11 βETA HYDROXYSTEROID DEHYDROGENASE AND HYPERTENSION

by

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DECLARATION

This thesis was composed by myself, and the work presented in it is my own. Materials and methodology performed by others in collaborative studies are indicated in the acknowledgements and text.

Paul M. Stewart

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Dedication

To Mum and Dad and my wife Susan

Abstract

11βeta hydroxysteroid dehydrogenase (11β-OHSD) is a microsomal enzyme responsible for the conversion of cortisol to cortisone, found principally in the liver and kidney. In man cortisol is an active steroid, cortisone inactive. Deficiency of the enzyme is a rare cause of mineralocorticoid hypertension and hypokalaemia; only 15 children with the defect have been described.

The first adult case of 11β-OHSD deficiency is described, a young male with severe hypertension, life-threatening hypokalaemia and suppressed plasma aldosterone and renin activity. During detailed metabolic balance studies on this index case, it seemed clear that cortisol was acting as a potent mineralocorticoid on the kidney and rectum. From these original observations I hypothesised that renal 11β-OHSD by converting cortisol to cortisone, was a critical paracrine mechanism dictating intra-renal cortisol. Deficiency or inhibition of this mechanism allows cortisol to act on the type I mineralocorticoid receptor. As I shall show, although there was a marked disturbance in cortisol metabolism, plasma circadian cortisol levels were normal, and the patient had no evidence of glucocorticoid excess.

The testing of this novel hypothesis forms the major basis of this thesis. 11β-OHSD has been characterised further, particularly in the kidney which plays a major role in cortisol metabolism. Inhibitors of 11β-OHSD have been found which may be of clinical relevance e.g. progesterone and alcohol. In particular I have shown that the sodium retention associated with liquorice administration is secondary to 11β-OHSD inhibition and not, as previously thought, to a direct action on the type I mineralocorticoid receptor. 11β-OHSD activity along with the activity of the renin-angiotensin-aldosterone system has been studied in a population of essential hypertensives and in alcohol withdrawal hypertension with interesting results.

I suggest that from our observations in an unusual case of mineralocorticoid hypertension, we have uncovered a previously unsuspected novel paracrine mechanism preventing cortisol from acting as a mineralocorticoid.

Abbreviations

The following abbreviations have been used in this thesis:

ACTH = adrenocorticotrophic hormone
AME = Syndrome of apparent mineralocorticoid excess
aldo = aldosterone
B = corticosterone
BSA = bovine serum albumin
Bq = becquerels
CBG = cortisol binding globulin
Ci = curies
cm = centimeters
cpm = counts per minute
DOC = deoxycorticosterone
DHF (DHE) = dihydrocortisol (dihydrocortisone)
E = cortisone
EDTA = ethylene diamine tetra-acetic acid
F = cortisol
GC/MS = gas chromatography/ mass spectrometry
GLC = gas liquid chromatography
Hg = mercury
HPLC = high pressure liquid chromatography
K^+ = potassium
kg = kilogrammes
Km = Michaelis constant
KRB = Krebs Ringer buffer
MCR = Metabolic clearance rate
mg = milligramme
mm = millimeters
mV = millivolts
Na^+ = sodium
NADP = nicotinamide adenine dinucleotide phosphate
NADPH = nicotinamide adenine dinucleotide phosphate hydrogen
ng = nanogrammes
PRA = plasma renin activity
rpm = revolutions per minute
s.d. = standard deviation
SEM = standard error of mean
THF (THE) = tetrahydrocortisol (tetrahydrocortisone)
µg = microgramme

The systematic nomenclature for "trivial" steroid names used in this thesis are as follows:

