.

3.4.1. 11ßHSD activities along the rat nephron

Of the 5 nephron segments investigated in the current study, the highest level of both $NADP^+$-dependent and NAD^+-dependent corticosterone oxidation was measured in the CCD. 11ßHSD activity in the presence of NAD^+ was higher in the CCD than that observed in the presence of $NADP^+$ although this difference was not statistically significant. While substantial NAD^+-dependent 11ßHSD activity was anticipated in the CCD on the basis of previous reports (see chapter 1), the finding that a significant level of $NADP^+$-dependent corticosterone oxidation also occurs in this region of the nephron was surprising for several reasons. As stated in chapter 1, on the basis of immunolocalisation studies, it is generally accepted that the more distal parts of the nephron are devoid of $NADP^+$-preferring type 1 11ßHSD. It would thus appear that the type 2 isoform of 11ßHSD, the presence of which has been firmly established in the CCD (see Chapter 1), may be employing $NADP^+$, in addition to NAD^+, as a cofactor. Contemporary literature, however, states that the type 2 isoform of 11ßHSD employs NAD^+ *exclusively* (as opposed to preferentially) as its cofactor (Agarwal *et al*,

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1994; Albiston *et al*, 1994). It is, of course, possible that the techniques employed in the early immunolocalisation studies (see chapter 1) were not able to detect type 1 11ßHSD in the CCD if, for example, the epitope of this enzyme was masked in one part of the nephron (the CCD) but not in another (the PCT). The use of polymerase chain reaction (PCR) techniques in the CCD would confirm or refute such a theory. If type 1 11ßHSD is absent from the PCT, as contended in the literature (Edwards *et al*, 1988; Rundle *et al*, 1989), it would appear that another $NADP^+$-utilising enzyme is responsible for some, if not all, of the $NADP^+$-dependent corticosterone oxidation observed in the CCD. Without further studies such a theory is merely speculative, although possible candidates include other SCAD enzymes such as PGDH or 9-keto-reductase, both of which are known to utilise $NADP^+$ as a cofactor (Lee and Levine, 1975) and are important moderators of renal function (See Levenson *et al*, 1982).

Since the role of both mineralocorticoids and glucocorticoids within the OMCD is uncertain (see chapter 1), it is difficult to speculate, without further studies as to what the role/s of the $NADP^+$- and NAD^+-dependent 11ßHSD activities in this segment may be. It is interesting to note, however, that the presence of $NADP^+$-dependent corticosterone oxidation in this distal part of the nephron supports the finding of $NADP^+$-dependent activity in the CCD.

The levels of both $NADP^+$- and NAD^+-dependent 11ßHSD activity observed in the OMCD are comparable to those measured in the PCT. The presence of type 1 ($NADP^+$-dependent) 11ßHSD activity has been demonstrated in the proximal tubule in both the current study and by others (see chapter 1). The functional significance of this low affinity isoform is unknown. However, given the importance of glucocorticoids (as opposed to mineralocorticoids) in the regulation of acid-base balance in the PCT, as detailed in chapter 1, it could be argued teleologically that the lower affinity isoform may be necessary to protect GR from supraphysiological glucocorticoid concentrations such as those associated with stress. Consistent with this hypothesis, the reported absence of the type 2 isoform from this region of

the nephron (see Chapter 1) would prevent the inactivation of glucocorticoids which are necessary for acid-base balance. Furthermore, given the reported absence of MR from the PCT (see Chapter 1), the presence of the type 2 11ßHSD enzyme in this nephron segment would seem unnecessary. Thus, it is likely that both the $NADP^+$- and NAD^+-dependent corticosterone oxidation observed in the PCT reflects type 1 11ßHSD activity, given that this isoform is promiscuous in its cofactor requirement.

In the thick ascending limb of Henle's loop, 11ßHSD activity was higher in the cTAL than in the mTAL (enzyme activities being below the limit of detection of the assay in the latter segment). The fact that levels of glucocorticoid oxidation in the mTAL were very low would argue against any major importance of the enzyme in this region of the nephron. Indeed, it has been reported that plasma aldosterone concentrations do not affect potassium transport in mTAL *in vitro*: Work and Jamison (1987) demonstrated that potassium transport in the isolated, perfused mTAL did not differ between sham-operated versus adrenalectomised or adrenalectomised (plus aldosterone treatment) rats.

The presence of low levels of NAD^+-dependent 11ßHSD activity in the cTAL is consistent with the findings of Smith *et al* (1997) who reported the expression of 11ßHSD2 in tubules thought to represent TAL. This suggests a minor protective role for this enzyme in this part of the nephron under normal physiological conditions. Whether or not this enzyme activity aquires a more active role in pathophysiological situations is unknown, but it is possible that inhibition of 11ßHSD in the loop of Henle by CBX may have contributed to the change in potassium delivery to the early distal tubule which was observed in the CBX-infused rats. Hence, a decrease in NAD^+-dependent 11ßHSD activity may have been expected to increase MR activation, and thus potassium reabsorption in the cTAL.

3.4.2. The free flow micropuncture technique

The development of techniques for the collection and analysis of nanolitre samples of tubular fluid has allowed the direct study of single-nephron function. As such, this technique has proved invaluable in determining renal function at the single nephron level, *in vivo*. However, it should be appreciated that the renal micropuncture technique has several limitations, the main one of which arises from the inaccessibility of certain nephron segments. In the adult rat, samples can only be obtained from the distal tubule and from the convoluted section of the proximal tubule, and as such, interpretation of data obtained from micropuncture studies has to be made with caution. In the series of micropuncture experiments described in this chapter, tubular fluid collections were made while each rat received either corticosterone alone or corticosterone plus CBX infusate. In order to allow the collection of an adequate number of samples, these infusions had to be maintained for several hours. One potential problem with this technique is that due to inherent variation between rats, any differences observed between the two groups of animals in the experimental period may not be caused by the different infusion. In order to demonstrate that the two groups were, in the first instance, comparable, a thirty-minute control period was included in the study. Comparisons made between the control and CBX treated rats, showed that prior to the infusion of the drug, overall renal function (MABP; 102 ± 3 in control animals *vs* 104 ± 2mmHg: GFR; 1.3 ± 0.2 in control animals *vs* 1.2 ± 0.2) in the two groups was similar, suggesting that any differences between groups observed during the experimental period are due to the differing infusates received by the animals during that time.

3.4.2.1. Animal and whole kidney data

The plasma data demonstrate that as observed in the adrenal intact rats (see Table 2.2; Chapter 2), the infusion of CBX did not alter plasma sodium or potassium concentrations, despite the longer infusion period of the

micropuncture protocol. Likewise, MABP and GFR were unaltered in the adrenalectomised animals receiving CBX compared to those infused with corticosterone only. Thus the animal parameters (plasma electrolyte data, GFR and MABP) described above demonstrate that the act of adrenalectomy and replacement of physiological levels of corticosteroids does not alter the general status of the animal. During the experimental period of the micropuncture study, urinary sodium excretion was lower in CBX-treated rats compared to control animals, although potassium excretion was unaffected by CBX infusion. All the above findings are consistent with those reported in chapter 2 in adrenal intact rats and demonstrate that the two studies are comparable regarding the general status of the animals and net urinary excretion.

3.4.2.2. Effects of CBX infusion on sodium and potassium transport in the distal tubule.

Infusion of CBX to adrenalectomised rats did not alter sodium delivery to the early distal tubule. Since sodium reabsorption by the TAL reduces sodium concentration in tubule fluid due to the low osmotic water permeability of this segment (see Chapter 1), the absence of any change in sodium concentration in the fluid entering the early distal tubule indicates that administration of CBX to these rats did not alter sodium reabsorption in the TAL. Since infusion of this drug would be expected to increase glucocorticoid activation of MR, an increase in sodium reabsorption by this portion of the nephron may be expected in light of the fact that both MR and 11ßHSD activities have been reported in the loop of Henle (see Chapter 1) and the presence of NAD^+-dependent 11ßHSD activity detected in the cTAL (reported in this chapter). Furthermore, studies performed by Stanton (1986) reported that the decreased sodium reabsorption in the rat loop of Henle following adrenalectomy was restored to normal (sham-operated) levels by the replacement of physiological doses of aldosterone. Although the results presented in this chapter do not show any change in sodium reabsorption by the loop following CBX administration, it is possible that the loop of Henle has a limited capacity for MR-mediated sodium reabsorption. Hence, whilst

the physiological replacement of aldosterone restores sodium reabsorption to normal values within this segment, the administration of CBX in the current study, which would be expected to increase intrarenal glucocorticoids to supraphysiological concentrations, would not necessarily increase sodium reabsorption any further.

The administration of CBX did not alter sodium transport between the accessible early and late parts of the distal tubule. Although a change in distal tubular reabsorption may have been expected (see Chapter 1) the lack of any effect is consistent with an earlier study (Field *et al* 1984). Field and co-workers reported that in the adrenalectomised rat, administration of an acute high physiological dose of aldosterone (comparable to that observed following the acute infusion of potassium to adrenal intact rats) did not alter sodium reabsorption in the distal nephron despite reducing fractional sodium excretion in the final urine. Thus, it would appear that the decrease in FE_{Na} reported both in the current and the preceding chapter occurs beyond this part of the tubule. As stated previously, one disadvantage of the *in vivo* micropuncture technique is the limited accessibility, namely to only the superficial nephrons of the kidney. Hence, in the micropuncture study reported herein, the cortical, outer and inner medullary collecting ducts were all inaccessible, but are all segments in which the reabsorption of sodium is likely to occur (see Chapter 1). Active MR-modulated sodium transport is likely to have increased in nephron segments beyond the distal tubule including not only the CCD, as would be anticipated (see Chapter 1), but also in the IMCD. In a recent study, ENaC mRNA levels were reported to have increased in rat renal IMCD cell cultures after 3 hours of aldosterone or dexamethasone treatment (Volk *et al*, 1995). Since 11ßHSD activities have been reported in this nephron segment (Nolan *et al*, 1996), it is possible that increased glucocorticoid access to both MR and GR in the IMCD following inhibition of 11ßHSD activities, contributed to the antinatriuresis observed in the CBX treated animals.

The delivery of potassium to the early distal part of the nephron was decreased in CBX-infused rats compared to control animals. This reduction in potassium reabsorption in the kidney following CBX administration is likely to occur in the loop of Henle since, as stated in Chapter 1, potassium transport in the PCT is not thought to be regulated by corticosteroids. Consistent with this finding, administration of aldosterone to adrenalectomised rats has previously been reported to reduce potassium delivery to the early distal nephron (Stanton, 1986). Moreover, Cortney (1969) and Hierholzer et al (1965), also using free-flow micropuncture studies, have previously demonstrated that adrenalectomy reduces potassium reabsorption in the rat superficial loop of Henle. However, another study (Higashihara and Kokko, 1985) observed no such effect of the acute infusion of aldosterone to adrenalectomised rats. The reasons for the discrepancies between the studies described above are probably due to the differing techniques employed (which would result in differing flow rates and composition of fluid entering the distal tubule), and length of exposure to the hormone.

In the distal nephron of adrenalectomised rats infused with high dose CBX potassium secretion was nearly 3 times higher than that measured in the same nephron site of vehicle-only infused adrenalectomised rats. This is again in accordance with the findings of Field et al (1984) (who demonstrated a two-fold increase in aldosterone-infused adrenalectomised rats) and of Stanton (1986) (who also showed restoration to control levels of distal tubular potassium secretion in aldosterone infused adrenalectomised rats). Hence, the results indicate that, as suspected from the studies described in Chapter 2, the lack of any demonstrable effect of CBX on net potassium urinary excretion, despite substantial inhibition of both renal 11ßHSD activities, was 'masked' in the urine. There are several possible reasons for this 'masking' effect, one of which is likely to reflect the opposing effects of CBX administration on potassium transport in the loop of Henle and the distal tubule. The combined effects of increased potassium reabsorption in the former (which, as stated earlier, is likely to reflect

increased reabsorption in the loop of Henle) while increased potassium secretion occurred in the distal tubule could result in no overall change in the final urinary potassium concentration. Furthermore, the flow rate of luminal fluid through the distal tubule is a major determinant of potassium secretion in the distal nephron (Good and Wright, 1979; Malnic *et al*, 1989); decreases in flow rate result in a decrease in potassium secretion and vice versa. Hence, the reduced flow rate observed in the CBX-treated rats, although failing to reach statistical significance, may nonetheless have been sufficient to oppose MR-stimulated potassium secretion in the distal tubule and CCD and therefore attenuate the stimulation of net potassium excretion. It is also possible that further downstream from the site of micropuncture, reabsorption along the OMCD and IMCD could occur (see Chapter 1) to counteract the increased potassium secretion between the ED and LD segments of the distal tubule.

3.4.3. 11ßHSD activities following i.v. CBX infusion

3.4.3.1. "Micropuncture" studies- (CBX infusion for 5hr)

$NADP^+$- and NAD^+-dependent renal 11ßHSD activities, measured in the contralateral kidneys of these CBX-infused adrenalectomised rats, were inhibited by 86% and 71%, respectively. The decrease in NAD^+-dependent 11ßHSD activity observed in the CBX-infused rats would be expected to enhance glucocorticoid access to MR, accounting for the increase in potassium secretion observed in the distal nephron, As such, this observation is consistent with the postulated role for 11ßHSD2 in protecting the MR from hyper-stimulation by glucocorticoids. The micropuncture experiments described herein also demonstrate that the intravenous infusion of CBX does alter potassium transport in at least one specific nephron segment despite there being no overall change in urinary potassium excretion. Moreover, it would appear that the antinatriuresis in rats observed in both the current and preceding chapters is not the result of enhanced sodium reabsorption in superficial distal nephrons. It should be noted, however, that although CBX inhibited both $NADP^+$-and NAD^+-dependent

renal 11ßHSD activities, the decreases in the adrenalectomised rats described in this chapter are less than those observed in the adrenal intact rats used in the studies described in Chapter 2 with the same dose of CBX, despite the longer CBX infusion period in the current study. Moreover, in Chapter 2, a greater inhibition (at least 80%) of renal 11ßHSD activities with the intermediate dose (0.6mg/hr) of CBX was not associated with a decrease in urinary sodium excretion, whereas the 71% inhibition of renal NAD^+-dependent 11ßHSD activity observed in the adrenalectomised rats described in this chapter caused a significant antinatriuresis. This discrepancy between the two studies is likely to be due to the differences in adrenal status of the animals and the different experimental procedures in the two studies (see next chapter).

3.4.3.2. 11ßHSD activities in PCT and CCD (CBX infusion for 1hr)

In the PCT of adrenalectomised rats, $NADP^+$-dependent 11ßHSD activity was inhibited by 43% and in the CCD, NAD^+-dependent corticosterone oxidation was inhibited by 33% following the infusion of CBX (6mg/hr for 1hr). In the whole kidneys of these same animals, 92% inhibition of $NADP^+$-dependent corticosterone oxidation was observed following CBX infusion; an observation in striking contrast to the fact that in the same kidneys, NAD^+-dependent 11ßHSD activity was unaffected by CBX infusion. In fact, if anything, CBX appeared to have increased rather than suppressed NAD^+-dependent 11ßHSD glucocorticoid oxidation in renal homogenates obtained from these adrenalectomised rats.

The contralateral kidneys obtained from both the micropuncture and the microdissection studies were not inhibited by CBX to the same extent as was observed in the studies described in Chapter 2 (>95%). One possible reason for this discrepancy was due to the duration of drug infusion. However, this is not likely to be a major factor since inhibition of $NADP^+$-dependent 11ßHSD activity in contralateral kidneys from both the micropuncture and microdissection studies were decreased to a similar degree (86 and 92%, respectively), regardless of the time course of CBX

infusion (5hr *vs* 1hr, respectively). It is also possible that infusion of CBX into the animals used in the current study was insufficient, due to an unnoticed blockage in the cannula or reduced arterial flow rate to internal organs. This again is unlikely, since hepatic $NADP^+$-dependent 11ßHSD activity measured in the livers obtained from the rats used in the microdissection studies were inhibited by 97%. In the previous chapter, performed in adrenal intact rats, 98% inhibition of renal $NADP^+$-dependent 11ßHSD activity was observed in both the kidney and liver.

The most obvious difference between the experiments described herein and in Chapter 2 is the adrenal status of the animals used in each study. The fact that the rats used in the present studies were adrenalectomised and received replacement corticosteroids, may for reasons as yet unknown, alter the response of renal 11ßHSD activities to CBX. There is a precedent for such a phenomenon; as stated in chapter 1, Latif *et al* (1992), reported that CBX administered to adrenalectomised rats did not result in inhibition of renal $NADP^+$-dependent corticosterone metabolism.

3.4.4. Conclusions

In summary, in adrenalectomised (steroid replaced) rats infused with CBX for 5hr, urinary sodium excretion was decreased but no kaliuresis was observed. Conversely, at the single nephron level, no change in sodium transport in either the loop of Henle or the distal tubule was observed, in contrast to demonstrable changes in potassium transport which occurred in both of these nephron segments. In adrenalectomised rats, the infusion of CBX for 1hr did not result in inhibition of NAD^+-dependent 11ßHSD activity in the 'whole' kidney, despite substantially decreasing $NADP^+$-dependent glucocorticoid oxidation in the same tissue. Since, in the experiments described in Chapter 2, substantial inhibition of NAD^+-dependent glucocorticoid oxidation was observed following CBX infusion to adrenal intact rats, the results of the current study suggest that adrenalectomy may impair the ability of CBX to inhibit 11ßHSD activity. A series of experiments, performed to investigate the effect of adrenalectomy on renal 11ßHSD activities are described in the following chapter.