aldosterone = 18,11-hemiacetal of 11β-21,dihydroxy-3,20-dioxo-pregn-4-en-18-al

corticosterone = 11β,21-dihydroxypregn-4-ene-3,20-dione

cortisol = 11β,17,21-trihydroxypregn-4-ene-3,20-dione

cortisone = 17,21-dihydroxypregn-4-ene-3,11,20-trione

11β-hydroxy-androsterone = 3α,11β,dihydroxy-5α-androstan-17-one

11β-hydroxy-etiocholanolone = 3α,11β,dihydroxy-5β-androstan-17-one

11-oxo-androsterone = 3α-hydroxy-5α-androstane-11,17-dione

11-oxo-etiocholanolone = 3α-hydroxy-5α-androstane-11,17-dione

tetrahydrocortisone = 3α,17α,21-trihydroxypregnane-11,20-dione

tetrahydrocortisol = 3α,11β,17α,21-tetrahydroxypregnan-20-one

allo-tetrahydrocortisol = 3α,11β,17α,21-tetrahydroxypregnan-20-one

cortols = 3α,11β,17α,20(αorβ),21-pentahydroxy-5β-pregnane

cortolones = 3α,17α,20(αorβ),21-tetrahydroxy-5β-pregnan-11-one

cortoic acids = all 17α-hydroxy-21-oic acid metabolites of F and E

cortolic acid = 3α,11β,17α,20(αorβ) -tetrahydroxypregnan-21-oic acid

cortolonic acid 3α,17α,20(αorβ),11-oxo-trihydroxypregnan-21-oic acid

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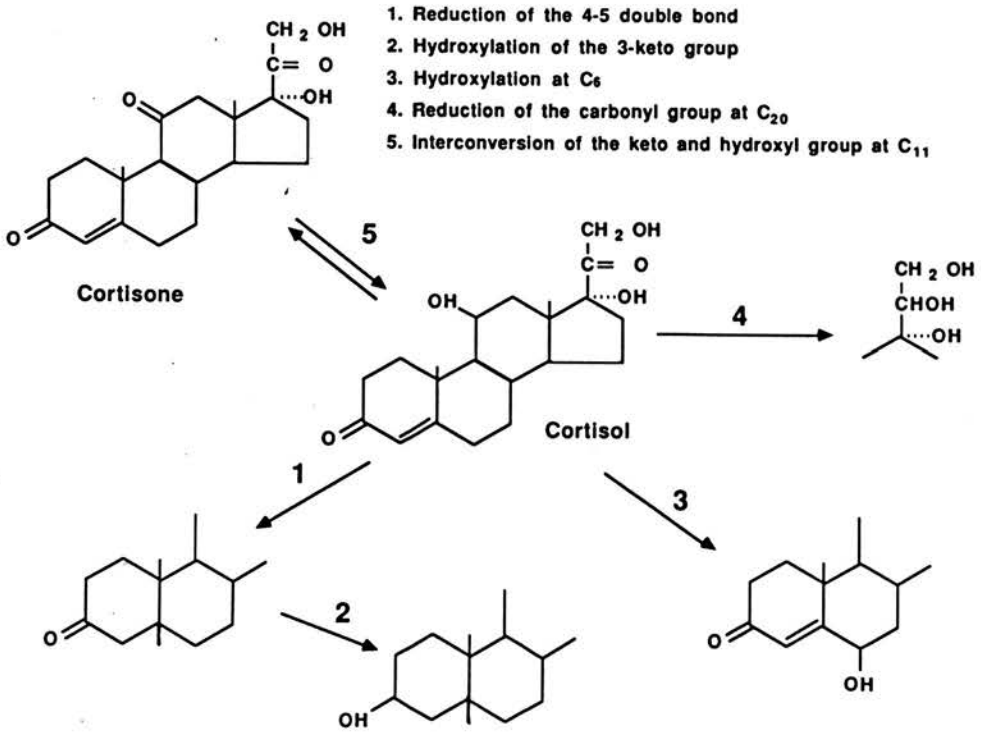
CHAPTER 1 INTRODUCTION

In diseases related to steroid excess, much greater emphasis has been placed on steroid secretion than on metabolism. Thus, even in conditions where steroids may have been implicated in their pathogenesis, normal plasma steroid levels and/or normal secretion rates have been interpreted as negative results.

Although cortisol was fully isolated and characterised from the urine of a patient with Cushing's syndrome in 1948 (Mason and Sprague 1948), it was in 1951 that cortisol was first identified using the recently developed paper chromatography method, as the principal circulating adrenocortical hormone in man (Nelson et al 1951). Prior to this only crude bioassay methods were available for detecting the "adrenocortical hormone," and these were based on the ability of the suitable extract to increase resistance to cold in adrenalectomised animals (Vogt 1943). It is now known that between 15-20 mg/day of cortisol is secreted by the normal adrenal glands (Cope and Black 1958). Between 90-95% of cortisol is bound to an α_2-globulin, cortisol binding globulin (also known as transcortin). There is also weak binding to albumin, leaving less than 2% of cortisol as "free" biologically active steroid. Until recently its main site of metabolic breakdown has been thought to be the liver, with the major metabolic pathways depicted in fig 1.1. Contrary to what may be expected, even marked changes in cortisol metabolism do not affect plasma levels (e.g. plasma cortisol is usually normal in patients with severe hepatic dysfunction). This is because the plasma cortisol concentration at any one time in the circulation is equal to the cortisol secretion rate/ metabolic clearance rate. Any change in

Figure 1.1 The major metabolic transformations of cortisol

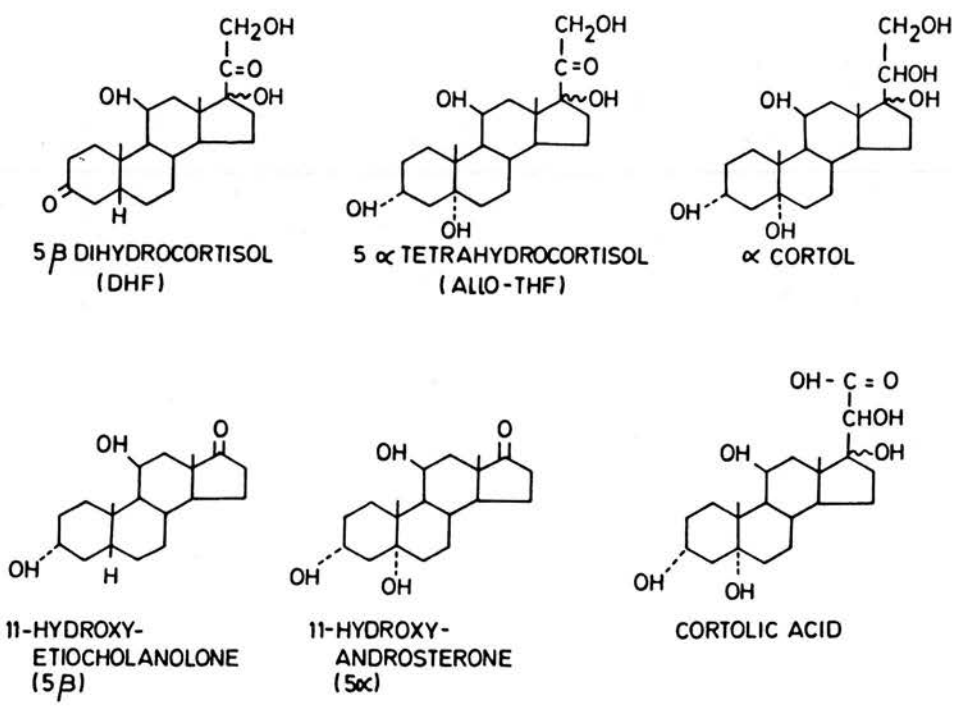


clearance is associated with an inverse change in cortisol secretion rate, brought about by the negative feedback control mechanism.

The plasma half-lives of cortisol and cortisone are 90 and 30 minutes respectively (Peterson 1959), cortisone having a much shorter half-life due, in part to the ease of which the 11-keto group is converted to the 11-hydroxy group of cortisol (step 5 in fig.1.1). In the liver metabolism of cortisol and cortisone commences with reduction of the 4-5 double bond and reduction of the 3-keto group to form initially dihydrocortisol (DHF) and dihydrocortisone (DHE), and then tetrahydrocortisol (THF) and tetrahydrocortisone (THE). This first step is carried out by either 5α-reductase (to form 5αDHF) or 5β-reductase (to form 5β-DHF), and is dependant upon NADPH (McGuire and Tomkins 1959). 5α-reductase is found in the microsomes, 5β-reductase in the cytoplasm. The structures of these steroids are depicted in fig.1.2, and the full nomenclature for these trivial steroid names appears in the "abbreviations" section. THF and THE are rapidly conjugated with glucuronic acid, and these being more water soluble are excreted in the urine. Between 1-2mg of each are excreted daily, but at most, only 35% of the daily cortisol production is excreted in this form (Cope 1972). Once formed there is negligible interconversion between THF and THE.

Reduction of the side chain (at C_{20}) of THF and THE is carried out by 20-hydroxysteroid dehydrogenase to produce cortol and cortolone respectively (fig.1.2). Containing an extra hydroxyl group at the C_{20} position, they are rapidly excreted following conjugation with glucuronic acid, and account for about 30% of the total daily cortisol turnover. As with THF and THE, both α and β isomeric forms are found.

Figure 1.2 The structures of the principal cortisol metabolites



20-hydroxylation can also occur prior to reduction of the 4-5 double bond to form 20-hydroxycortisol. This is also true of hydroxylation at the 6 position. 6β-hydroxycortisol is a main cortisol metabolite in guinea-pig urine, from where it was first recognised (Nadel et al 1959). In man 300μg (males) and 400μg (females) of 6β-hydroxycortisol are excreted daily, this value increasing in states of cortisol excess (e.g. Cushing's syndrome, Voccia et al 1979), or with induction of 6β-hydroxylase e.g. by oestrogens, (Katz et al 1962), phenytoin (Werk et al 1964), and rifampicin (Edwards et al 1974, Kyriazopoulou et al 1984).

Following reduction of the 4-5 double bond described above, side chain cleavage of both 5α-THF, 5β-THF and 5α-THE, 5β-THE can occur converting these C_{21} steroids into C_{19} steroids (fig.1.2). Respectively these are 11-hydroxy-androsterone, 11-hydroxy-etiocholanolone, 11-oxo-androsterone, and 11-oxo-etiocholanolone, and are known to form 8-10% of the total cortisol metabolites, with 5β metabolites being formed in preference to 5α metabolites. Urinary free cortisol and cortisone make up less than 3% of the total cortisol turnover. Much of this data comes from Fukushima's studies in the late 1950's (Fukushima et al 1960). He administered 0.25mg of ^{14}C-labelled cortisol to normal men. Urine was collected for the subsequent 48hr and the metabolites identified after hydrolysis. The distribution of the isotope was as follows (%):

Cortol + β-cortol	6.6
Cortolone + β-cortolone	19.8
Tetrahydrocortisol	17.8
Allo-tetrahydrocortisol	9.5
Tetrahydrocortisone	24.1

11-hydroxy-etiocholanolone	3.9
11-oxo-etiocholanolone	3.1
11-hydroxy-androsterone	1.0
Cortisone	1.7
Cortisol	1.5
Total recovery	90.2%

In these early studies only 80-90% of the total radioactivity administered could be identified as known metabolites. More recently, studies from Monder and Bradlow have isolated carboxylic acid derivatives of cortisol and cortisone. These are extremely polar and could not be extracted from body fluids using organic solvents (Bradlow et al 1977, Monder and Bradlow 1980). Cortolic acid is formed from cortol, cortolonic acid from cortolone by oxidation at the C_{21} position (fig.1.2). Together they account for up to 10% of the total cortisol metabolites.

The above metabolic transformations have been largely thought to be carried out in the liver. Extrahepatic metabolism has been thought to be unimportant. However, as I will show, it is clear that the conversion of cortisol to cortisone occurs to a large extent in the kidney. This is almost certainly the case for some of the other metabolic tranformations described above. For example 5α and 5β-reductase activity is clearly present in the kidney (Hierholzer et al 1984, Morris and Brem 1987), as is 6β-hydroxylase (Lipman et al 1962), and 20 hydroxysteroid dehydrogenase (Ganis et al 1956, Reach et al 1977, Hoyer et al 1984). The kidney also seems capable of cleaving the side chain of cortisol leaving a C_{19} steroid (Ganis et al 1956).