Chapter 4:

The effect of adrenalectomy on the inhibition of rat renal 11ß-hydroxysteroid dehydrogenase activities by carbenoxolone.

4.1. Introduction

As stated in Chapters 1 and 2, the renal effects of CBX are assumed to depend upon the access of glucocorticoids to the MR (following inhibition of 11ßHSD2), rather than a direct interaction of the drug with the MR itself. It remains to be seen however, whether the presence of the adrenal glands and/or endogenous glucocorticoids are required for the inhibition of 11ßHSD activities by carbenoxolone. The experiments described in chapter 3, in which CBX was administered to adrenalectomised rats, indicated that adrenalectomy may affect the inhibition of 11ßHSD activities by CBX (reported in Chapter 2). Whilst $NADP^+$-dependent glucocorticoid oxidation was inhibited by 78% in the contralateral kidney of rats prepared for microdissection, NAD^+-dependent 11ßHSD activity was not inhibited at all by the administration of a dose of CBX which had previously been associated with 95% inhibition of NAD^+-dependent glucocorticoid oxidation in adrenal intact rats. Together these findings suggest that adrenalectomy and/or the replacement of exogenous dexamethasone and aldosterone to adrenalectomised rats, may affect the ability of intravenously administered CBX to inhibit renal 11ßHSD activities, particularly that of the NAD^+-dependent isoform.

The experiments reported in this chapter were designed to examine the effects of adrenalectomy on inhibition of renal 11ßHSD activities by CBX. This was achieved by comparing the *in vivo* and *in vitro* effects of CBX on $NADP^+$-dependent and NAD^+-dependent 11ßHSD activities in kidneys obtained from adrenal intact versus adrenalectomised rats.

4.2. Materials and Methods

4.2.1. Adrenal intact rats: Determination of *ex vivo* 11ßHSD activities in PCT, CCD, the contralateral ('whole') kidney and the liver following CBX infusion

The infusion of high dose CBX (6mg/hr) was performed exactly as described in section 3.2.4.2, such that all rats received corticosterone for 30 min. Thereafter, one group of animals received corticosterone and CBX (6mg/hr) whilst the second group of rats (control animals) continued to receive corticosterone only for 1hr. Microdissection of nephron segments, and assays of 11ßHSD activities in PCT and CCD segments were performed as described in 3.2.1.6), in 5 adrenal intact rats.

The contralateral kidney and the liver were excised for: i) *ex vivo* determination of renal and hepatic 11ßHSD activities and ii) assessment of the effects of CBX on renal 11ßHSD activities *in vitro.*

Renal 11ßHSD activities were determined as described in section 3.2.4.4; hepatic 11ßHSD activities were determined as described in section 2.2.4, except that 500nmol/l B was used as substrate instead of 500nmol/l F.

4.2.2. Determination of 11ßHSD activities in contralateral kidneys obtained from adrenalectomised and adrenal intact rats in the presence of CBX *in vitro.*

Kidney biopsies obtained from both adrenal intact and adrenalectomised rats (n=5 kidneys from each group) were incubated with 8 concentrations (serial dilutions from 10^{-9} to 10^{-5} mol/l) of CBX in PBS *in vitro* under the assay conditions described in section 2.2.1.3.

4.2.3. Statistics

Data presented are means ± sem. Due to the relatively small number of animals used in this series of studies, experimental data sets were tested for normality by the use of regression analysis of n score transformations. All data were found to conform to normal distributions. As such, the use of the parametric Student's unpaired *t*-test was employed for statistical comparisons to assess differences in renal 11ßHSD activities between i) the adrenal intact and adrenalectomised control animals (to establish whether adrenalectomy affects basal 11ßHSD activities), and ii) for comparison between CBX-treated *vs* control adrenal intact or adrenalectomised rats. Statistical significance was taken as *P*<0.05.

4.3. Results

4.3.1. Inhibition of 11ßHSD activities in the PCT and CCD by CBX *in vivo*

There was no difference in NADP^{+}-dependent 11ßHSD activity in the PCT between adrenal intact and adrenalectomised control animals (Table 4.1). In the PCT of adrenal intact rats, CBX significantly inhibited NADP^{+}-dependent 11ßHSD activity *in vivo* by 78 ± 7%, compared to control rats (Figure 4.1a; *P*<0.05). This compared to an attenuated effect (43 ± 14% inhibition) of CBX *in vivo* on NADP^{+}-dependent 11ßHSD activity in the PCT of adrenalectomised rats; enzyme activities were not significantly different from those of the control adrenalectomised rats (Table 4.1; Figure 4.1a).

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Table 4.1. Effects of adrenalectomy on $NADP^+$-dependent 11ßHSD activities (fmol B oxidised/mm.60min) measured *ex vivo* in the presence of 1mmol/l $NADP^+$ and 1μmol/l B in the PCT of control and CBX-treated (6mg/hr) rats. Adrenalectomised (ADX) rat data are taken from Chapter 3. All values are mean (± sem) n=5 animals in each group. *$P<0.05$ CBX-treated *vs* respective control animal.

	Control	CBX
Adrenal intact	236 ± 63	52 ± 28*
ADX	311 ± 49	178 ± 45

As observed in PCT, there were no differences in NAD^+-dependent 11ßHSD activity in the CCD between adrenal intact and adrenalectomised control animals (Table 4.2). In the CCD of adrenal intact and adrenalectomised rats, CBX inhibited NAD^+-dependent corticosterone oxidation by 35 ± 6% and 31 ± 21% respectively, decreases which were not significant compared to 11ßHSD activities in the adrenal intact and adrenalectomised control rats (Table 4.2; Figure 4.1b).

Table 4.2. Effects of adrenalectomy on NAD^+-dependent 11ßHSD activities (fmol B oxidised/mm.60min) measured *ex vivo* in the presence of 1mmol/l NAD^+ and 1μmol/l B in the CCD of control and CBX-treated (6mg/hr) rats. Adrenalectomised (ADX) rat data are taken from Chapter 3. All values are mean (± sem) n=5 animals in each group.

	Control	CBX
Adrenal intact	405 ± 132	263 ± 68
ADX	572 ± 130	394 ± 162

Figure 4.1: a) $NADP^+$-dependent 11ßHSD activity in PCT and b) NAD^+-dependent 11ßHSD activity in CCD obtained from adrenal intact and adrenalectomised rats rats infused with CBX (6mg/hr for 1hr), measured *ex vivo* in the presence of 1mmol/l $NADP^+$ or NAD^+ and 1µmol/l B. 11ßHSD activities are presented as % of respective control value. Adrenalectomised rat data is taken from that presented in chapter 3 for comparison. All statistical analyses were performed on the non-standardised data presented in tables 4.1 (a) and 4.2 (b). *$P<0.05$ (CBX *vs* control rats).

PCT
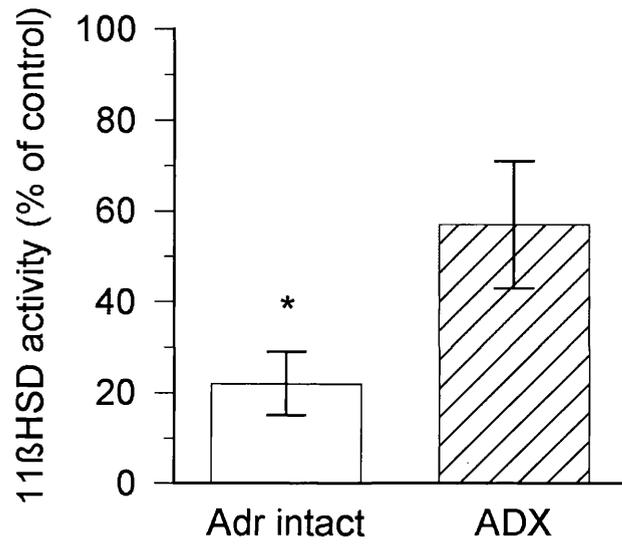
CCD
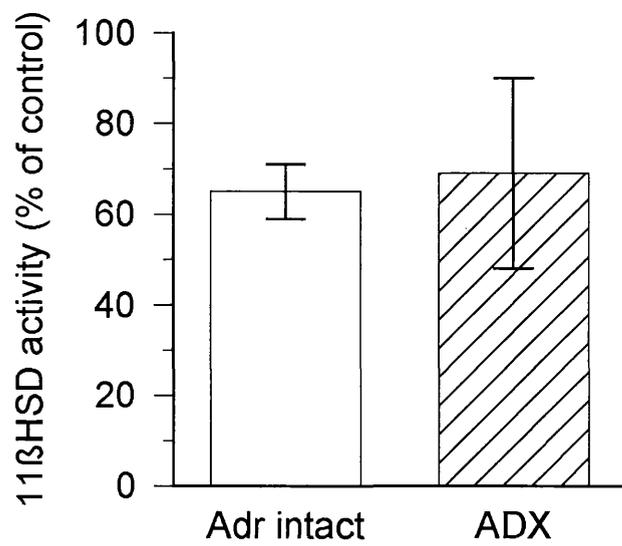
4.3.2. Inhibition of 11ßHSD activities by CBX *in vivo* in homogenates of whole kidney and the liver

In the contralateral kidney, both $NADP^+$- and NAD^+-dependent 11ßHSD activities were lower in adrenalectomised control rats compared to adrenal intact control animals (Tables 4.3 and 4.4), although only the latter decrease was significant (Table 4.4; $P<0.05$).

Table 4.3. Effects of adrenalectomy on $NADP^+$-dependent 11ßHSD activities (pmol B oxidised/mg protein.30min) in biopsies of kidneys taken from control and CBX-treated (6mg/hr) rats measured *ex vivo* in the presence of 400µmol/l $NADP^+$ and 500nmol/l B. Adrenalectomised (ADX) rat data are taken from chapter 3. All values are mean (± sem) n=5 animals in each group. $*P<0.05$, $***P<0.001$ CBX-treated *vs* respective control animal.

	Control	CBX
Adrenal intact	232 ± 20	4 ± 1***
ADX	169 ± 44	33 ± 14*

Table 4.4. Effects of adrenalectomy on NAD^+-dependent 11ßHSD activities (pmol B oxidised/mg protein.30min) in biopsies of kidneys taken from control and CBX-treated (6mg/hr) rats measured *ex vivo* in the presence of 400µmol/l NAD^+ and 500nmol/l B. Adrenalectomised (ADX) rat data are taken from chapter 3. All values are mean (± sem) n=5 animals in each group. [a]$P<0.05$ ADX *vs* adrenal intact control rats; $***P<0.001$ CBX-treated *vs* respective control animal.

	Control	CBX
Adrenal intact	122 ± 11	10 ± 5***
ADX	73 ± 10[a]	97 ± 22

In 'whole' kidneys, CBX significantly inhibited $NADP^+$-dependent 11ßHSD activity by 80 ± 8% in the adrenalectomised rats (Figure 4.2; $P<0.05$) and by 98 ± 1% in the adrenal intact animals as compared to respective control animals (Figure 4.2; $P<0.001$). Likewise, NAD^+-dependent corticosterone oxidation was significantly inhibited (92 ± 3%) in the contralateral kidneys of the adrenal intact rats ($P<0.001$), whereas CBX failed to inhibit renal NAD^+-dependent 11ßHSD activity in the adrenalectomised rats (Figure 4.2).

In liver, there was no difference in $NADP^+$-dependent 11ßHSD activity between adrenal intact or adrenalectomised control rats (Table 4.5). CBX significantly inhibited rat hepatic $NADP^+$-dependent 11ßHSD activity in both groups of animals by 97 ± 1% (Figure 4.3; $P<0.001$).

Table 4.5. Effects of adrenalectomy on $NADP^+$-dependent 11ßHSD activities (pmol B oxidised/mg protein.30min) in liver taken from control or CBX-treated (6mg/hr) rats, measured *ex vivo* in the presence of 400μmol/l $NADP^+$ and 100nmol/l B. Adrenalectomised (ADX) rat data are taken from chapter 3. All values are mean (± sem) n=5 animals in each group. ***$P<0.001$ CBX-treated *vs* respective control animal.

	Control	CBX
Adrenal intact	593 ± 89	18 ± 6***
ADX	672 ± 139	23 ± 10***

Figure 4.2: a) $NADP^+$ and b) NAD^+-dependent 11ßHSD activities in homogenates of whole kidney obtained from adrenal intact and adrenalectomised rats rats infused with CBX (6mg/hr for 1hr), measured *ex vivo* in the presence of 400μmol/l $NADP^+$ or NAD^+ and 500nmol/l B. 11ßHSD activities are presented as % of control value. Adrenalectomised rat data is taken from chapter 3 for comparison. All statistical analyses were performed on the non-standardised data presented in tables 4.3 (a) and 4.4 (b). *P<0.05, ***P<0.001 (CBX-treated *vs* control rats)

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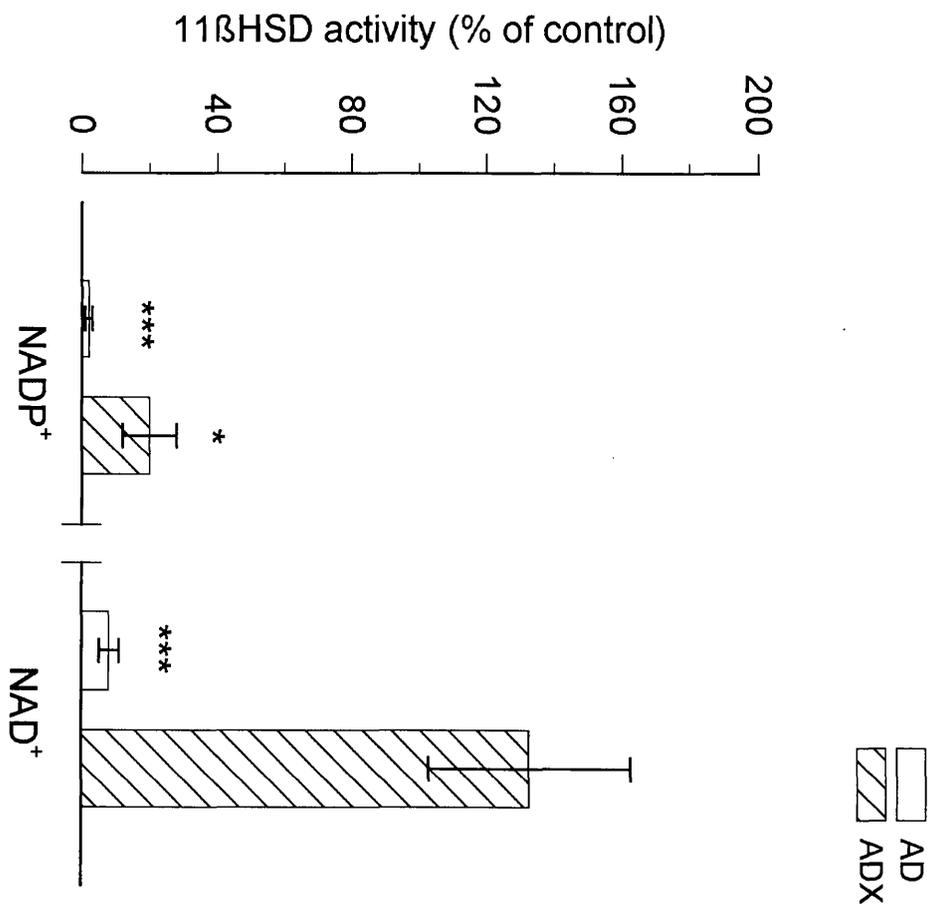
Figure 4.3: NADP$^+$-dependent 11ßHSD activity in liver obtained from adrenal intact and adrenalectomised rats infused with CBX (6mg/hr for 1hr), measured *ex vivo* in the presence of of 400µmol/l NADP$^+$ and 100nmol/l B. 11ßHSD activities are presented as % of control value. Adrenalectomised rat data is taken from chapter 3 for comparison. All statistical analyses were performed on the non-standardised data presented in table 4.5 ***$P<0.001$ (CBX-treated *vs* control rats).

11ßHSD activity (% of control)

Adr. Intact ***

ADX ***
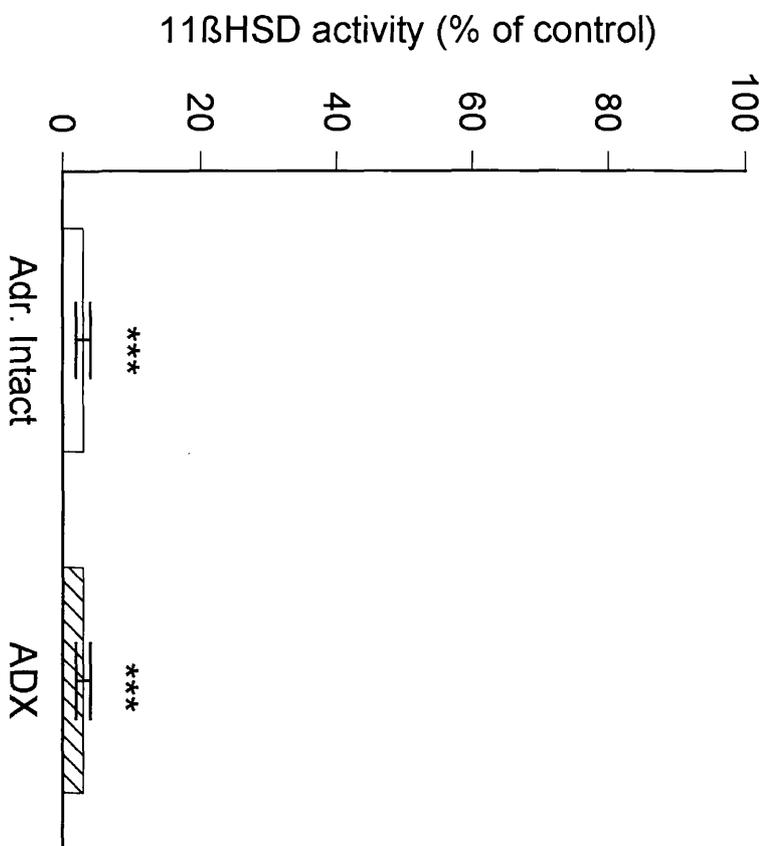
4.3.4. 11ßHSD activities in kidneys obtained from adrenal intact and adrenalectomised rat kidney in the presence of CBX *in vitro*.

The *in vitro* concentration-response curves shown in Figures 4.4 and 4.5, demonstrate that both $NADP^+$-dependent and NAD^+-dependent 11ßHSD activities in the kidneys obtained from adrenalectomised rats were more sensitive to CBX *in vitro* than were the kidneys obtained from adrenal intact animals. As shown by the EC_{50} values (Table 4.6), adrenalectomy had a greater impact on the sensitivity of $NADP^+$-dependent corticosterone oxidation to CBX than on the susceptibility of NAD^+-dependent 11ßHSD activity to inhibition by CBX.

Table 4.6. EC_{50} values for *in vitro* inhibition of renal $NADP^+$-dependent and NAD^+-dependent 11ßHSD activities by CBX, measured in the presence of 400μmol/l $NADP^+$ or NAD^+ and 500nmol/l B, in kidneys obtained from adrenal intact and adrenalectomised (ADX) rats (mean and 95% confidence interval; *$P<0.05$ relative to adrenal intact), n=5 kidneys for each group.

EC_{50} (nmol/l) for $NADP^+$-dependent activity		EC_{50} (nmol/l) for NAD^+-dependent activity	
adrenal intact	ADX	adrenal intact	ADX
105 (28-390)	7* (3-18)	80 (44-142)	26* (11-62)

Figure 4.4: Concentration-response curves for *in vitro* inhibition of renal NADP$^+$-dependent 11ßHSD activities by CBX in kidneys obtained from a) adrenal intact and b) adrenalectomised rats. 11ßHSD activity is presented as % of control (0µmol/l CBX concentration) value for individual kidneys.
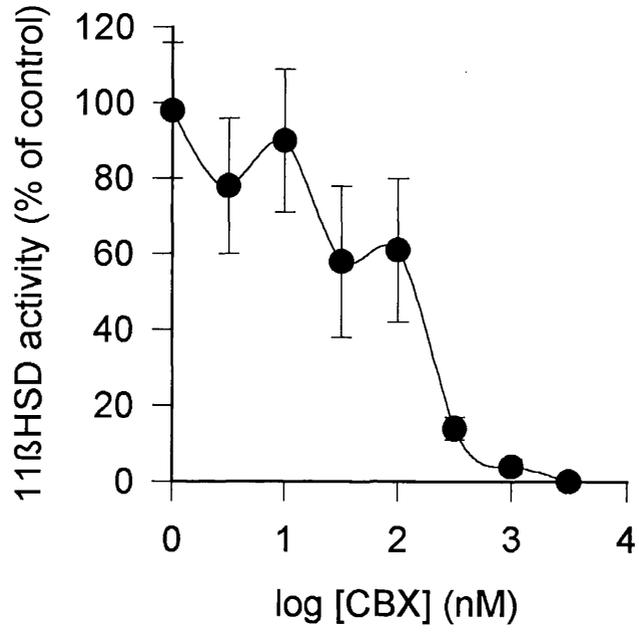
a) Adrenal intact

b) ADX
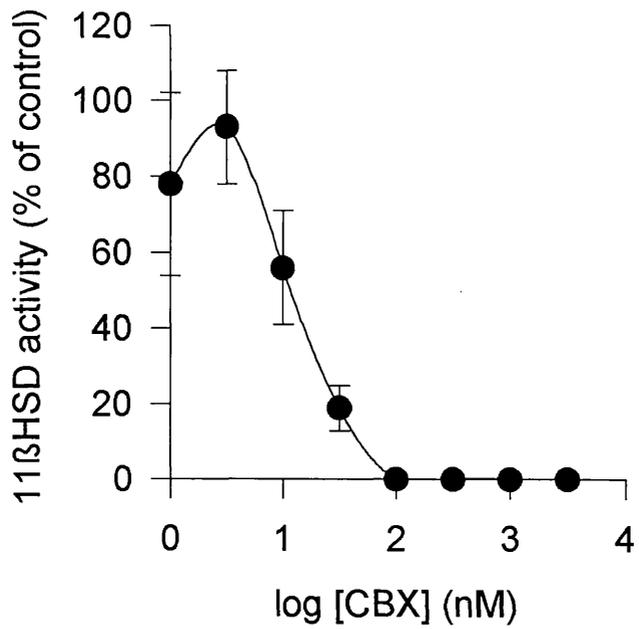
Figure 4.5: Concentration-response curves for *in vitro* inhibition of renal NAD^+-dependent 11ßHSD activity by CBX in kidneys obtained from a) adrenal intact and b) adrenalectomised rats. 11ßHSD activity is presented as % of control (0µM CBX concentration) value for individual kidneys.

a) Adrenal intact
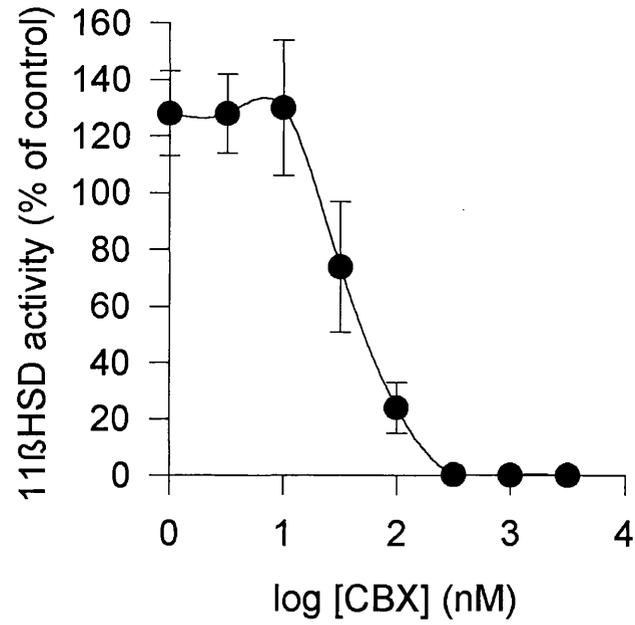
b) ADX
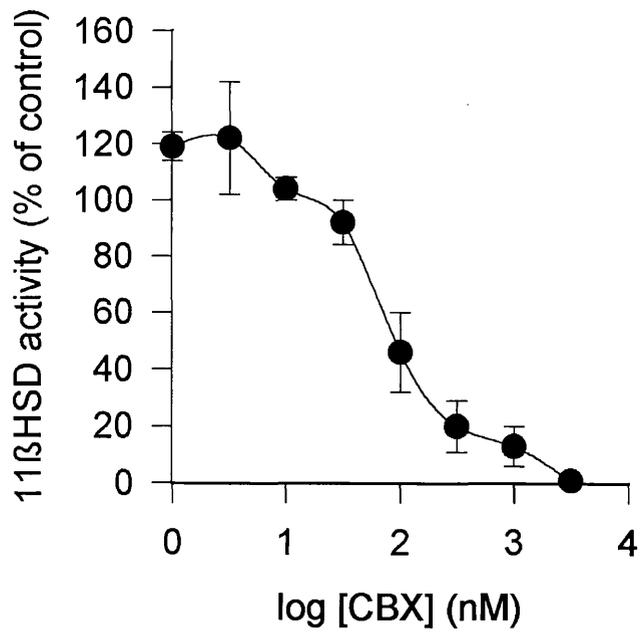
4.4. Discussion

The experiments described in Chapter 3, in which CBX was administered to adrenalectomised rats, indicated that adrenalectomy may affect the ability of CBX to inhibit renal 11ßHSD activities. In the studies described in this chapter, 11ßHSD activities following CBX infusion were determined in nephron segments and the whole kidneys of adrenal intact rats and were compared to the 11ßHSD activities of CBX treated adrenalectomised rats described in Chapter 3.

4.4.1. Effect of adrenalectomy on renal 11ßHSD activities in control animals

There were no differences in the $NADP^+$- or NAD^+-dependent 11ßHSD activities in the PCT and CCD, respectively, of control animals between adrenal intact *vs* adrenalectomised rats. In contrast, despite $NADP^+$-dependent activities being similar in both adrenal intact and adrenalectomised rats, NAD^+-dependent 11ßHSD activity in whole kidneys was 1.7-fold higher in adrenal intact rats compared to adrenalectomised animals. It should be noted that the NAD^+-dependent activity observed in the whole kidney of the adrenal intact rats in the current study is higher than that previously reported in Chapters 2 and 3. The reason for this discrepancy is unclear although it may reflect that fact that the adrenal intact rats used in the current study were infused with high dose corticosterone on the day of experimentation (to control for the acute infusion of corticosterone to adrenalectomised rats).

The discrepancy between control 11ßHSD activities in the whole kidney data and those of the microdissected segments may reflect the small amount of tissue used in the measurement of the latter: such variability is inherent in assays of microdissected segments. Furthermore, this inherent variability appears to be exacerbated by adrenalectomy; a greater variance in 11ßHSD activities between individual animals is apparent in the adrenalectomised group compared to the adrenal intact animals (Figures 4.1

and 4.2). In consequence, despite the fact that CBX inhibition of NADP$^+$- and NAD$^+$-dependent 11ßHSD activities was, on average, attenuated in segments dissected from adrenalectomised rats compared to adrenal intact animals, this effect did not reach statistical significance. Given the data variability between the microdissected samples, it is probable that the results obtained in the homogenates of contralateral kidneys of these rats are the more reliable indicators of the effect of adrenalectomy on renal 11ßHSD activities. As such, the remainder of this discussion will be limited to comparisons made between the contralateral kidneys of adrenal intact and adrenalectomised rats.

4.4.2. Effect of CBX infusion in homogenates of whole kidneys and liver

Data obtained using the whole kidney show that both NADP$^+$- and NAD$^+$-dependent enzyme activities are less susceptible to inhibition by CBX *in vivo* when animals are adrenalectomised compared to adrenal intact animals. This difference was particularly apparent for the NAD$^+$-dependent 11ßHSD activity which, in the adrenalectomised rat, appeared to have been stimulated (33% increase) following CBX administration, as opposed to the substantial inhibition (92%) seen in the adrenal intact rat. (The latter value was consistent with the level of 11ßHSD activity inhibition observed with 6mg/hr CBX in Chapter 2).

The findings reported in this chapter are consistent with a prior report by Latif *et al* (1992) which found no effect of s.c. CBX administration on renal NADP$^+$-dependent corticosterone oxidation in adrenalectomised male rats, despite substantial inhibition of NADP$^+$-dependent glucocorticoid oxidation when the tissue was incubated with CBX *in vitro*. Latif and co-workers attributed the lack of effect of 2.5mg and 10mg CBX to the subcutaneous route of administration: due to its predominantly hydrocarbon nature (see Figure 1.10), CBX might be expected to be absorbed into subcutaneous adipose tissue. In the experiments reported herein, despite the fact that this issue was circumvented by direct i.v. infusion of CBX, the ability of CBX to

inhibit renal 11ßHSD activities was likewise compromised, suggesting that adrenalectomy may have been the confounding factor in the aforementioned study performed in David Morris's laboratory.

4.4.3. Possible causes of altered response of renal 11ßHSD activities to CBX

The mechanisms by which the inhibition of renal 11ßHSD activities by CBX are attenuated in adrenalectomised animals are unknown. One conceivable explanation for the altered response was that adrenalectomy may inherently change the properties of 11ßHSD such that its physicochemical interaction with CBX is altered. This possibility was addressed by incubating biopsies of kidneys removed from both the adrenal intact and adrenalectomised animals with increasing concentrations of CBX *in vitro*. The fact that both $NADP^+$- and NAD^+-dependent renal 11ßHSD activities obtained from adrenalectomised animals were more (rather than less) sensitive to CBX *in vitro*, compared to renal tissue obtained from adrenal intact animals, suggests adrenalectomy does not compromise the inhibitory action of CBX (Table 4.6 and Figures 4.4 and 4.5).

The finding that adrenalectomy does not alter the response of renal 11ßHSD activities to CBX *in vitro* suggests that the decreased inhibition of enzyme activities following this surgery is a result of altered accessibility of the drug to the enzyme *in situ*. Clearly, however, this is not the case for the inhibition of hepatic 11ßHSD activity, since $NADP^+$-dependent hepatic 11ßHSD activity was inhibited by 97% in both adrenal intact and adrenalectomised rats. One possible reason for the different effects of CBX on the liver and kidney *in vivo* concerns the access of the drug to its site of action. Since the liver, which has a high perfusion rate, is the predominant route for conjugation and clearance of CBX, it is likely that a high concentration of the drug would be delivered to this organ regardless of adrenal status. In contrast, a lower concentration of the active drug may have been delivered to the kidney, with a further decrease in drug delivery following

adrenalectomy. Another possible explanation for the differences in the inhibition of NADP$^+$-dependent 11ßHSD activity in the liver compared to the kidney is due to different localisation of the 3 known transcripts for the enzyme. 11ßHSD1A (which encodes the fully active enzyme) is the only transcript thus far to have been localised to the liver, whereas transcripts 1A, 1B and 1C have all been found in the sheep and rat kidney (Yang, 1994; White *et al*, 1997). Although 11ßHSD1B and C are both truncated inactive enzymes, it is possible that they still bind both glucocorticoids and CBX and, as such, lower the concentration of CBX available to inhibit the active 11ßHSD1A isoform in the kidney. Since the 11ßHSD1B and 11ßHSD1C transcripts are not present in the liver, a higher concentration of CBX would be available to inhibit the active hepatic 11ßHSD1A enzyme, all else being equal. The question remains whether adrenalectomy would affect the relative exposure of each of these transcripts to CBX in the kidney.

It is possible that much of the CBX administered to the adrenalectomised rats did not reach the renal circulation, or if it did, CBX was not transported or taken up (in sufficient concentrations) by the cells and subcellular compartments containing the 11ßHSD to cause inhibition of this renal enzyme. A change in blood flow to the kidney is a possible cause of modified CBX delivery to its site of action, since the loss of catecholamines following excision of the adrenal medulla may impair blood flow not only to the kidney itself but also to the more distal regions of the nephron where the NAD$^+$-dependent type 2 isoform is localised. This explanation is unlikely, however, for several reasons. The capillary network, and, therefore, blood supply throughout the kidney is equally distributed amongst all nephron segments. Hence, there is no logical reason why CBX in the blood should access the proximal region of the nephron more readily than the distal component. Moreover, as reported in the preceding chapters, the intravenous administration of CBX for 3 or 5 hours did not affect GFR or arterial blood pressure whatever the adrenal status of the animal. Although renal blood flow is unlikely to be responsible for the altered response of renal 11ßHSD to CBX *in vivo*, it is possible that the removal of

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catecholamines may, for example, affect the cellular uptake of the drug. In order to determine whether changes in corticosteroid versus catecholamine concentrations are responsible for the altered response of renal 11ßHSD activities to CBX *in vivo*, a 'partial' adrenalectomy could be achieved pharmacologically with aminoglutethimide. Since this drug inhibits all cytochrome P450 enzymes required for steroidogenesis, the synthesis of corticosteroids could be prevented following the administration of aminoglutethimide to adrenal intact animals while catecholamine synthesis remained unaffected.

Aside from the loss of corticosteroids and/or catecholamines following adrenalectomy, other physiological factors may play a role in the altered response of renal 11ßHSD to CBX in *vivo*: ACTH for example, is not only influenced by corticosteroid concentrations, but is also reported to affect the activity of 11ßHSD itself. Circulating ACTH levels have been reported to be increased in adrenalectomised animals (Akana *et al*, 1985 and 1988). ACTH is reputed to inhibit renal 11ßHSD activities: a marked increase of the cortisol/cortisone ratio in urine or plasma has been reported during ACTH infusion but not following cortisol infusion in humans (Eisenschmid *et al*, 1987; Walker *et al*, 1992). It is thus possible that adrenalectomy alters ACTH concentrations, which in turn may decrease control 11ßHSD activities *and* affect the interaction between renal 11ßHSD and CBX. However, even if ACTH does affect such an interaction, ACTH concentrations in the adrenalectomised (steroid replaced) rats used in the current study are unlikely to be elevated since dexamethasone replacement has been reported to lower adrenalectomy-induced increases in ACTH concentrations to levels observed in adrenal intact rats (Akana *et al*, 1985 and 1988). Thus, alterations in circulating ACTH levels are not likely to be a major contributing factor to the altered response of renal 11ßHSD activities to CBX *in vivo* in adrenalectomised rats.