For a steroid to possess activity (either as a glucocorticoid or mineralocorticoid) it must have a hydroxyl group at the C-11 position. A steroid having a keto group at C-11 has no biological activity. Step 5 in fig 1.1. involves this shuttle between the C-11 hydroxyl and keto groups and is carried out by an enzyme complex, 11βeta-hydroxysteroid dehydrogenase (11β-OHSD). Thus in man 11β-OHSD is responsible for the reversible conversion of cortisol (Kendall's compound F) to cortisone (Kendall's compound E). Current evidence suggests that the enzyme complex exists as at least two separate enzymes (Abramovitz et al 1982, Lakshmi and Monder 1985), one responsible for the "oxidation" of cortisol to cortisone, the second for the reverse or "reductase" reaction. The enzyme is found in the microsomes in many tissues in man, principally the liver (Bush 1968, Koerner 1969, Wortman et al 1971, Lax et al 1978) and kidney (Burton 1965, Jenkins 1966), but also the placenta (Bernal et al 1980, Murphy 1981a), lung (Murphy 1978, Nicholas and Lugg 1982, Abramovitz et al 1984), gastrointestinal tract (Burton and Anderson 1983), adrenal gland (Whitehouse et al 1967, O'Hare 1973, Sahura et al 1986), heart (Kolanowski et al 1981), testis (Koerner 1966), prostate, thyroid and skeletal muscle (Jenkins 1966). The enzyme has also been studied in fetal tissues (Murphy 1981b). Activity differs markedly from tissue to tissue; in the liver activity is strongly reductive (i.e. E → F), in the kidney strongly oxidative (i.e. F → E). Liver 11β-OHSD is therefore essential for cortisone to possess activity, enabling it to be converted to the active cortisol. It is known that the above reactions are co-factor dependant, F → E occurring in the presence of NADP, E → F requiring NADPH. Different activity of 11β-OHSD from tissue to tissue may therefore reflect altered redox potential therein (hence NADP:NADPH ratio) or may

reflect a different enzyme. With the enzyme microsomal and hence membrane bound this problem has remained unsolved (Bush 1969, Lakshmi and Monder 1985).

11β-OHSD is thus widespread throughout many tissues, and exerts a critical role in determining the amount of active steroid exposed to a tissue, by controlling the shuttle between active steroid possessing the C-11 hydroxyl group and inactive steroid possessing the C-11 keto group. Despite its simplicity and knowledge of both the existance and fuction of 11β-OHSD since the early 1960's this mechanism has received little attention.

Although cortisol isotopic studies have suggested that the kidney is responsible for only 10% of the total cortisone production (Hellman et al 1971), clinical investigations have indicated that the principal site of conversion of F → E is in the kidney (Srivastava et al 1973). In this thesis I will explore the role of renal 11β-OHSD further. The impetus for this research came from our observations during investigation of the first adult case of 11β-OHSD deficiency (described in Chapter 2), from which it was clear that renal 11β-OHSD in man had a vital role in protecting the kidney from high intrarenal cortisol levels which could then act as mineralocorticoid. In Chapter 3, the enzymology and further characterisation of 11β-OHSD is discussed. In the remainder of this thesis I have investigated the role of this "protective" mechanism in several areas of both scientific and clinical relevance, and have documented that a failure of this pathway of cortisol metabolism may be of considerable relevance, not only as a physiological paracrine mechanism but also in the pathogenesis of some hypertensive states.

CHAPTER 2 G.B. : THE FIRST ADULT CASE OF 11β HYDROXYSTEROID DEHYDROGENASE DEFICIENCY

INTRODUCTION

Impaired conversion of cortisol (compound F) to cortisone (compound E) has been described in a small group of children with hypertension and hypokalaemia, low renin, and low aldosterone and has been called the syndrome of "apparent mineralocorticoid excess" (Ulick et al 1979, New et al 1982, Monder et al 1986). At the time of writing this thesis and prior to the studies described here, 18 children worldwide have been found to have 11β-OHSD deficiency. 15 of these have been reported in the world literature (Table 2.1).

The first case was described in 1974 by Werder et al in Zurich (Werder et al 1974); a 3 year old girl with short stature, polyuria and polydipsia. Blood pressure was elevated at 175/115 mmHg, plasma potassium was low at 2.8 mmol/l. Urinary steroid profiles revealed low levels of aldosterone and its metabolites. Cortisone metabolites (principally THE) were low and failed to rise following ACTH. However it was not until 1979 that workers in New York studying two similar hypertensive children (Ulick et al 1979), first demonstrated that there was a defect in the peripheral conversion of cortisol to cortisone (detailed further in previous (Ulick et al 1977, New et al 1977) and subsequent publications (New et al 1982, Oberfield et al 1983)). Another five cases have been documented by Shackleton and co-workers (Shackleton et al 1980, Shackleton et al 1985), with individual cases from Fiselier (Fiselier et al 1982), Honour (Honour

et al 1983) and Harinck (Harinck et al 1984) and most recently three further cases by Monder, New and co-workers (Monder et al 1986). As shown in Table 2.1, all the reported subjects had marked hypertension and hypokalaemia on presentation. Many presented with polyuria and polydipsia, presumably secondary to nephrogenic diabetes insipidus, a well known effect of hypokalaemia.

The characteristic urinary steroid metabolite profile is well described (Shackleton et al 1980 and 1985, Monder et al 1986). Patients have limited ability to metabolise cortisol to cortisone but can carry out the reverse reaction. As a result the urinary 11β-hydroxy metabolites of cortisol (cortols, tetrahydrocortisol (THF) and the C-19 steroids, principally 11-hydroxy-androsterone and 11-hydroxy-etiocholanolone) are elevated, whilst the 11-oxo metabolites (cortolones and tetrahydrocortisone (THE)) are diminished. In addition there appears to be a reduction in activity of 5β reductase with 5α THF (allo-THF) being formed in preference to 5β THF (Chapter 1 and Fig 2.1).