The most interesting finding of this particular study is that NAD^+-dependent 11ßHSD activity is far less susceptible to inhibition by CBX *in vivo* following

adrenalectomy than is the $NADP^+$-dependent activity. The different effect of CBX on the two isoforms following adrenalectomy may reflect the intracellular delivery of the GA derivative to the two isoforms within the target cell, rather than changes to the mechanism of CBX action (conclusions about which assume that once inside the cell, CBX has equal access to both isoforms). Several groups have investigated the intracellular localisation of the type 1 and type 2 isoforms within the target cell and found both isoforms of 11ßHSD in the endoplasmic reticulum (ER) with type 1 11ßHSD localised to the luminal membrane (Ozols, 1995) and type 2 in the cytoplasmic side of the ER (Fejes-Toth, 1996). As such there is no apparent reason why CBX should reach the $NADP^+$-dependent but not the NAD^+-dependent isoform within the cell. In the rat kidney, nuclear 11ßHSD activity has previously been reported (Kobayashi *et al*, 1987). However, these studies were performed on crude subcellular preparations prior to the description of the two distinct isoforms of 11ßHSD. Nonetheless, more recently, Shimojo *et al*, (1997) reported that, in addition to cytoplasmic staining, about 30-40% of the total 11ßHSD2 immunoreactivity in human tissues localises to the periphery of nuclei. However, the issue of intracellular 11ßHSD localisation has yet to be resolved since several studies have reported no such staining in either rabbit (Naray-Fejes-Toth, 1996 and 1998) or human nuclei (Kyossev, 1996) of aldosterone target cells.

The conclusions which can be drawn from the studies reported in this chapter are limited. The interpretation of the effects of adrenalectomy on renal 11ßHSD activities are confounded by the fact that several experimental variables occurred between the two groups of animals studied. The effect of adrenalectomy is difficult to interpret since adrenalectomised rats received chronic physiological doses of dexamethasone. Since this synthetic glucocorticoid is reported to be metabolised preferentially by the type 2 isoform of 11ßHSD (Siebe, 1993; Albiston, 1994; Ferrari *et al*, 1996), the profoundly different effects of CBX infusion on $NADP^+$ verus NAD^+-dependent 11ßHSD activities (in the current study taken to represent the type 1 and type 2 isoforms, respectively) could be due to the presence of

dexamethasone rather than to the adrenalectomy itself. Repetition of the studies without the replacement of dexamethasone would eliminate this possibility. In addition, the use of high doses of corticosterone administered on the day of experimentation may also have affected basal 11ßHSD activities and/or the interaction of CBX with the enzyme. Repetition of these experiments in adrenalectomised rats without the acute high dose corticosterone infusion, if performed in conjunction with the aforementioned proposed experiments (without dexamethasone replacement) would eliminate these confounding factors.

4.4.4. Conclusions

In conclusion, the present study has demonstrated that adrenalectomy and/or steroid replacement with dexamethasone or corticosterone, impairs the ability of CBX to inhibit renal 11ßHSD activities *in vivo*. The fact that this phenomenon occurs predominantly with NAD^+-dependent glucocorticoid oxidation is significant for several reasons: not only does it suggest that the mechanism of CBX action is more complex than had previously been anticipated, but that more functional differences between the $NADP^+$- and NAD^+-dependent isoforms of 11ßHSD may exist than are currently recognised.

Chapter 5:

Effects of changes in dietary intake of sodium and potassium, and of metabolic acidosis, on 11ß-hydroxysteroid dehydrogenase activities in rat kidney

5.1. Introduction

Adrenal steroids are important in maintaining normal sodium, potassium and acid-base balance. Increased mineralocorticoid activity, mainly as an elevation of plasma aldosterone concentration, is an important homeostatic response to chronic acid or potassium loads, and to sodium depletion (Kurtzman *et al*, 1990, see Laragh and Sealey, 1992). In Chapters 2 and 3, it was demonstrated that sodium and potassium transport within the kidney were altered when renal 11ßHSD activities were inhibited by the administration of CBX. Given the postulated role of renal 11ßHSD2 in regulating glucocorticoid access to MR, it was hypothesised that changes in renal 11ßHSD activities may be a feature of the renal adaptation to changes in potassium, sodium and acid-base balance. Increased potassium and/or decreased sodium intake (which necessitate increased mineralocorticoid action) could, for example, be associated with decreased 11ßHSD activity, effecting greater access of glucocorticoids to renal MR. The aim of the experiments described in the current chapter was, therefore, to investigate the effects of changes in dietary intake of potassium and sodium, and of metabolic acidosis, on $NADP^+$- and NAD^+-dependent 11ßHSD activities in the rat kidney.

5.2. Materials and Methods

5.2.1. Dietary changes in potassium and sodium intake, and induction of acidosis

Male Sprague-Dawley rats (initial weight range 130-160g) were maintained on either a standard chow rat diet containing 140mmol/l sodium and 180mmol/l potassium per kg dry weight, a potassium-deficient diet (TD 88239, Harlan Teklad, Bicester, UK; <750μmol/l potassium per kg dry weight), a high potassium diet (TD 94121; 1.25 mol potassium per kg dry weight) or a low sodium diet (821653, Special Diet Services, Witham, UK; 13 mmol/l sodium per kg dry weight) for 10 days. On day 9, animals were placed in individual metabolism cages and urine was collected over 24 hours for the measurement of urinary sodium and potassium concentrations. On day 10, each rat was anaesthetized with pentobarbitone sodium (60mg/kg, i.p.; Sagatal, Rhone Merieux, Harlow, UK) and the right femoral artery was cannulated with polyethylene tubing. A 1ml sample of arterial blood was then taken for the measurement of plasma sodium, potassium and aldosterone concentrations and, after sacrifice, the kidneys were rapidly excised and snap-frozen in liquid nitrogen.

Acidosis was induced by the addition of 1.5% (w/v) NH_4Cl to the drinking water of rats maintained on a standard chow diet. One group of animals was given NH_4Cl for 3 days and another group for 10 days (to allow longer adaptation). Arterial blood samples were taken as already described and total plasma CO_2 concentrations (TCO_2) were also measured.

5.2.2. Radiometric conversion assay of renal 11ßHSD activities

Biopsies of all samples were assayed for $NADP^+$- and NAD^+-dependent 11ßHSD activities as described in section 2.2.1.3 using a quarter of each kidney prepared by sequentially bisecting the kidney in the sagittal and transverse planes.

5.2.3. Effects of changes in sodium and potassium concentration *in vitro* on renal 11ßHSD activities

Male Sprague Dawley rats (300g) were sacrificed by cervical dislocation and kidneys rapidly excised and snap-frozen in liquid nitrogen. 11ßHSD activity was determined using a modification of the method described above. In brief, after preparation in the hypotonic lysis buffer, the homogenate was divided into two equal volumes. One was restored to isotonicity by the addition of 1.5mol NaCl whilst the other was made isotonic with 1.5mol KCl. The first homogenate (with NaCl) was diluted 7:1 with a simple salts buffer (1mmol/l $MgSO_4$, 10mmol/l HEPES, 0.1% (w/v) BSA, 10mmol/l glucose, 140mmol/l NaCl, 5mmol/l KCl, 1.8mmol/l calcium acetate). The second aliquot of homogenate (with KCl) was diluted in a similar buffer but with a reversed ratio of NaCl to KCl (simple salts buffer as above but 5mmol/l NaCl and 140mmol/l KCl). Thereafter, the samples were referred to as low potassium/high sodium and high potassium/low sodium samples respectively. The $[^3H]$-F and enzyme cofactors were prepared in double distilled water (rather than PBS) and added as described in section 2.2.1.3. Incubations were performed over 1, 4 and 24 hr periods, instead of the 30 minute incubations carried out for the preceding enzyme assays. These assay durations were selected to establish the time-dependent nature of any effects of the K^+:Na^+ ratio on 11ßHSD activities *in vitro*. Whereas enzyme activities obeyed first order Michaelis-Menten kinetics at 1 hr (for both $NADP^+$- and NAD^+-dependent glucocorticoid oxidation) and at 4 hr (for the

NAD^+-dependent 11ßHSD activity only), rates of cortisol metabolism ceased to be linear after 4 hr.

5.2.4. Analyses

Urinary and plasma sodium and potassium concentrations were measured by flame photometry (model 543, Instrumentation Laboratory, Warrington, UK). Plasma aldosterone concentrations were measured by immunoassay (Coated-tube Aldosterone kit, DPC Ltd, Caenarfon, UK; within assay coefficient of variation = 7.2%; between assay coefficient of variation = 8.9%). TCO_2 measurements were made on a total CO_2 analyzer (model 965, Ciba-Corning, Halstead, UK) to indicate plasma bicarbonate concentrations.

5.2.5. Statistics

All data are presented as means ± sem. For all *in vivo* experiments, statistical differences were assessed using ANOVA and post hoc Tukey-Kramer multiple comparison tests to compare mean values for (1) animals fed the control diet *vs* high and low potassium, and low sodium rats; and (2) control acidotic animals (0 day) to 3 and 10 day acidotic animals. For *in vitro* experiments, statistical comparisons were assessed using Student's unpaired *t*-test for comparisons between high potassium/low sodium *vs* high sodium/low potassium concentrations. In all analyses, a difference was considered statistically significant if $P<0.05$.

5.3. Results

5.3.1. Effect of changes in sodium and potassium intake

5.3.1.1. Plasma and urine data

Animals maintained on a potassium-deficient diet were hypokalaemic, though the plasma aldosterone concentration was not suppressed (Table 5.1). Rats maintained on a high potassium diet were slightly hyperkalaemic and had an increased plasma aldosterone concentration (3.6-fold; $P<0.05$); rats fed a low sodium diet had an even greater increase in the plasma aldosterone concentration (7-fold; $P<0.01$) with no change in the plasma sodium concentration (Table 5.1).

Table 5.1: Plasma electrolyte and aldosterone concentrations in rats maintained on either a control, low or high potassium, or low sodium diet. P_K, P_{Na} and P_{aldo} are the plasma concentrations of potassium, sodium and aldosterone, respectively. All values are mean ($\pm$ sem) n=5 in all groups, except low potassium where n=6. $*P<0.05$ vs control, $**P<0.01$ vs control, $***P<0.001$ vs control.

	Control	Low K	High K	Low Na
P_K (mmol/l)	4.0 ± 0.1	$1.8 \pm 0.1***$	$4.7 \pm 0.1**$	4.0 ± 0.1
P_{Na} (mmol/l)	140 ± 0.8	141 ± 1.6	140 ± 1.0	141 ± 0.5
P_{aldo} (pmol/l)	780 ± 103	1083 ± 110	$2815 \pm 704*$	$5402 \pm 713**$

Although rats maintained on the low potassium diet were smaller than control animals ($166 \pm 9g$ *vs* $195 \pm 6g$ body weight, respectively; $P<0.01$), there was no change in kidney weights (0.81 ± 0.1 *vs* $0.76 \pm 0.1g$, respectively) between these two groups of animals, due to renal hypertrophy in the potassium-depletion. Thus all urinary data are presented as total urinary electrolyte excretion over the 24 hr collection period. Urinary excretion of potassium was decreased in potassium-depleted rats (Figure 5.1a; $P<0.001$) and increased in potassium-loaded animals (Figure 5.1a; $P<0.001$). Sodium excretion was reduced in rats fed a low sodium diet (Figure 5.1b; $P<0.05$).

Figure. 5.1: 24hr potassium and sodium urinary excretion (U_xV) in rats maintained on (a) low (L), control (C), and high (H) potassium diets or (b) control (C) or low (L) sodium diets. *$P<0.05$, **$P<0.01$ compared with control values.

a)

b)

Dietary potassium

Dietary Sodium

K⁺ excretion

Na⁺ excretion
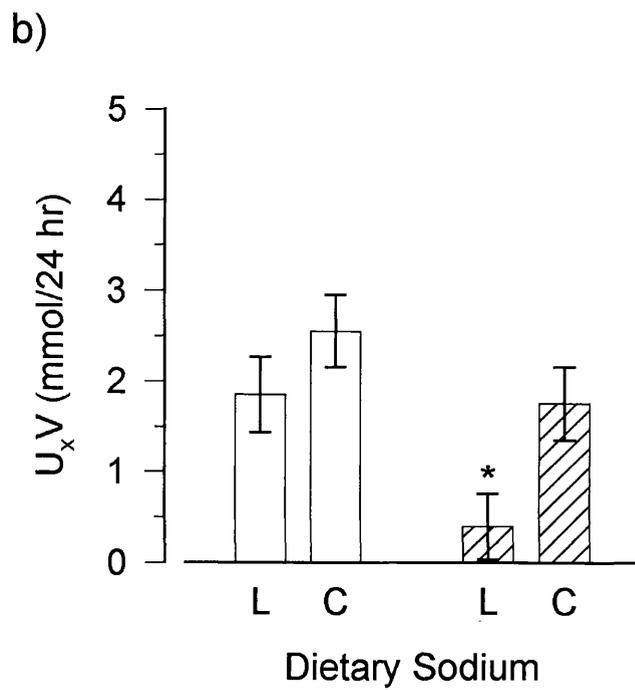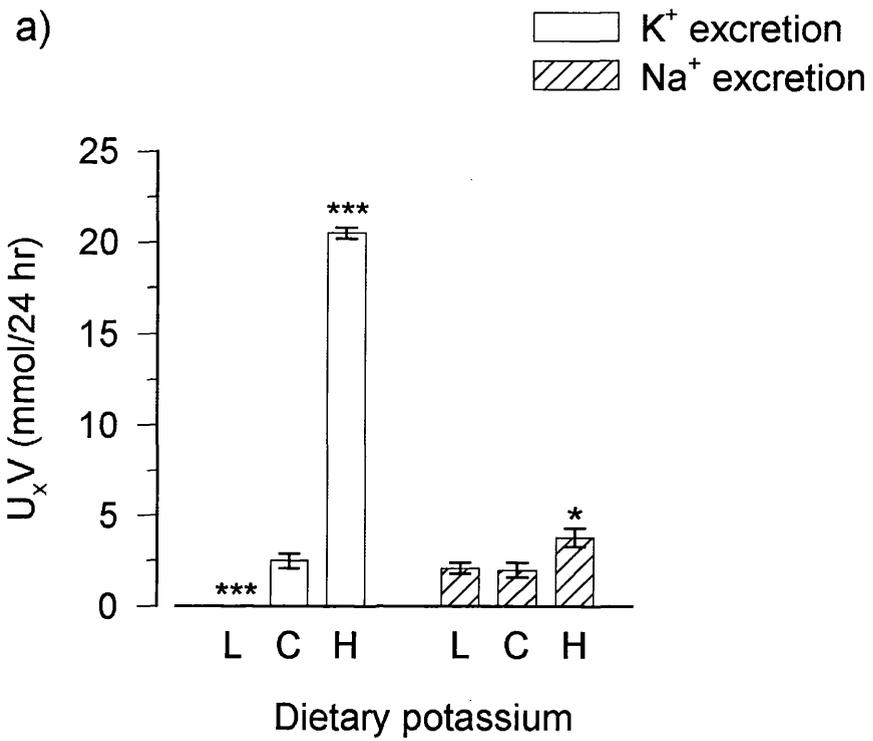
5.3.1.2. 11ßHSD activities

In animals maintained on a high potassium diet, NAD^+-dependent 11ßHSD activity was decreased by 59% compared with control (Figure 5.2; $P<0.01$), whereas $NADP^+$-dependent cortisol oxidation was unaltered (Figure 5.2). In contrast, decreasing dietary potassium intake had no effect on either $NADP^+$- or NAD^+-dependent 11ßHSD activities (Figure 5.2). In animals maintained on a low sodium diet, NAD^+-dependent 11ßHSD activity was decreased by 28% compared with control animals (Figure 5.3; $P<0.05$); $NADP^+$-dependent cortisol oxidation was lower in sodium depleted *vs* control rats but this decrease was not significant (Figure 5.3).

Figure.5.2: 11ßHSD activities (pmol F oxidised/mg protein.30 min) in renal tissue obtained from rats maintained on low (L), control (C) and high (H) potassium diets, measured *ex vivo* in the presence of 400µmol/l NADP$^+$ or NAD$^+$ and 500nmol/l F. *P<0.05, **P<0.01.
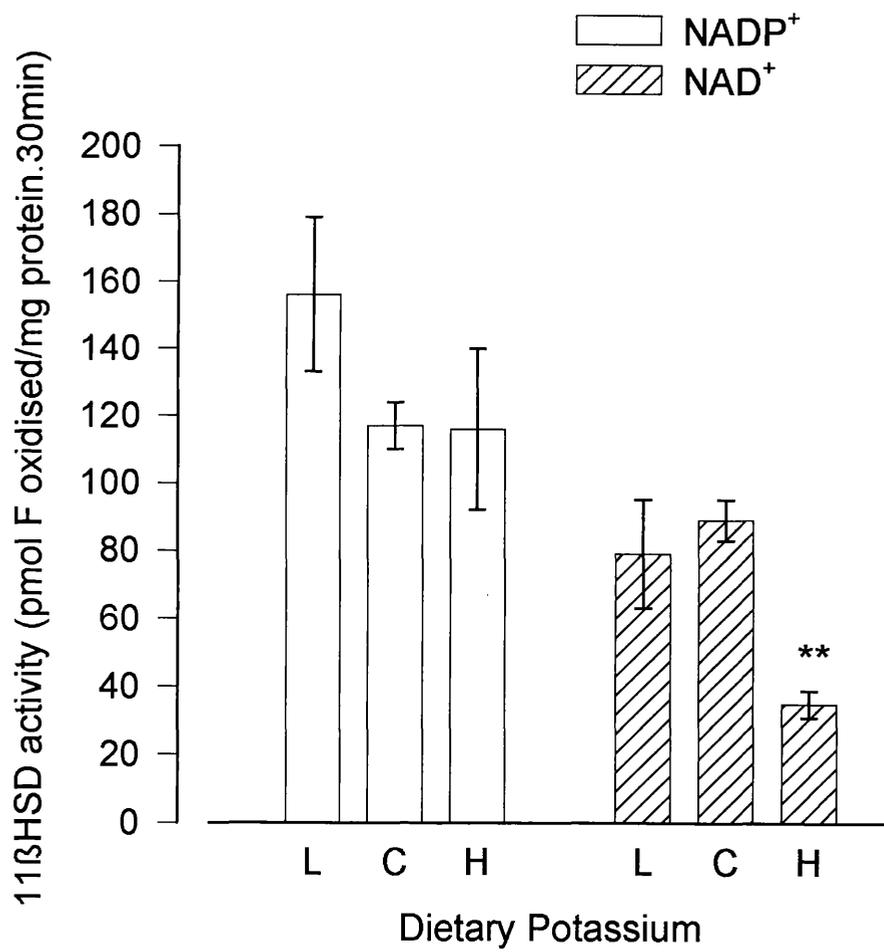
Figure. 5.3: 11ßHSD activities (pmol F oxidised/mg protein.30 min) in renal tissue obtained from rats maintained on low (L) and control (C) sodium diets, measured *ex vivo* in the presence of 400µmol/l $NADP^+$ or NAD^+ and 500nmol/l F. *$P<0.05$ compared with control values.
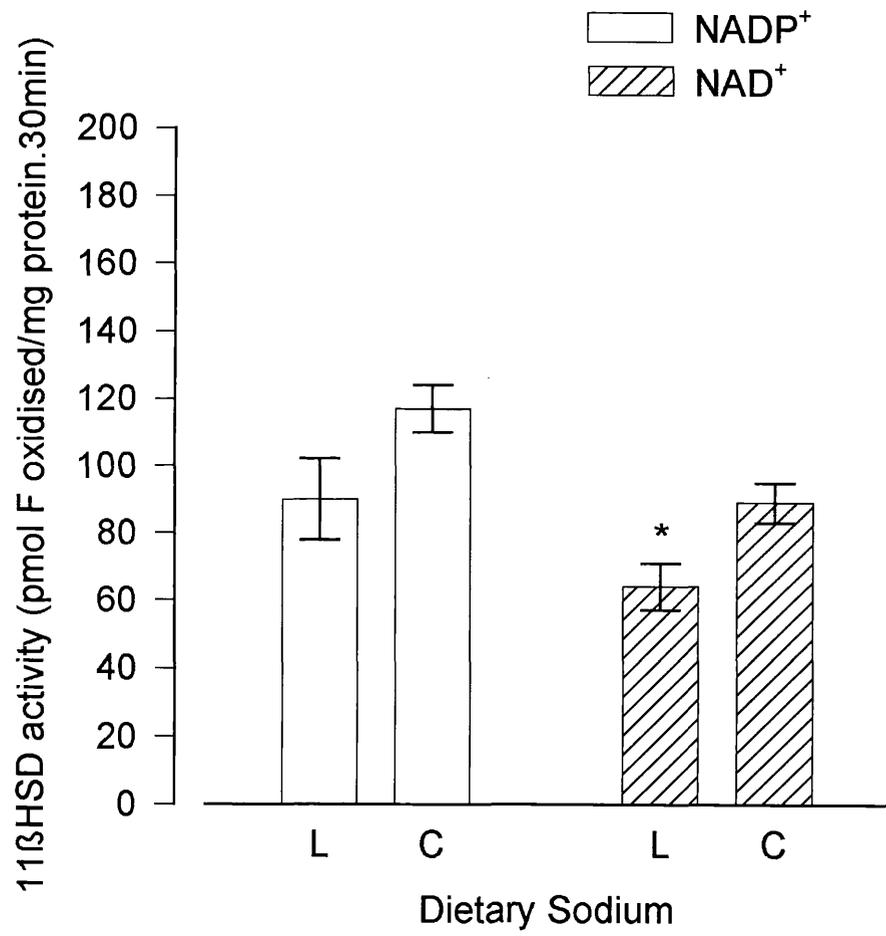
5.3.2. Effect of changes in sodium and potassium concentrations *in vitro*

There were no differences in either $NADP^+$- or NAD^+-dependent 11ßHSD activities between incubations in high potassium/low sodium *vs* high sodium/low potassium concentrations *in vitro*, irrespective of assay duration (Table 5.2).

Table 5.2. 11ßHSD activities (pmol F oxidised/mg protein.unit time) in renal homogenates incubated *in vitro* in high potassium/low sodium (High K^+) or low potassium/high sodium (Low K^+) concentration assay buffers in the presence of 400µmol/l $NADP^+$ or NAD^+ and 500nmol/l F. All values are mean ($\pm$ sem) n=4 kidneys per group.

	$NADP^+$		NAD^+	
	High K^+	Low K^+	High K^+	Low K^+
1hr	714 ± 92	731 ± 67	101 ± 16	102 ± 9
4hr	2025 ± 160	1983 ± 143	346 ± 42	403 ± 74
24hr	2899 ± 126	2892 ± 109	470 ± 58	471 ± 42

5.3.3. Effect of metabolic acidosis

5.3.3.1. Plasma and urine data

Rats maintained on the 1.5% (w/v) NH_4Cl drinking water for either 3 or 10 days were moderately acidotic compared with control rats (Table 5.3; $P<0.01$) although plasma aldosterone concentrations were only significantly higher in the 10-day acidotic rats (Table 5.3; $P<0.01$). Both short-term and longer-term acidotic animals exhibited antikaliuresis and antinatriuresis (Table 5.3; $P<0.01$; $P<0.001$, respectively).

Table 5.3. Plasma total carbon dioxide (TCO_2) concentration, plasma aldosterone (P_{aldo}) concentration and urinary potassium and sodium excretion (U_KV and $U_{Na}V$, respectively) in normal, 3-day and 10-day NH_4Cl-treated acidotic rats. All values are mean ($\pm$ sem) n=5 animals per group. **$P<0.01$; ***$P<0.001$ vs Day 0.

	Duration of acidosis (days)		
	0	3	10
TCO_2 (mmol/l)	27.2 ± 1.0	21.5 ± 0.8**	21.3 ± 0.8**
P_{aldo} (pmol/l)	834 ± 155	2417 ± 469	3162 ± 592**
U_KV (μmol/24hr)	218 ± 15	87 ± 7***	148 ± 9**
$U_{Na}V$ (μmol/24hr)	101 ± 2	42 ± 4***	77 ± 5**

5.3.3.2. 11ßHSD activities

In both 3-day and 10-day acidotic rats, NAD^+-dependent 11ßHSD activity was decreased by 50% and 52% respectively (Figure 5.4; $P<0.01$ for both). $NADP^+$-dependent cortisol oxidation in both short-term and longer-term acidotic rats was unaltered (Figure 5.4).

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Figure. 5.4: 11ßHSD activities (pmol F oxidised/mg protein.30 min) in renal tissue obtained from rats maintained on NH_4Cl drinking water for 0, 3 or 10 days, measured *ex vivo* in the presence of 400μmol/l $NADP^+$ or NAD^+ and 500nmol/l F. **P<0.01 compared with control values.
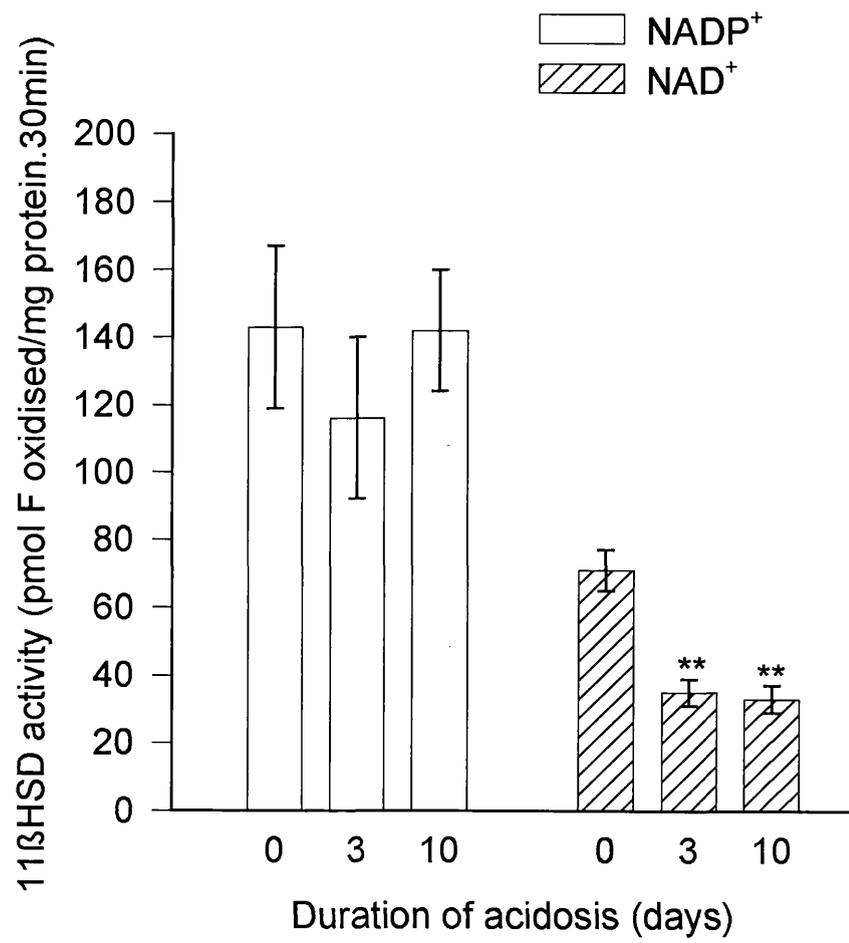
5.4. Discussion

The experiments described in the current chapter were designed to investigate whether changes in sodium and potassium balance and acid/base status, may evoke alterations in the activity of renal 11ßHSD that would contribute to the adaptive renal response by affecting the access of glucocorticoids to renal corticosteroid receptors.

5.4.1. The experimental model

In the present study, as for those described in Chapter 2, it is assumed that $NADP^+$-dependent 11βHSD activity represents the proximal isoform (11ßHSD1), whereas NAD^+-dependent activity is indicative of changes in the distal nephron (11ßHSD2). It is recognised that the use of different cofactors does not allow absolute discrimination between the activities of the specific isoforms of 11βHSD, since 11βHSD1, though preferentially utilising $NADP^+$ as an oxidant co-factor, can also employ NAD^+. This being the case, effects of dietary manipulations on the activity of 11ßHSD1 should be reflected in comparable changes to both $NADP^+$-dependent and NAD^+-dependent glucocorticoid oxidation. In contrast, changes in NAD^+-dependent 11ßHSD activity that are not accompanied by parallel changes in $NADP^+$-dependent cortisol oxidation can be assumed to indicate selective effects on 11ßHSD2 activity. In the present study, the fact that potassium and acid loading and sodium depletion substantially decreased NAD^+-dependent 11βHSD activity without affecting $NADP^+$-dependent cortisol metabolism suggests that these effects reflect decreases in 11βHSD2 activity.

It must also be emphasised that the altered electrolyte and water handling which occurred in response to the dietary manipulations reported herein, can occur independently of adrenal steroids. In this context, changes in sodium, potassium and acid excretion cannot be attributed unambiguously to altered corticosteroid concentrations alone. For example, in rats maintained on the high potassium diet, polydipsia and polyuria would be

expected (and indeed occurred); an important consideration, since it is well documented that increased renal flow rate results in increased potassium secretion in the distal tubules (Good and Wright, 1979, Malnic et al, 1989) independent of adrenal steroids. Hence, in the *in vivo* model used in the current study, the access of glucocorticoids to renal MR and GR cannot be assessed using urinary electrolyte measurements.

5.4.2. Altered dietary potassium intake.

As expected, increased dietary potassium intake was associated with a marked increase in the circulating aldosterone concentration; urinary potassium excretion was substantially elevated with only a moderate rise in the plasma potassium concentration. In these potassium-adapted animals, a 59% decrease in NAD^+-dependent 11ßHSD activity was observed, whereas $NADP^+$-dependent activity was unchanged. This is consistent with a decrease in 11βHSD2 activity in the distal nephron and no alteration in 11βHSD1 activity. Hence, decreased 11ßHSD2 activity may enhance the mineralocorticoid effects of circulating glucocorticoids such that these adrenal steroids could act in concert with aldosterone to promote potassium excretion.

Although dietary potassium deprivation is typically associated with hypokalaemia, reduced urinary potassium secretion and metabolic alkalosis (Wright and Giebisch, 1985), the effect on the plasma aldosterone concentration is variable. In the present study, rats maintained on a low potassium diet were hypokalaemic and exhibited significantly reduced potassium excretion but plasma aldosterone was little different from that of rats on a control diet. Although it might be anticipated that suppressed plasma aldosterone would promote renal potassium conservation, it may not be a prerequisite. During potassium depletion, net reabsorption in the proximal nephron is largely unchanged but collecting duct function can be altered from net secretion to net reabsorption of potassium. The main mechanisms involved are a reduction in potassium channel density in the

apical membrane of the principal cell (Lang and Rehwald, 1992) and an increase in H^+/K^+-ATPase-mediated potassium reabsorption in the intercalated cell (Eiam-Ong *et al*, 1993); both can occur independently of mineralocorticoids. Given the lack of effect of the low potassium diet on the plasma aldosterone concentration in the present study, it is perhaps not surprising that renal 11βHSD activities were unaffected by this dietary manipulation. Under normal conditions, 11βHSD2 appears to be highly efficient in protecting the MR from activation by glucocorticoids and, as such there may be little need to increase NAD^+-dependent 11βHSD activity in the distal nephron in response to potassium deprivation. Furthermore, it has been reported that a high potassium diet increases MR expression in adrenalectomised rats, whereas potassium depletion has no down-regulatory effect on MR expression (Rafestin-Oblin, 1984).

5.4.3. Low sodium intake.

In rats fed a low sodium diet, urinary sodium excretion was significantly reduced and the plasma aldosterone concentration was seven-fold higher than in control animals. In such a state of renal sodium conservation, suppression of 11βHSD2 activity might have been expected, to permit glucocorticoid activation of renal MR and further enhance renal sodium retention. Indeed, NAD^+-dependent 11βHSD activity was decreased by 28% whereas $NADP^+$-dependent glucocorticoid oxidation was not decreased significantly by this dietary regime. The effect of *increasing* dietary sodium intake was not investigated in the current study since the standard rat chow used as the control diet already has a high sodium content. However, a recent study has shown that a high sodium diet stimulates $NADP^+$-dependent 11βHSD activity in the proximal tubule of the dog (Brem *et al*, 1997); although the functional significance of this finding is unclear.

5.4.4. Metabolic acidosis.

Since acidosis is a common feature of adrenal insufficiency and mineralocorticoids promote acid excretion (see Chapter 1), it was postulated that acidosis may result in inhibition of renal 11βHSD2 activity, leading to activation of MR by glucocorticoids and enhanced net acid excretion. Rats maintained on 1.5% NH_4Cl for 3 and 10 days were acidotic, as indicated by their reduced plasma TCO_2 content (an index of plasma bicarbonate concentration). As anticipated, plasma aldosterone concentrations were higher in both 3 and 10 day acidotic rats compared with control animals, and NAD^+-dependent 11βHSD activity was significantly decreased at each time point (50 and 52%, respectively). $NADP^+$-dependent glucocorticoid oxidation was unaffected in either case. These data suggest that acidosis promotes renal acid excretion by stimulation of the MR due to both an increase in the plasma aldosterone concentration and greater access of glucocorticoids to MR. Furthermore, as stated in chapter 1, the role of glucocorticoids in the maintenance of acid base balance in the collecting duct is well known, and it is thus likely that the increased access of glucocorticoids to the GR also plays an important role in the renal adaptation to metabolic acidosis. The data presented herein are consistent with the 26% decrease in glucocorticoid oxidation in the IMCD of 1-day acid-loaded rats recently reported by Nolan *et al* (1997). Together, the two studies suggest that the suppression of type 2 11ßHSD activity in response to acidosis is of rapid onset and progressively increases to a certain level (seen in the current study after 3 days of acid loading) whereafter enzyme activity is maintained at a constant (albeit a decreased) level.

5.4.5. Possible mechanisms causing decreases in 11ßHSD activities

The mechanisms by which the observed decreases in NAD^+-dependent 11ßHSD activity occur in response to potassium and acid loading or sodium deprivation remain unclear. The possibility of *direct* effects of increased potassium ion concentration or decreased sodium ion concentration on the

enzymes was investigated *in vitro*. However, a reversal of the sodium:potassium concentration ratio from 28:1 to 1:28 had no effect on either NADP$^+$- or NAD$^+$-dependent glucocorticoid metabolism over 24hr.