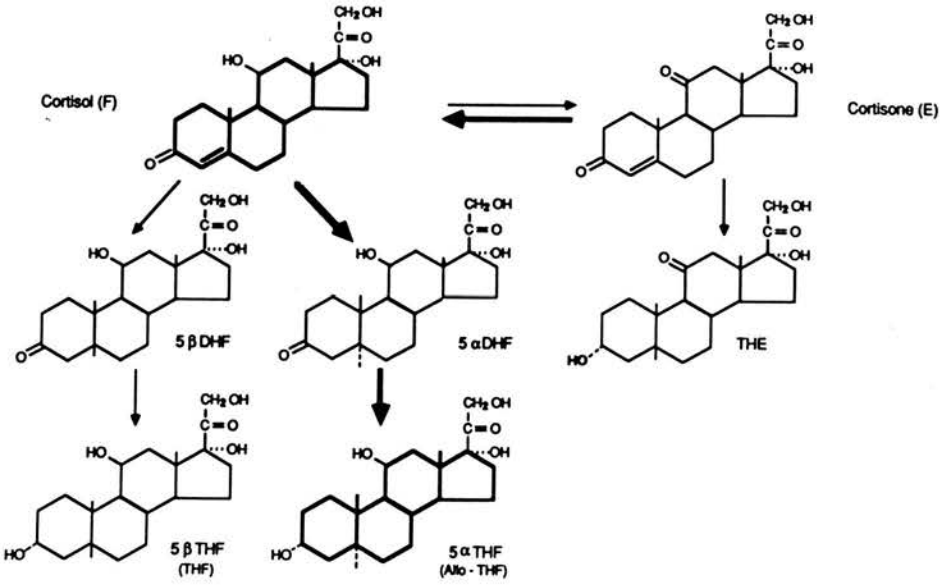
Defective peripheral cortisol metabolism results in a prolonged plasma cortisol half-life. This was first shown in subjects LK and YK in Table 2.1 from Ulick's group (Ulick et al 1979), these children having plasma cortisol half-lives of 187 and 122 minutes respectively (normal range 80-90 min). This is also the case in other cases (e.g. 109 min, Harinck et al 1984, 113, 128 and 137 min in the cases described by Dimartino-Nardi et al 1987). Normal plasma cortisol levels are maintained by a reduction in the daily secretion rate, operating through the negative feedback mechanism on the hypothalamo-pituitary axis. Thus using double isotope dilution methods, the

Table 2.1

Reported Cases of 11Beta Hydroxysteroid Dehydrogenase Deficiency

No	Identity	Sex	Age at presentation (yr)	Age In 1988 or (death) (yr)	Blood pressure (mmHg)	Plasma K^+ (mmol l^{-1})	THF + allo THF / THE ratio	Presenting Features	Referenced
1	T	F	3	17	17/115	2.8	10.2	Polyuria, short stature	Werder et al 1974
2	LK	F	0.75	(13)	180/140	2.7	16.2	Failure to thrive, short stature	New et al, 1977., Ulick et al, 1977.,
3	YK	M	7	18	250/180	2.3	10.0	Left hemiparesis	Ulick et al, 1979
									New et al, 1982
									Oberfield et al, 1982
4	O	M	2	16	140/90	2.2	15.9	Polyuria, polydipsia, failure to thrive	Shackleton et al, 1980
5	MT	F	3	(12)	180/120	2.5	9.8	Polyuria, hypertension	Winter & M^{c}Kenzie, 1977
6	LC	M	1.5	15	170/110	2.3	19.8	Polyuria, polydipsia, vomiting	Shackleton et al, 1985
7	AE	F	3	15	170/110	2.3	31.2	Polyuria, polydipsia, failure to thrive	
8	RE (sister of AE)	F	0.25	7	200/130	3.0	13.4	Polyuria, polydipsia, failure to thrive	Shackleton et al, 1985
9	UE	M	6	(6)	210/130	2.7	-	Died age 6. R. hemiparesis.	
	(brother of AE, RE)							(Presumed case)	
10	JH	M	0.5	(0.5)	200/100	2.7	68.8	Diarrhoea, vomiting	Honour et al, 1983
11	TF	M	1.5	9	150/110	1.8	32.5	Polydipsia, failure to thrive	Fiselier et al, 1984
12	HN	F	0.75	23	150/100	3.0	15.5	Failure to thrive, hypertension	Harinck et al, 1984
13	SW	F	2	13	130/90	1.4	8.9	L. leg weakness, failure to thrive	Monder et al, 1986
14	TW(Sibling of SW)	F	2	8	142/98	3.0	20.1	Cerebral palsy, hypertension	Dimartino-Nardi et al , 1987
15	JB	M	0.75	10	140/90	3.1	14.9	Polyuria, polydipsia, failure to thrive	
16	GB	M	21	26	200/140	1.7	13.5	Failing vision, polyuria	Stewart & Edwards, 1986
									Stewart et al, 1988
17	MS	M	6	11	-	2.8	29.8	-	Patient of Dr C. Kater, Brazil
18	DC	F	3	6	200/110	2.2	7.4	Failure to thrive	Patient of Dr. D. Peskovitz, Mexico Shackleton et al, 1987
19	GP	F	1.5	12	-	-	40.0	-	Patient of Dr G. Phillipou, Australia

Figure 2.1 Cortisol metabolism in 11β-OHSD deficiency. Broad arrows indicate major pathways of metabolism.



cortisol secretion rate was only 2 and 5 mg/day (normal range 14-20 mg/day) in patients L.K. and Y.K. (Ulick et al 1979), and 8 mg/day in Harinck's patient (Harinck et al 1984). That this is mediated through ACTH is shown by the fact that plasma levels and secretion rates of other ACTH dependant steroids e.g. corticosterone, deoxycorticosterone and 11-deoxycortisol are reduced (New et al 1977). Many of the cases reported have elevated urinary free cortisol levels (Shackleton et al 1980 and 1985, Harinck et al 1984).