It seems likely, therefore, that the inhibition of 11βHSD2 activity in potassium loaded or sodium deprived rats is dependent on some other factor(s) involved in the response to hyperkalaemia or dietary sodium deficiency. Whereas the elevation in the aldosterone concentration is an obvious candidate, this seems unlikely since the reduction in dietary sodium intake, which produced a greater increase in the circulating aldosterone concentration than did either potassium or acid loading, had less of an effect on NAD$^+$-dependent renal 11ßHSD activity (28% decrease) than did potassium loading (59% decrease) or acidosis (50-52% decrease). Moreover, administration of aldosterone to adrenalectomised rats has been shown to *increase* rather than decrease 11βHSD activity in the distal nephron (Alfaidy *et al*, 1997) The same authors also reported that vasopressin, which is also a known stimulus to distal nephron potassium secretion (Schafer *et al*, 1990), stimulated 11ßHSD activity (Alfaidy *et al*, 1997). Since NAD$^+$-dependent 11ßHSD activity was only ever decreased in response to the dietary manipulations employed in the current study, AVP concentrations, (not measured in the current study), are unlikely to have been the cause of the altered glucocorticoid oxidation. It has also been reported, recently, that increased potassium excretion is associated with elevated urinary levels of endogenous inhibitors of renal 11ßHSD activities, so-called 'Glycyrrhetinic Acid Like Factors' (Morris *et al*, 1998). It is possible that increased levels of these factors may have contributed to the observed decrease in NAD$^+$-dependent 11ßHSD activity in potassium-loaded rats.

It is possible that altered acid-base status itself may be responsible for the decreases in NAD$^+$-dependent enzyme activity observed in both potassium- and acid-loaded rats. Both conditions cause metabolic acidosis and both cloned 11βHSD isoforms are equally pH sensitive *in vitro* with low pH decreasing glucocorticoid oxidation by 11ßHSD1 and 11ßHSD2 (Monder

and Lakshmi, 1989, Lakshmi *et al*, 1993; Nolan *et al*, 1997). However, in the present study, $NADP^+$-dependent activity was unaffected by *in vivo* acidosis, suggesting that systemic pH, under these conditions, does not alter enzyme activity. Similarly, the intracellular acidosis associated with systemic acid-loading does not appear to regulate 11βHSD2 activity since potassium-depletion, a condition also associated with a fall in intracellular pH (Adam *et al*, 1986) did not alter the activity of either enzyme isoform.

From the experiments described herein, it is unknown whether changes in the level of NAD^+-dependent glucocorticoid oxidation represent decreased activity of existing enzyme or altered levels of enzyme expression. However, a recent study (Brem *et al*, 1997) has reported that the increased $NADP^+$-dependent 11ßHSD activity in the proximal tubule of dogs on a high dietary sodium intake was due to both a 4-fold increase in the V_{max} of 11ßHSD and a 2-fold increase in 11ßHSD1 mRNA expression, suggesting that regulation of this enzyme probably involves both altered enzyme kinetics and levels of enzyme expression.

5.4.6. Conclusions

In conclusion, adaptations to both high potassium and low sodium diets, and to metabolic acidosis, involve not only increased mineralocorticoid secretion but also decreased renal NAD^+-dependent 11βHSD activity. The decreases in enzyme activity in response to these dietary manipulations would facilitate the binding of endogenous glucocorticoids to both GR and MR, which would be expected to complement the aldosterone-mediated regulation of potassium, sodium and proton excretion.

A major limitation of the current study is that the use of 'whole kidney' homogenates does not provide information with regard to the response of individual nephron segments to these dietary manipulations. It is possible that 11ßHSD activities in certain parts of the nephron respond to certain

physiological stimuli whilst glucocorticoid oxidation in other parts of the kidney remains unaltered. The experiments described in the following chapter were designed to investigate this possibility.

Chapter 6:

Effect of dietary potassium loading on 11ß-hydroxysteroid dehydrogenase activities along the rat nephron

6.1. Introduction

From the experiments described in Chapter 5, it is apparent that renal 11ßHSD activities can be altered by certain physiological stimuli. The fact that only NAD^+-dependent glucocorticoid oxidation was decreased in response to potassium and acid loading and sodium depletion, is consistent with the proposed role for 11ßHSD2 in 'protecting' the MR. An enhancement of increased activation of MR would be beneficial in these circumstances.

A major limitation of the studies described in the previous chapter, however, was that the use of renal homogenates does not provide information relating to the region of the nephron affected by physiological alterations. In view of the proposed role for NAD^+-dependent 11ßHSD, it may be anticipated that enzyme activities in more distal nephron sites (ie, mineralocorticoid target sites) may be preferentially altered in response to these physiological changes. Thus, the series of experiments described in this chapter were designed to investigate the effects of potassium loading on 11ßHSD activities in the rat CCD, a known mineralocorticoid target site. In addition to this nephron segment, $NADP^+$-dependent and NAD^+-dependent 11ßHSD activities were also measured in PCT, medullary and cortical TAL, and OMCD; areas of the nephron in which the roles of 11ßHSD are poorly elucidated.

6.2. Materials and Methods

6.2.1. Dietary changes in potassium intake

Male Sprague-Dawley rats (initial weight range 90-98g) were maintained on either a standard chow rat diet as described in section 2.2.2.1, or on a high potassium diet (see 5.2.1). On day 9, animals were placed in individual metabolism cages, water consumption monitored and urine collected over 24 hours for the measurement of potassium and sodium concentrations, osmolality and pH. On day 10, each rat was anaesthetised and prepared for microdissection surgery as described in 3.2.1 except that prior to the perfusion of the left kidney, a 1ml sample of blood was obtained from the aorta for the measurement of plasma potassium, sodium, aldosterone, TCO_2 and osmolality. After sacrifice the contralateral kidney was rapidly removed and snap-frozen in liquid nitrogen.

6.2.2. Determination of *ex vivo* 11ßHSD activities in single nephron segments

Microdissection of nephron segments and subsequent assays for the measurement of 11ßHSD activities in PCT, mTAL, cTAL, CCD and OMCD segments were performed as described in Chapter 3 (3.2.7).

6.2.3. Statistics

Data are presented as means ± sem. Student's unpaired *t*-test was employed for statistical comparisons to assess differences in 11ßHSD activities between rats maintained on the normal and high potassium diets in individual nephron segments. Mann-Whitney U non-parametric test was employed for statistical comparisons to assess differences in $NAD^+/NADP^+$ 11ßHSD activity ratios between rats maintained on the normal and high potassium diets in individual nephron segments (since ratios are not

normally distributed). Statistical significance for both tests was taken as $P<0.05$.

6.3 Results

6.3.1. Plasma and urine data

Rats maintained on the high potassium diet became hyperkalaemic and acidotic and had an elevated (2-fold higher) circulating aldosterone concentration compared to those animals maintained on the control diet. Plasma sodium concentration and osmolality was similar in the two groups of animals (Table 6.1).

Table 6.1. Plasma electrolyte and aldosterone concentrations in rats maintained on either a control or high potassium diet. P_K, P_{Na}, P_{aldo}, and TCO_2 are the plasma concentrations of potassium, sodium, aldosterone and total carbon dioxide, respectively; P_{OSM}. is the plasma osmolality. All values are means ($\pm$ sem) n=8 animals per group ***$P<0.001$ compared to control animals.

	Control	High Potassium
P_K (mmol/l)	3.5 ± 0.2	4.7 ± 0.2***
P_{Na} (mmol/l)	139.8 ± 0.9	141.7 ± 1.2
P_{aldo} (pmol/l)	1736 ± 143	3813 ± 240***
TCO_2 (mmol/l)	23.2 ± 0.6	18.8 ± 0.8***
P_{OSM} (mosm/kg H_2O)	305 ± 5	300 ± 2

Potassium-loaded rats exhibited polydipsia (80 ± 2ml *vs* 28 ± 2ml water drank/24hr, $P<0.001$) and polyuria (Table 6.2, $P<0.001$). Urinary osmolality and pH were lower in the potassium-loaded rats (Table 6.2, $P<0.001$ for both); urinary potassium excretion was increased (Figure 6.1, $P<0.001$) but urinary sodium excretion was unchanged (Figure 6.1).

Figure 6.1: 24hr potassium and sodium urinary excretion (U_xV) in rats maintained on control (C) and high potassium (HK) diets. Values are mean ± sem. ***P<0.001 compared with control values.
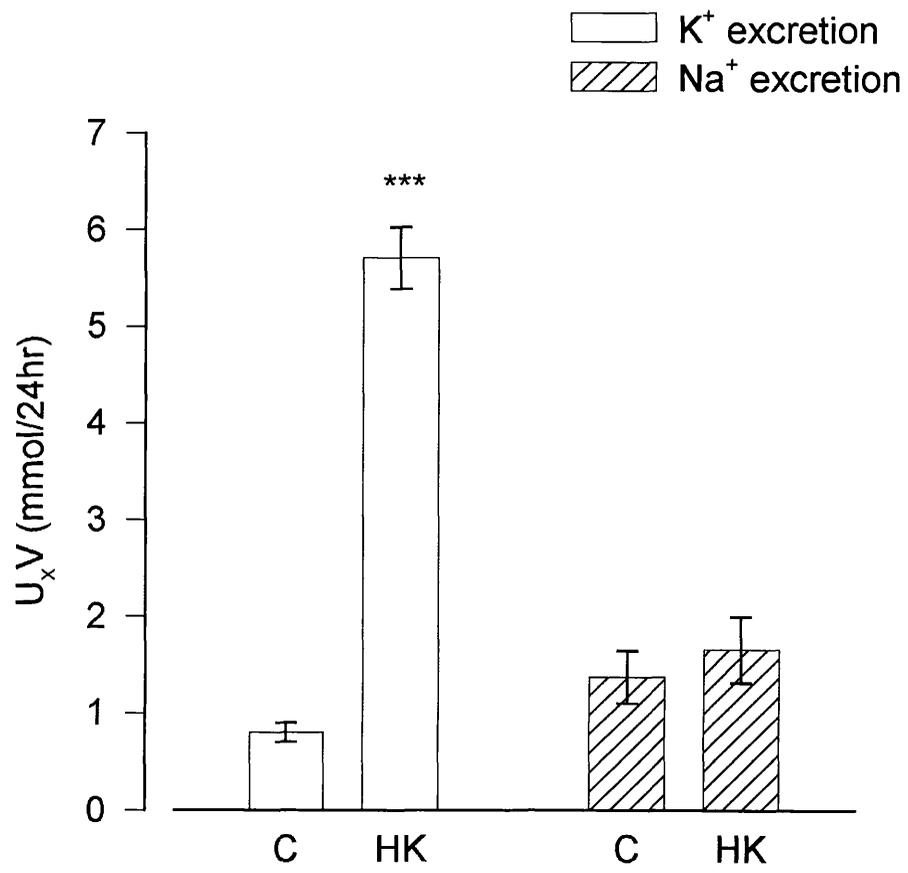
Table 6.2. Urine data in rats maintained on either a control or high potassium diet. V is urine flow rate, U[K$^+$] and U[Na$^+$] are urinary potassium and sodium concentrations, respectively; U$_{OSM}$ is the urine osmolality. **$P<0.01$, ***$P<0.001$ compared to control animals. n = 8 animals per group.

	Control	High Potassium
V (ml/24hr)	9 ± 1	63 ± 2***
U[K$^+$] (mmol/l)	88 ± 8	91 ± 3
U[Na$^+$] (mmol/l)	180 ± 18	23 ± 3***
U$_{OSM}$ (mosm/kg H$_2$O)	1622 ± 187	824 ± 29**
pH	8.2 ± 0.2	6.2 ± 0.2**

6.3.2. 11ßHSD activities in single nephron segments

In the PCT, dietary potassium loading was associated with an increase in NADP$^+$- and a decrease in NAD$^+$-dependent 11ßHSD activity, although neither change in enzyme activity was statistically significant (Figures 6.2 and 6.3, respectively). The ratio of NAD$^+$/NADP$^+$-dependent 11ßHSD activities in the PCT was significantly decreased in potassium loaded rats (Table 6.3; $P<0.05$).

Dietary potassium loading did not increase either NADP$^+$-dependent or NAD$^+$-dependent 11ßHSD activity in the mTAL to detectable levels (Figures 6.2 and 6.3, respectively).

In the cTAL of animals fed the high potassium diet, NADP$^+$-dependent 11ßHSD activity was unaffected (Figure 6.2) while NAD$^+$-dependent corticosterone oxidation was decreased compared to rats maintained on the control diet (Figure 6.3; $P<0.05$). The ratio of NAD$^+$/NADP$^+$-dependent activities was unchanged in the cTAL of potassium loaded rats (Table 6.3).

In the CCD, dietary potassium loading was associated with an increase in NADP$^+$- and a decrease in NAD$^+$- dependent 11ßHSD activity, although neither change in enzyme activity was statistically significant (Figures 6.2 and 6.3, respectively). The ratio of NAD$^+$/NADP$^+$-dependent 11ßHSD activities in this segment was decreased in the potassium-adapted rats compared to animals maintained on the control diet (Table 6.3; $P<0.05$).

In the OMCD of rats maintained on the high potassium diet, both NADP$^+$ and NAD$^+$-dependent corticosterone oxidation were increased compared to animals fed the control diet (Figure 6.2; $P<0.05$ and Figure 6.3; $P<0.01$, respectively). The ratio of NAD$^+$/NADP$^+$-dependent corticosterone oxidation in OMCD was unaffected by dietary potassium loading (Table 6.3).

Table 6.3. Ratio of NAD$^+$-dependent/NADP$^+$-dependent 11ßHSD activities along the nephron. Data are mean ($\pm$ sem) n=8 rats per group. *$P<0.05$ compared to control ratio

Nephron segment	Control	High K$^+$
PCT	1.4 ± 0.5	0.5 ± 0.1*
cTAL	2.3 ± 1.0	1.9 ± 0.7
CCD	1.7 ± 0.4	0.9 ± 0.1*
OMCD	1.5 ± 0.1	1.6 ± 0.3

Figure 6.2: NADP$^+$-dependent 11ßHSD activity (fmol B oxidised/mm.60 min) in PCT, mTAL, cTAL, CCD and OMCD, obtained from rats maintained on control or high potassium diets for 10 days, measured *ex vivo* in the presence of 1mmol/l NADP$^+$ and 1µmol/l B. *$P<0.05$ compared with control value. UD denotes undetectable 11ßHSD activity.
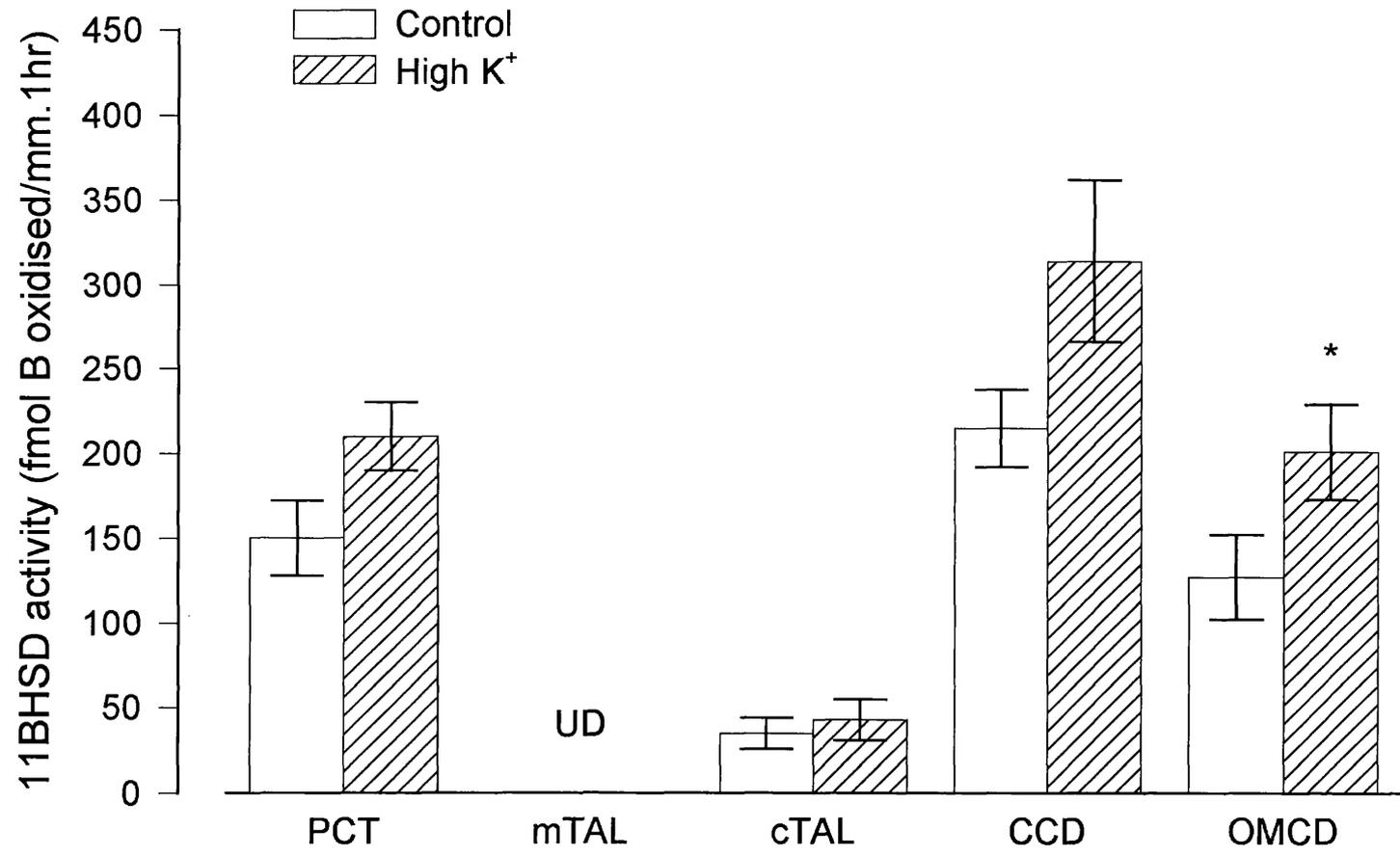
Figure 6.3: NAD$^+$-dependent 11ßHSD activity (fmol B oxidised/mm.60min) in PCT, mTAL, cTAL, CCD, OMCD, obtained from rats maintained on control or high potassium diets for 10 days, measured *ex vivo* in the presence of 1mmol/l NAD$^+$ and 1µmol/l B. *P<0.05 **P<0.01 compared with control value. UD denotes undetectable 11ßHSD activity.
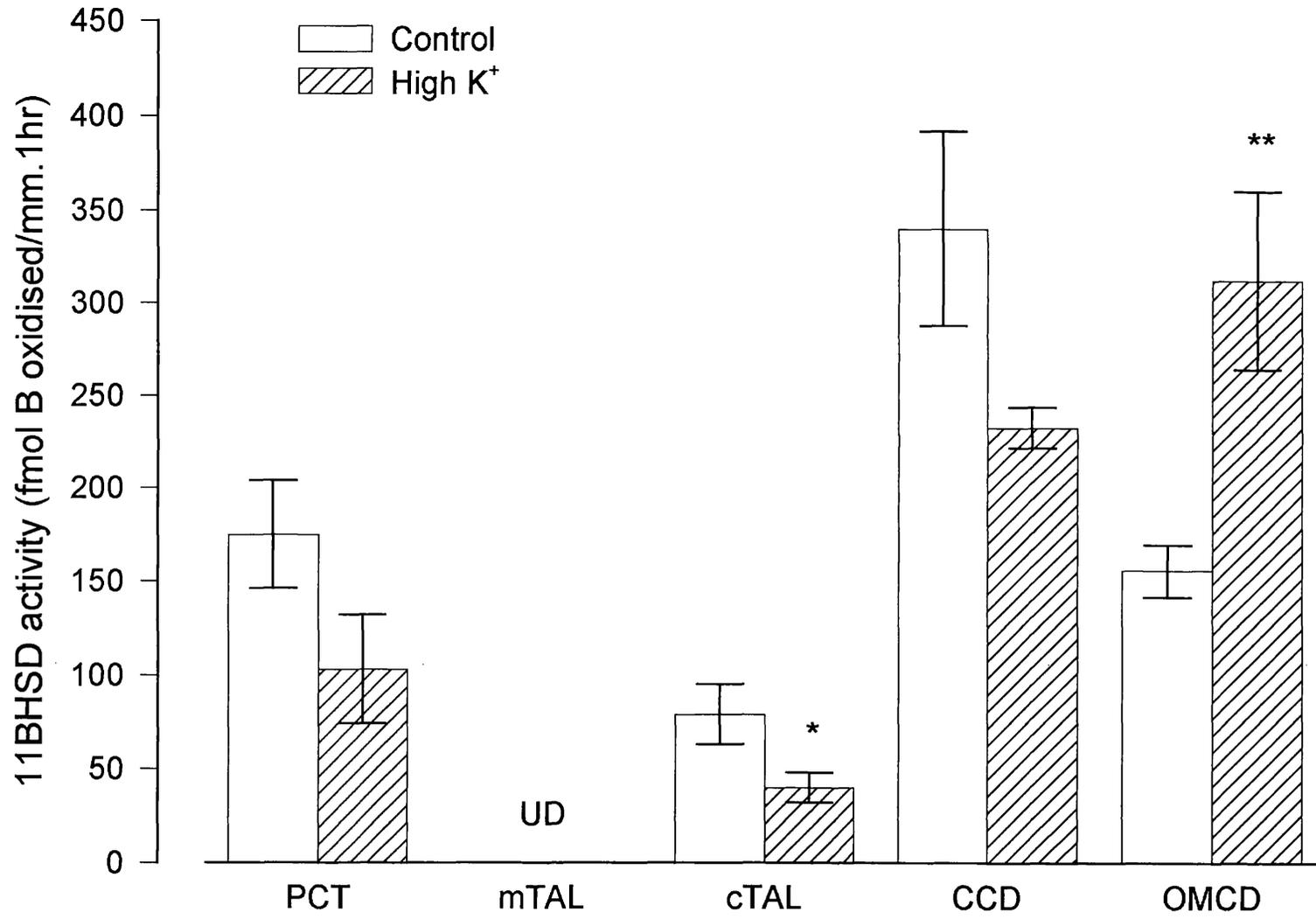
6.4 Discussion

In Chapter 5 it was shown that dietary potassium loading was associated with a substantial decrease in renal NAD$^+$-dependent 11ßHSD activity. The aim of the experiments described in this chapter was to establish in which segments of the nephron these changes in glucocorticoid metabolism were occurring.

6.4.1. Plasma and urinary data

Rats maintained on the high potassium diet had an elevated circulating aldosterone concentration and urinary potassium excretion was substantially increased with only a moderate increase in plasma potassium concentration. These changes were consistent with those observed in the studies described in Chapter 5. The increased urine flow rate observed in both studies was associated with polydipsia as anticipated, and this enhanced flow rate would contribute to the enhanced urinary potassium excretion (see Chapter 3). In the current study it has also been demonstrated that, as expected, potassium-loaded animals became mildly acidotic and produced an acid urine. All of the above changes in plasma and urine confirm that the animals were adapted to the high potassium diet.

6.4.2. 11ßHSD activities in single nephron segments

In general, in the potassium-adapted rats, NADP$^+$-dependent 11ßHSD activity tended to increase in each of the nephron segments studied. In contrast, NAD$^+$-dependent glucocorticoid oxidation tended to decrease along the nephron of the potassium adapted rats, with the notable exception of the OMCD, in which a substantial increase in NAD$^+$-dependent corticosterone oxidation was observed.

6.4.2.1. 11ßHSD activities in the PCT

As anticipated, dietary potassium loading did not significantly alter either $NADP^+$ or NAD^+-dependent 11ßHSD activities in the PCT. In general however, $NADP^+$-dependent glucocorticoid oxidation in potassium loaded rats was increased while 11ßHSD activity in the presence of NAD^+ was decreased in the PCT of the potassium loaded rat, resulting in a significant decrease in the $NAD^+/NADP^+$ 11ßHSD activity ratio. The significance of such a finding is unknown since the PCT, despite being responsive to glucocorticoids (see Chapter 1), is not thought to play a role in the 'fine tuning' of urinary electrolyte excretion. The observation that 11ßHSD activities are responsive to the high potassium diet, is however, in accordance with a recent study which reported that a high sodium diet stimulates $NADP^+$-dependent 11ßHSD activity in the proximal tubule of the dog (Brem *et al*, 1997): the functional significance of the changes in $NADP^+$-dependent 11ßHSD activity in the PCT reported by both Brem *et al* and in the present chapter remain unclear.

6.4.2.2. 11ßHSD activities in the TAL

The fact that dietary potassium loading did not increase either $NADP^+$- or NAD^+-dependent 11ßHSD activities in the mTAL to detectable levels (see Chapter 3) would suggest that glucocorticoid metabolism in this segment is of little importance in both the normal rat or in the renal response to dietary potassium loading.

In the present study, the significant decrease in NAD^+-dependent 11ßHSD activity observed in the cTAL of the potassium loaded rat supports the hypothesis that corticosteroids may play a role in the modulation of electrolyte transport in the superficial loops of Henle. Indeed, increased MR activation in the loop of Henle, either as a result of elevated aldosterone concentrations (Stanton, 1986) or following pharmacological inhibition of renal 11ßHSD activities (section 3.3.2), is associated with increased potassium reabsorption in this region of the nephron *in vivo*. It would therefore be anticipated that the decrease in NAD^+-dependent glucocorticoid

metabolism observed in the potassium loaded rats in this study, would also be associated with enhanced potassium reabsorption by the cTAL. However, a study in which *in vivo* perfusion of Henle's loop was performed on dietary potassium-loaded rats (Unwin *et al*, 1994) indicated that potassium reabsorption in the loop of Henle was slightly (but significantly) decreased. It must be emphasised that in these *in vivo* studies net potassium reabsorption is calculated from the loop of Henle as a whole and not for individual components of the loop. It is therefore possible that subtle changes in potassium transport in the TAL might be masked by those occurring in the descending limb. Indeed, Unwin *et al* (1994) reported that in addition to the effect on potassium reabsorption, dietary potassium loading was also associated with a reduction in fluid reabsorption in the anatomical loop, which would suggest an effect on the PST as the thick ascending limb is generally considered to be impermeable to water.

6.4.2.3. 11ßHSD activities in the CCD

Neither $NADP^+$- nor NAD^+-dependent activities in the CCD of potassium loaded rats were significantly different from those measured in control animals. However, an interesting observation in the CCD of these potassium adapted animals was that $NADP^+$-dependent 11ßHSD activity tended to increase, while corticosterone oxidation in the presence of NAD^+ was decreased, resulting in a significantly decreased ratio of $NAD^+/NADP^+$-dependent activities. The fact that dietary potassium loading was associated with opposite changes in $NADP^+$- and NAD^+-dependent 11ßHSD activities in the CCD is important for several reasons. The observations support the finding reported in Chapter 3, that there appears to be $NADP^+$-dependent 11ßHSD activity in the CCD. Such a finding contradicts the widely accepted view that no $NADP^+$ utilising 11ßHSD isoform is present in this region of the nephron. However, as stated in Chapter 1, there have been several studies performed over the past decade which have provided indirect evidence for the presence of more than two isoforms of 11ßHSD in the kidney (Stewart *et al*, 1991; Walker *et al* 1992; Alfaidy *et al* 1995). In their study, Walker and colleagues (1992) attributed the lack of a demonstrable difference between

cofactor utilisation in proximal versus distal renal tubules to inadequate purity of the two fractions. In the current study, the microdissection of individual nephron segments ensured that no such impurities could occur in any of the nephron segments. Hence, the observation that substantial levels of $NADP^+$-dependent glucocorticoid oxidation are present in the 'pure' CCD provides evidence that the initial observations by Walker et al (1992) were not due to contamination. Moreover, the fact that dietary potassium loading alters $NADP^+$-dependent activity in the opposite direction to that of the NAD^+-dependent glucocorticoid oxidation suggests that the observed $NADP^+$-dependent 11ßHSD activity is not merely an artefact and does in fact reflect the presence of an $NADP^+$-dependent enzyme, capable of oxidising corticosterone to 11-dehydrocorticosterone, in the CCD.

A recent study (Kotelevstev et al, 1999) has reported that mice with targeted disruption of the 11ßHSD2 gene exhibit hypertension, hypotonic polyuria and hypokalaemia; all of which are consistent with increased access of glucocorticoids to renal MR. However, it is interesting to note that the morphological changes along the nephron, which are consistent with enhanced MR activation, notably hyperplasia and hypertrophy of the tubular epithelium, were confined to the distal nephron only; the epithelium of the CCD was unchanged. Given the significance of the proposed protective role of type 2 11ßHSD, it is surprising that the only segment of the nephron which appears to be affected morphologically in the type 2 11ßHSD knock out mouse, is the DCT. The fact that the CCD was unchanged in these 11ßHSD type 2 deficient mice could be consistent with the hypothesis that another enzyme may be responsible for the observed glucocorticoid metabolism in this segment.

An increase in $NADP^+$-dependent glucocorticoid oxidation, which would be expected to decrease glucocorticoid access to renal corticosteroid receptors, would seem at odds with the decrease observed in the NAD^+-dependent 11ßHSD activity which has been proposed to enhance glucocorticoid access to renal MR and GR. However, the increase in

NADP$^+$-dependent glucocorticoid oxidation does not necessarily reflect the activity of 11ßHSD; other members of the SCAD family may be able to oxidise glucocorticoids, including, as stated in Chapter 3, prostaglandin metabolising enzymes. Indeed it has been previously reported that a high potassium diet decreased the urinary excretion of PGF2α (Nasjletti *et al*, 1985). Although the authors suggested that increased 9-keto reductase activity (a bi-directional enzyme utilising NADPH or NADP$^+$) was responsible for this observation, urinary PGE$_2$ excretion was unchanged, suggesting that other factors may have been responsible for the altered renal excretion of the PGF2α. In this context it is worth remembering the high degree of structural and functional homology between 11ßHSD1 and PGDH (Baker, 1991); the latter could contribute to renal glucocorticoid oxidation.

6.4.2.4. 11ßHSD activities in the OMCD

In the OMCD of rats maintained on a high potassium diet, NADP$^+$- and NAD$^+$-dependent 11ßHSD activities were both significantly increased compared to control animals. Since both activities were increased, it is possible that the observed glucocorticoid oxidation in the presence of both cofactors reflects the sole activity of type 1 11ßHSD using NAD$^+$ promiscuously. Alternatively, the observed increase in NAD$^+$-dependent glucocorticoid metabolism in response to dietary potassium loading could reflect type 2 11ßHSD activity; in the current study it is not possible to determine which isoform may be responsible. Nevertheless, the fact that glucocorticoid metabolism was increased in the presence of both cofactors in the OMCD was an unexpected finding for several reasons. First, as stated in Chapter 1, it is generally perceived that active transport of potassium diminishes as the collecting duct descends toward the papilla; as such it may have been anticipated that the role of glucocorticoid metabolism in the OMCD would be minimal. However, the fact that dietary potassium loading resulted in a doubling of NAD$^+$-dependent 11ßHSD activity in the OMCD, suggests that, as in the cTAL, the presence of this enzyme may play an active role in the renal response to a high potassium diet. Secondly, it is not clear why NAD$^+$-dependent 11ßHSD activities of the collecting ducts of the

nephron would respond differently in the cortical and medullary regions. It would seem likely that these differences may be due to the fact that transport mechanisms, and their regulation by corticosteroids, within these two regions of the collecting duct differ (see Chapter 1). It should be noted that although the OMCD is reputed to contain an isoform of H^+/K^+-ATPase which is absent from the CCD (see Wingo and Smolka, 1995), evidence suggests that, in the rat, this transporter is not regulated by mineralocorticoids (Eiam-Ong et al, 1993). To date, there is no evidence for a corticosteroid-modulated electrolyte transporter in the OMCD. However, while renal transport processes continue to be discovered, the substantial increase in 11ßHSD activity in the OMCD may reflect an important potassium transport system in this region of the collecting duct which has yet to be described.

Although the role of 11ßHSD in the cTAL and OMCD remains uncertain, it would appear that the mediators responsible for the observed changes in 11ßHSD activities in potassium adapted animals differ between the cortex and medullary regions of the kidney. Examination of Figure 6.3 reveals that NAD^+-dependent activity in potassium adapted rats was decreased in cortical regions of the nephron, but increased in the medullary nephron segment. The decrease in NAD^+-dependent glucocorticoid oxidation in cortical regions could be mediated by elevated production of an endogenous inhibitor of type 2 11ßHSD activity in response to dietary potassium loading. Similarly, the increase in $NADP^+$-dependent glucocorticoid metabolism observed in the OMCD could reflect increased concentrations of a paracrine stimulus to this enzyme activity in the renal medulla. The presence of both endogenous inhibitors (for example, GALFs) and stimulators (for example, AVP) of 11ßHSD activities has been reported in renal tissue (see Chapter 5). It is unknown however, whether the concentrations of these substances would be altered in the whole kidneys of dietary potassium loaded rats or whether they would differ between the cortical and medullary regions of the kidneys of potassium-adapted animals. Further experiments are required to identify any endogenous substances that are elevated in the kidneys of

potassium adapted rats and to investigate the effects of these substances on 11ßHSD activities *in vitro.*

6.4.3. Changes in 11ßHSD expression

From the current experiments it is not possible to establish whether the observed changes in renal 11ßHSD activities in response to dietary potassium loading reflect altered activity of existing enzyme or altered levels of enzyme expression. The use of PCR would provide further information on how the changes in 11ßHSD activities are elicited. PCR techniques could likewise be employed to establish if the observed $NADP^+$-dependent glucocorticoid oxidation in the CCD and OMCD reflects 11ßHSD1 activity or is due to the action of another enzyme. As stated in Chapter 3, it is possible that type 1 11ßHSD does exist in the more distal parts of the nephron, but that the immunohistochemical techniques employed to date have not been able to detect it.