In summary the steroid metabolic disorders seen in 11β-OHSD deficiency are:

1) Low aldosterone production (New et al 1977) and tetrahydro-aldosterone excretion.
2) An elevated THF + allo-THF/THE ratio.
3) Low THF/allo-THF ratio suggesting reduced 5β reductase activity (Monder et al 1986).
4) High excretion of cortisol and unconjugated cortisol metabolites such as 20β-hydroxycortisol and 6β-hydroxycortisol.

CASE REPORT

G.B., a Caucasian male, presented aged 21 with a two week history of altered vision. During the preceding two years he had had three episodes of tonsillitis, each being associated with carpopedal spasm and peri-oral paraesthesiae. He had noticed polydipsia and nocturia for several years. There was no other past medical history; he did not smoke, consume alcohol or take any regular medications. Consumption of exogenous mineralocorticoids such as liquorice was denied. A strong family history of hypertension was noted (see below).

Examination revealed a blood pressure of 200/145 mmHg (right arm), 192/140 mmHg (left arm), sinus rhythm with no radial-femoral delay or vascular bruits. The apex beat was positioned normally but heaving in quality. The second heart sound was loud. Fundoscopy showed grade III hypertensive changes. Height was 173 cms; weight 53 kg; secondary sexual characteristics were normal.

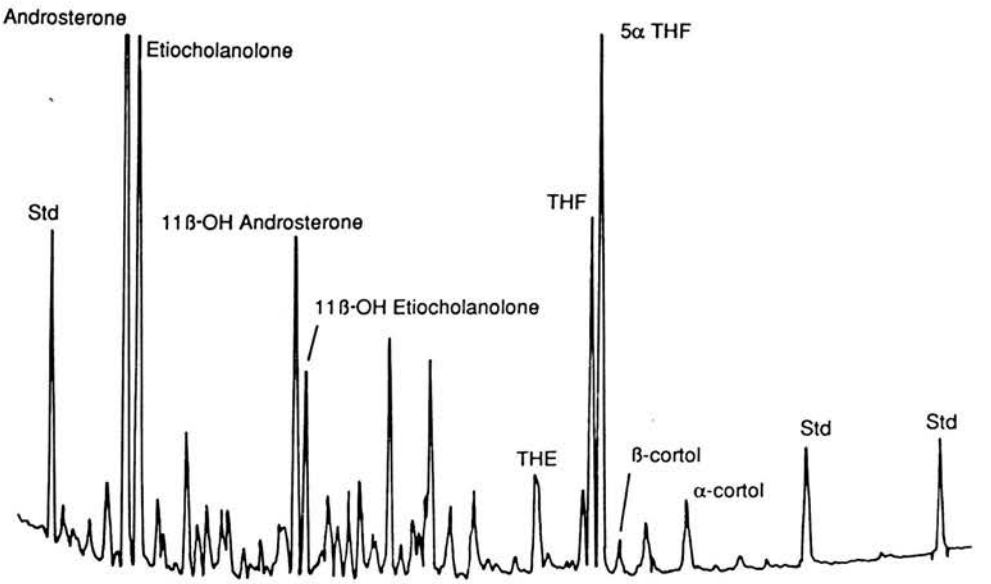
Chest x-ray was normal; ECG showed voltage criteria for left ventricular hypertrophy. During an intravenous urogram (IVU) 24 hours after admission he sustained two episodes of ventricular fibrillation treated with D.C. shock. Biochemistry on recovery showed a plasma Na^+ 148 $mmol.l^{-1}$, K^+1.7 $mmol.l^{-1}$, bicarbonate 32 $mmol.l^{-1}$. Creatinine clearance and 24 hour urinary metanephrines were normal. The IVU showed mild bilateral nephrocalcinosis with two renal cysts confirmed on subsequent ultrasonography and computerised tomography. Serum and 24 hr urinary calcium were normal. Supine plasma renin activity and aldosterone levels were supressed at 0.1 ng $ml^{-1}hr^{-1}$ (reference range 0.5-1.5) and <100 pmol l^{-1} (reference range 150-500pmol l^{-1}) respectively.

Over the next few months control of his blood pressure and hypokalaemia was poor despite a variety of antihypertensive medications (atenolol 300 mg, captopril 150 mg, hydralazine 200 mg, amiloride 10 mg, prazosin 25 mg, nifedipine retard 40 mg and Sando K 48 mmol per day). He was then referred to the Western General Hospital, Edinburgh and a diagnosis of 11β-OHSD deficiency made from urinary steroid metabolites as measured by gas chromatography/mass spectrometry (Table 2.2, Fig 2.2).

Table 2.2 Blood pressures and urinary steroid metabolites in GB, his parents and siblings as compared to 22 normal controls aged 18-55 (16 males)

		GB	SB	PB	ND	TB	EB
			Father	Mother	Sister	Brother	Brother
Blood pressure (mmHg)		235/125	166/102	165/92	125/82	134/84	135/85
Steroid	reference range (mean ± SD) µg/24 hrs. n = 22						
Androsterone	2068 ± 892	10176	700	470	1966	7018	5741
Etiocholanolone	1690 ± 874	4427	1137	767	2379	6606	6333
11-oxo-etiocholanolone	426 ± 461	537	680	534	1059	1257	1155
11β-hydroxyandrosterone	1058 ± 875	1555	852	799	1821	3510	2513
11β-hydroxyetiocholanolone	396 ± 272	1075	309	686	1037	1270	972
Tetrahydrocortisone (THE)	2589 ± 1292	537	2435	4414	2545	4619	3675
Tetrahydrocortisol (THF)	1469 ± 585	2472	2076	3019	1380	3679	2456
Allo tetrahydrocortisol (allo-THF)	1033 ± 525	4819	1019	1860	839	3716	2367
α-cortolone	913 ± 386	182	513	778	622	874	662
α-cortol	303 ± 100	422	342	338	310	690	610
β-cortolone β-cortol	920 ± 414	537	241	916	310	765	512
THF + allo THF/THE ratio	0.97 ± 0.30	13.57	1.27	1.10	0.87	1.60	1.31

Figure 2.2 The urinary steroids of GB separated as methyloxime/trimethylsilyl ethers. The high excretion of THF and 5α-THF relative to THE is notable. The peaks labelled "Std" are internal standards added to permit quantitation.



The elevated THF and allo THF:THE ratio is diagnostic of 11β-OHSD deficiency (Table 2.1), the 5α THF (allo THF):5β THF ratio suggesting 5β-reductase deficiency also documented in the syndrome.

G.B. was then admitted to the Metabolic Unit, Western General Hospital under the care of Professor C.R.W. Edwards for further studies; local Ethical Committee and ARSAC approval was obtained for administration of radio-isotopes.

Materials and Methods

The patient was weighed at 0830 h each morning after emptying his bladder. Blood pressure was recorded automatically using a commercially available automatic Copal sphygmomanometer. During all metabolic studies BP was recorded in the supine position at 5 minute intervals from 1400 h to 1500 h each day. The Copal was checked against a random zero sphygmomanometer at weekly intervals.

Statistical analysis was performed on consecutive readings from three days (i.e. 30 measurements) from each metabolic study, using Student's paired 't' test.

ASSAYS

Plasma and urinary free cortisol were measured by radio-immunoassay, using the commercially available Amerlex kit (Amersham International) (Gough and Ellis 1981). Corticosterone was measured by modification of a previously reported method (Al-Dujaili et al 1981). Plasma and urinary electrolytes and creatinine were measured by an ion selective method using an Astra 4.

Aldosterone

Plasma aldosterone was measured by the direct radioimmunoassay of Al-Dujaili (Al-Dujaili and Edwards 1981a). Plasma was collected into heparinised tubes and centrifuged at 2400 rpm for 20 min. To 50µl of sample/standard/quality control, 200µl of ^{125}I-aldosterone (approx 3500 c.p.m) and 200µl of specific antibody (final dilution 1:400,000), diluted in phosphate/citrate buffer (0.05M), pH 4.0, was added. Incubation proceeded overnight at 4°C. Samples were mixed with 600µl of charcoal separation media (1% charcoal, 0.1% dextran, 0.1% gelatin in phosphate buffer (0.1M), pH 7.4), and centrifuged at 2400 rpm for 20 min. The supernatant was removed to waste and the charcoal pellet then counted in a gamma counter (Hydragamma 16, Innotron, Oxford, England). The bound fraction was calculated by subtracting the free counts from the total counts (i.e. tubes with label but no sample added). Using a standard curve (i.e. a plot of % bound from the known aldosterone samples), unknown values can be read off. Charcoal separation rather than a double antibody separation as described in the initial Al-Dujaili manuscript has considerably improved the sensitivity at lower values. The lowest detection limit for the assay is now 100 $pmoll^{-1}$.

Urinary aldosterone was measured using the same direct radioimmunoassay (Al-Dujaili and Edwards 1981b). 50µl of urine specimen was hydrolysed with 50µl of 1M hydrochloric acid at room temperature for 24h. This was then diluted to a volume of 2 ml with 0.05M phosphate/citrate buffer (pH 4). Samples were then assayed as for the plasma assay described above.

Plasma Renin Activity

The method is a modification of that described by Haber et al (Haber et al 1969). Plasma was collected on ice into tubes containing the inhibitor Na EDTA (10% final concentration), and centrifuged at 4°C at 3000 rpm for 20 min. Plasma was then buffered and incubated at 37°C in the presence of angiotensin I converting enzyme inhibitor for 2hr. Angiotensin I is generated during the incubation by the action of renin on angiotensinogen (renin substrate), and this was measured by radioimmunoassay. To 25µl of sample/standard/quality control 200µl of ^{125}I-angiotensin I label (approx. 3000 c.p.m.) and stock antiserum (final dilution 1:10,000) was added, mixed and incubated for 24hr at 4°C. 500µl of charcoal separation medium was then added to the tubes and the tubes centrifuged for 20 min at 4°C (3000 rpm). Antibody counts were then determined by subtracting the free counts (charcoal pellet) from total counts. Having constructed a standard curve by plotting % bound counts using samples with a known amount of angiotensin I, angiotensin I levels can be determined in the unknown samples. Plasma renin activity in ng/ml/hr is then calculated having corrected for the incubation period.

Deoxycorticosterone (DOC) assay

Plasma was extracted with 5 volumes of freshly distilled dichloromethane G.P.R. (BDH Chemicals, Poole), the extract washed with 0.1M NaOH and with water before aliquots (equivalent to 100µl plasma) were evaporated to dryness under nitrogen at 45°C.

The assay used rabbit antiserum (R7-6, St. Bartholomews Hospital) and tritium label (Amersham International 52 Ci/mmol). Because of cross reactivity with testosterone and progesterone,

extracts were purified further by HPLC (Waters Associates Inc., Mass.) using a μBondapak C_{18} reverse phase column with 60:40 (v/v) methanol:water as mobile phase. The appropriate fraction of eluate was collected, methanol evaporated off under nitrogen and the aqueous solution back extracted with dichloromethane. This extract was evaporated to dryness under nitrogen, dissolved in 100μl assay diluent (0.05 M phosphate buffer pH 7 containing 0.1% BSA), ^{3}H DOC (approx. 2000 c.p.m.) and antiserum in 100μl assay diluent added; final antiserum dilution 1:20,000. After overnight incubation at $4^{o}C$, bound and unbound DOC were separated using dextran coated charcoal and supernatant (following centrifugation) counted in Scintran T (BDH Chemicals, Poole).