6.4.4. Conclusions

In summary, the experiments described in the present chapter suggest that decreased NAD^+-dependent glucocorticoid metabolism in the cTAL and CCD, accompanied by an increase in NAD^+-dependent 11ßHSD activity in the OMCD, may play a role in the renal adaptation to dietary potassium loading. The studies also provide further evidence for the presence of a novel $NADP^+$-dependent glucocorticoid metabolising enzyme in the collecting duct of the rat kidney.

Chapter 7: General discussion

7.1. Aims of the experiments described in this thesis

A decade ago, the hypothesis was advanced that the enzyme 11ßHSD was responsible for conferring aldosterone specificity on the MR under physiological circumstances, converting cortisol and corticosterone to their inactive 11-keto metabolites and thus excluding them from epithelial MR. Following the cloning of the human sequence for 11ßHSD2, mutations in the gene sequence of this high affinity, NAD^+-dependent, essentially unidirectional isoform of 11ßHSD have been found in all patients examined to date with the syndrome of AME. Thus, the pivotal role of this protective mechanism in *in vivo* aldosterone selectivity of epithelial MR has been clearly established. That said, there are discrepancies in the findings of studies in which attempts have been made to correlate the pharmacological inhibition of renal 11ßHSD activities with changes in urinary electrolyte transport. The aim of the experiments described in this thesis was to improve the understanding of the physiological roles for isoforms of 11ßHSD and of glucocorticoids in the rat kidney.

7.2. The pharmacological inhibition of renal 11ßHSD activities: consequences for renal function

The experiments in which CBX was administered i.v. to rats supported the proposed role for 11ßHSD in conferring specificity on renal MR: substantial inhibition of renal glucocorticoid metabolism was associated with antinatriuresis and enhanced potassium secretion in the distal tubule (see Chapters 2 and 3). However, several observations resulting from the experiments described in this thesis provide important evidence for why discrepancies exist in previously published studies investigating the pharmacological inhibition of renal glucocorticoid metabolism. The two major observations, namely that urinary electrolyte excretion was not directly correlated with inhibition of renal 11ßHSD activities, and that the effects of CBX administration on renal 11ßHSD activities differed according to the

adrenal status of the rats, are important for several reasons as discussed below.

7.2.1. The validity of urine electrolyte excretion as a marker of renal mineralocorticoid activity

In Chapter 3 it was reported that increased glucocorticoid access to renal MR (as would be expected following the inhibition of renal 11ßHSD activities by <70%) was associated with increased potassium secretion in the distal tubule but did not affect overall potassium excretion. Such a finding is consistent with Field *et al* (1984) who reported that the administration of aldosterone increased potassium secretion in the distal tubule but had no effect on overall potassium excretion. Together, the two studies demonstrate that the overall urinary potassium excretion is an insensitive marker of altered potassium transport in the distal tubule and provide an explanation for the discrepancies in human studies in which antinatriuresis, but no kaliuresis, was observed in subjects administered CBX (Baron *et al*, 1969) or liquorice (Stewart *et al*, 1987).

7.2.2. The validity of the use of adrenalectomised animals for the investigation into pharmacological 11ßHSD inhibition

The experimental models used in the CBX experiments also indicate that the use of adrenalectomised rats, currently used in many studies, may provide misleading data on the effects of CBX on renal function. At present it is unknown whether it is the act of adrenalectomy itself which was responsible for the impaired ability of CBX to inhibit renal 11ßHSD activities *in vivo* (particularly that of the NAD^+-dependent isoform), or the use of chronic dexamethasone and/or acute corticosterone as replacement corticosteroids. Whatever the reason for the altered response of renal 11ßHSD to CBX in adrenalectomised rats, the findings reported in Chapter 4 are important in highlighting a potential contributor to the discrepancies reported in studies in which the adrenalectomised (adrenal steroid replaced) rat is used to investigate the effects of pharmacological inhibition of 11ßHSD

on renal function (Souness and Morris, 1989; Morris and Souness, 1990; Latif *et al*, 1992).

7.2.3. Is 11ßHSD the only mechanism responsible for conferring aldosterone specificity on renal MR?

An interesting observation in the pharmacological studies was the observation that <75% inhibition of both $NADP^+$- and NAD^+-dependent renal 11ßHSD activities, did not affect either urinary sodium excretion or sodium transport in the distal tubule of adrenal intact rats. Given the postulated role of mineralocorticoids and renal 11ßHSD activities in the regulation of sodium transport, such a finding was unexpected. One possible explanation for this particular observation is that 11ßHSD may not be the only factor involved in conferring aldosterone specificity on renal MR. Current research in this field, as discussed below, is providing evidence for this proposal including i) the presence of another MR 'protector' in the kidney and ii) support for the hypothesis that MR is inherently able to confer aldosterone specificity upon itself.

Morris *et al* (1998) have recently proposed that another renal enzyme in addition to 11ßHSD may contribute to corticosteroid receptor 'protection'. In A6 cells which express both MR and GR but no demonstrable 11ßHSD activity, corticosterone is oxidised to 6ß-hydroxycorticosterone which acts as an antagonist of MR, allowing the mediation of sodium transport in these cells via GR only. Inhibition of 6ß-hydroxylase leads to elevated corticosterone concentrations, increased occupation of both GR and MR and a consequent increase in sodium transport. 6ß-hydroxylase is present mainly in the liver of mammals (Brem *et al*, 1993) but is also expressed, albeit at lower levels, in human and rat kidneys (Schemtz *et al*, 1992). The observation that 11ßHSD inhibitors have also been found to inhibit 6ß-hydroxylase activity (Morris *et al*, 1998), may offer an explanation for the inconsistencies in experimental models of the 'MR protective hypothesis' in mammals (described both in this thesis and elsewhere).

Aside from other enzymes which may potentially affect the actions of glucocorticoids within the kidney, current research is focused upon the growing evidence that 11ßHSD2 activity is just one aspect of a sequential series of selectivity filters leading to hormone-specific and cell-specific aldosterone regulation of cellular function. Intrinsic properties of the MR are now thought to play a vital role in its discrimination between corticosteroid ligands. The initial studies on cloned and expressed MR showed that aldosterone and glucocorticoids had equal affinity (Arriza *et al*, 1987) for this receptor, but that aldosterone was an order of magnitude more potent than cortisol in regulating transcription via MR (Arriza *et al*, 1988). Indeed, there is recent evidence to suggest that MR behave differently depending on whether they have bound aldosterone or glucocorticoids: 1) aldosterone-MR complexes are much more stable than cortisol-MR complexes, as shown by their distinct half-lives (Lombes *et al*, 1994); 2) the efficiency and rate of nuclear translocation of the ligand-MR complexes are lower with glucocorticoids than with aldosterone (Lombes *et al*, 1994) and 3) the dissociation of the ligand from the hsp90 from MR is also ligand-dependent (Couette *et al*, 1992). Together, the observations suggest that the active MR conformation is ligand-dependent, and that these properties lead to the distinct transactivation efficiency of MR when bound to mineralocorticoids or to glucocorticoids.

Another degree of complexity arises from the possibility of heterodimerisation between GR and MR (Trapp *et al*, 1994; Trapp *et al*, 1996). The dimerisation of steroid receptors is required for binding to hormone response elements of target genes (Trapp *et al*, 1996). Several recent studies have demonstrated that in addition to homodimers (MR-MR or GR-GR), heterodimers (MR-GR) can form, which differ considerably in terms of transactivation potency compared with homodimers. Some groups have reported that MR and GR activate transcription synergistically through heterodimer formation, whereas others have shown that MR inhibits GR transcriptional activity through heterodimer formation (Trapp *et al*, 1994; Liu *et al*, 1995; Liu *et al*, 1996; Trapp *et al*, 1996). These opposite effects have

yet to be explained, and may depend on the different cell types and reporter genes used, suggesting that the cell context in which heterodimers are expressed may be important.

Hence, there is growing evidence for mechanisms which confer aldosterone specificity on the MR besides the colocalisation of 11ßHSD2 with this receptor. Future research is likely to focus on whether variations in the level of expression of MR, GR or other interacting transcription factors and of 11ßHSD drive specific cell functions to distinct states, in which specific aldosterone effects are amplified or minimised. Given the evidence for such complex regulation of MR activity at both the cellular and nuclear level, it is perhaps not surprising that experiments in which whole animals are infused with non-specific inhibitors of 11ßHSD and attempts made to correlate such inhibition to renal function, yield conflicting observations.

7.3. Putative regulators of renal 11ßHSD activities

The discrepant findings arising from the studies investigating the effects of pharmacological inhibition of 11ßHSD activities on renal function could question the importance of the role of this enzyme in regulating renal electrolyte transport. However, whilst it becomes increasingly apparent that 11ßHSD2 is unlikely to be the sole mechanism responsible for conferring aldosterone specificity on renal MR, the fact that NAD^+-dependent renal 11ßHSD activity appears to be physiologically regulated indicates that this enzyme is by no means redundant. On the contrary, data presented within this thesis provide evidence that 11ßHSD2 may, in fact, play a more active role in the homeostatic response of the kidney to certain physiological scenarios.

7.3.1. Putative regulators of renal 11ßHSD activities

Dietary potassium and acid loading and sodium depletion were all associated with substantial decreases in renal NAD^+-dependent glucocorticoid oxidation in the whole kidney; a finding consistent with the

Figure 7.1: Role for altered 11ßHSD2 activity in adaptive responses of the rat kidney to Na⁺, K⁺ and pH.

K^+

H^+

Na^+

ALDO

MR

B

$\oplus$

$(?)$

GR

A

11ßHSD2

K^+

$\ominus$

$\ominus$

H^+

$\oplus$

Na^+

TUBULAR FLUID

PLASMA
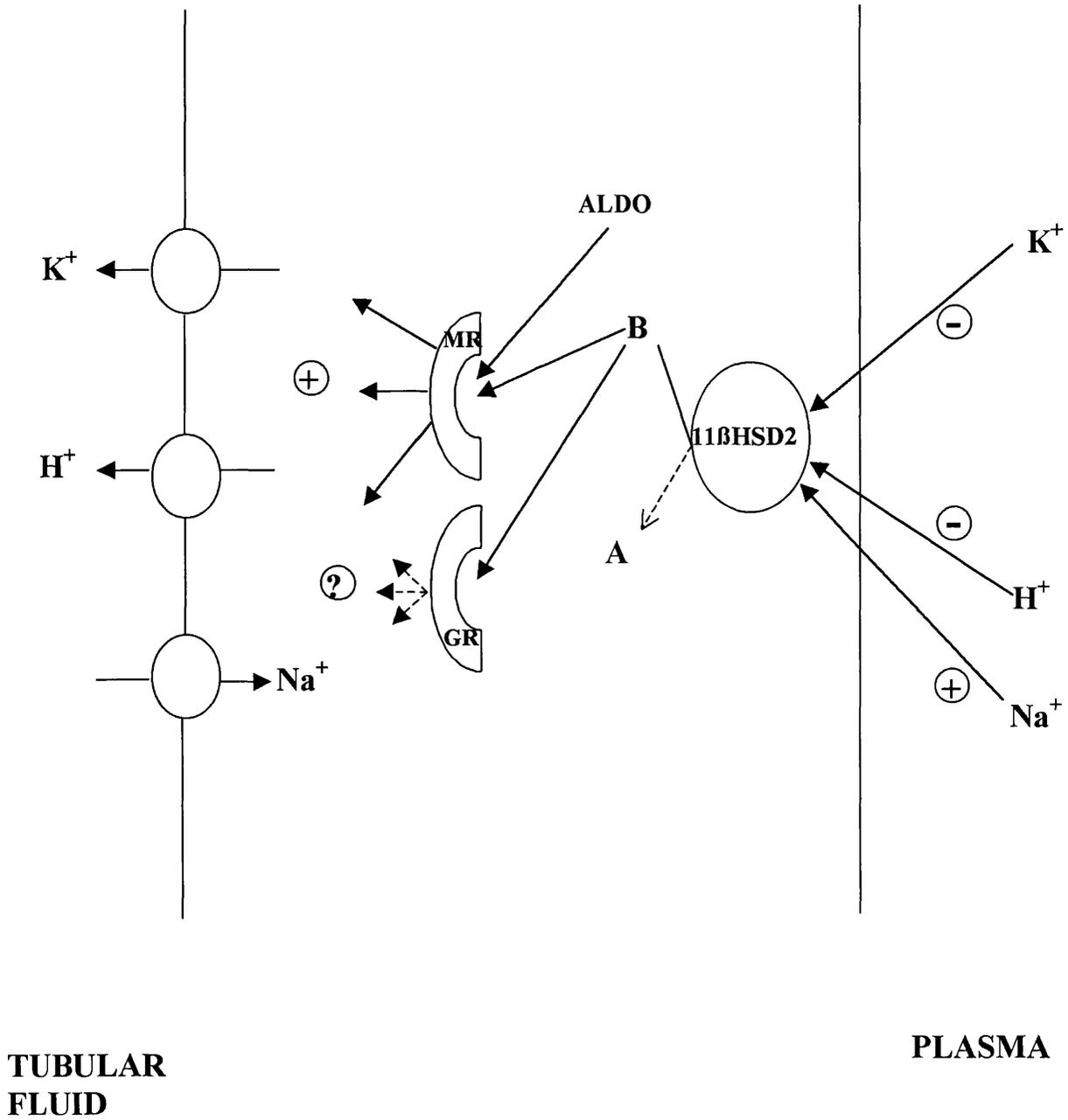
need to increase glucocorticoid access to renal MR (Figure 7.1). The fact that all these dietary manipulations are known to increase aldosterone concentrations indicates that these changes are more than a mere coincidence. Moreover, the fact that only NAD^+-dependent glucocorticoid metabolism was affected by these physiological conditions supports the theory that it is NAD^+-dependent type 2 11ßHSD which is the predominant isoform responsible for modulating glucocorticoid access to renal corticosteroids. To date, the functional role of renal 11ßHSD1 remains uncertain. Interest in the putative regulation of 11ßHSD has prompted other laboratories to investigate the effects of certain physiological scenarios on renal glucocorticoid metabolism (Nolan *et al*, 1997; Brem *et al*, 1997; Alfaidy *et al*, 1997; Escher *et al*, 1998). The results of these studies suggest that acid base balance, dietary sodium loading, AVP concentrations and uninephrectomy can all affect renal 11ßHSD activities.

7.3.2. The effects of dietary potassium loading on 11ßHSD activities along the rat nephron

The investigation into the effects of dietary potassium loading at the level of the single nephron provided evidence for a possible homeostatic role of corticosteroids in the cTAL and OMCD; areas of the nephron in which their function remains poorly elucidated. The findings in this particular study suggest that significant levels of corticosteroid-regulated potassium transport occurs in areas of the kidney aside from the distal nephron: a finding which supports the earlier conclusion that urinary potassium excretion is an insensitive index of mineralocorticoid activity in the distal tubule.

The presence of $NADP^+$-dependent glucocorticoid metabolism in the CCD suggests that another $NADP^+$-dependent glucocorticoid metabolising enzyme is present in these regions of the nephron. Moreover, the fact that the activity of this enzyme not only increases following dietary potassium loading but responds in the *opposite* direction to that of NAD^+-dependent glucocorticoid oxidation in the CCD, suggests that the observed $NADP^+$-

dependent glucocorticoid metabolism reflects the activity of an enzyme which is distinct from type 2 11ßHSD. In the context of this thesis, it is unknown whether this enzyme activity represents 11ßHSD types 1 or 2 (although this is unlikely), a third isoform of 11ßHSD or a different enzyme altogether.

Evidence for a third, intermediate affinity, $NADP^+$-dependent isoform of 11ßHSD has been suggested not only in the kidney (Walker *et al*, 1992) but also in other tissues (Gomez-Sanchez *et al*, 1996; Ge *et al*, 1997; Michael *et al*, 1997). The postulated role for a third 11ßHSD isoform could be the modulation of local glucocorticoid concentrations for binding the GR. Glucocorticoids have a wide variety of actions in a wide range of tissue. It would thus seem unlikely that a single high serum concentration of ligand can satisfy the particular needs for regulation of glucocorticoids at the tissue and cell level. Moreover, the K_m of 11ßHSD1 is in excess of normal circulating levels of corticosteroids, making it unlikely that this isoform of 11ßHSD is responsible for the regulation of glucocorticoid concentrations at the GR: the presence of a third, intermediate affinity isoform with a K_m within the concentration range of circulating glucocorticoids would however be able to fulfil this role.

There has been much attention focused on the various mechanisms involved in the 'protection' of the MR. Until recently, the mechanisms responsible for the modulation of glucocorticoid concentrations and GR activation had been somewhat overlooked. However, the recent advances in techniques which enable more detailed and specific investigation of the MR and GR, and the evidence for the existence of a third corticosteroid metabolising enzyme, indicates that further complexities in the regulation of both mineralocorticoid and glucocorticoid activity exist than are currently recognised.

7.4. Conclusions

In conclusion, the series of experiments described in this thesis have provided evidence for regulation of renal 11ßHSD activities by certain physiological regulators, suggesting an important homeostatic role for this enzyme above and beyond that of the 'passive' protector of MR. The studies in which the effects of CBX administration were related to renal electrolyte transport have highlighted potential factors which may be responsible for the discrepancies described in earlier 11ßHSD pharmacological inhibition studies. Finally, the fact that dietary potassium loading has opposing effects on $NADP^+$- and NAD^+-dependent glucocorticoid metabolism in the CCD provides further evidence for the existence of a novel isoform of 11ßHSD in the kidney.

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