Aliquots from 24h urine collections were stored at $-20^{o}C$ and the urinary steroid metabolites analysed using gas chromatography and gas chromatography/mass spectrometry.

Urinary steroids were measured by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) using previously reported methods (Shackleton 1985 and 1986).

Synthesis and metabolism of [11α^{3}H]- cortisol

The synthesis of this isotope was carried out by Dr. John Corrie in our Department (MRC scientist) [11α^{3}H]- cortisol was prepared as described by Hellman (Hellman et al 1971), but with modifications to improve the radiochemical yield and define the specific activity. The main changes were a 3-fold increase in the ratio of cortisone-3, 20-bisethylene eetal to sodium ^{3}H-borohydride and purification of the final product by thin layer chromatography on silica gel GF_{254}

(20 x 20 cm x 0.25 mm) in chloroform-methanol (9:1 by volume). The cortisol band was located under UV light, scraped from the plate and eluted with ethanol (1 x 5 ml; 6 x 1 ml). The cortisol concentration in recovered solution (total volume 9 ml) as determined by quantitative high pressure liquid chromatography (μBondapak C_{18}; mobile phase methanol-water 1:1 by volume; calibrated against a standard cortisol solution) was 1.48 mg/ml, and the radioactive concentration determined by scintillation counting was 5.91 MBq/ml. The specific activity was therefore 1.44 GBq/mmol. No cortisone or 11-epicortisol was detected in the purified product (lower limit of detection of either compound was 0.5%). The ethanol solution was passed through a 0.2μ filter, aliquoted into sterile vials (1.19 MBq/vial) and stored at 4°C.

The measured specific activity was substantially lower than that of the sodium borohydride (2.6 GBq per hydride) due to the well-known primary isotope effect. This effect was not considered by Hellman et al who assumed that the specific activity of the product could be calculated from that of the ^{3}H-borohydride.

With G.B. fasted overnight diuresis was established by an oral water load. When a constant urinary flow rate was achieved, 1.19 MBq [11α^{3}H]-cortisol in 0.36 ml ethanol was injected IV as a bolus in 15 ml water. Urine was collected at 15 minute intervals, blood at 15 minute intervals during the first 60 minutes, then every 30 minutes for 120 minutes. The samples were counted for total ^{3}H and for ^{3}H-H_2O following sublimation using a Packard Tricarb 4330 series liquid scintillation beta counter after correction for quench. Cocktail T (BDH Chemicals) was used as scintillant. Subtraction of ^{3}H-H_2O from

total ^{3}H gave a value in counts/min representing activity of the tritiated steroid. Plots of $\log_{10}$ [11α^{3}H]-cortisol versus time were made and the slope analysed by linear regression. Only the elimination phase (time points 45-120 min) was studied and expressed as a half-life.

Subtraction potential difference (p.d.)

As an index of "in vivo" mineralocorticoid activity substraction p.d. was measured using an Adrenosonde measuring the p.d. across a steroid insensitive mucosa (buccal mucosa) and subtracting this from a steroid sensitive mucosa (rectum) (Skrabal et al 1978). Subtraction of buccal from rectal p.d. eliminates the non-steroidal influences affecting rectal p.d.

The reference electrode was placed on the forearm over an intracutaneous injection of 0.3 ml 0.9% saline to eliminate skin p.d. The potential difference was read with the probe electrode placed on either the buccal mucosa 3 cms from the oral orifice or on the rectal mucosa 8 cms from the anal margin, 20 ml of 0.9% saline having been injected into the rectum. This was performed on every second day during the metabolic studies at 10.00 h. Two recordings (which never differed by more than 4 mV) were made at 20 min intervals and the mean recorded. The normal subtraction p.d. is -5 to +25 mV, with values of 40-80 mV occurring in states of mineralocorticoid excess such as primary aldosteronism (Skrabal et al 1983).

Metabolic balance studies

In order to investigate the role of cortisol in producing the

hypertension and hypokalaemia in our patient metabolic balance studies were performed on a fixed Na^+/K^+ diet (118 mmol Na^+, 78 mmol K^+). In balance it was possible to withdraw all drugs except for nifedipine, amiloride and Sando K (48 mmol) with no ill effects apart from mild elevation of blood pressure. The metabolic balance was divided into several studies.

Study 1 involved baseline investigation of the circadian rhythm of cortisol, measurement of other adrenal and gonadal steroids, ACTH and the oral administration of dexamethasone, initially 0.5 mg 6 hourly for 48 hours, then 1.5 mg/day.

Study 2 was performed with the patient in balance taking 1.5 mg dexamethasone daily. Hydrocortisone (10 mg/day) was then given subcutaneously via a pulsatile NIMR Millhill pump delivering steroid at 15 minute pulses. Ten mg was chosen as this was the sum of free and conjugated cortisol metabolites excreted in the urine in a 24h period as determined by GC/MS.

Study 3 was performed with the patient on no treatment for 8 days to reproduce the syndrome.

Study 4 Finally cortisone acetate was given orally (25 mg at 09.00 h, 12.5 mg at 17.00 h).

Study 5 looked at the metabolism of [11α^3H]-cortisol given intravenously as a bolus.

Haemodynamic assessment

With the patient supine on no treatment and fasted overnight, a thermodilution flow directed balloon catheter was sited in the pulmonary artery via the femoral vein and its position confirmed by fluoroscopy. Right atrial pressure (RAP) and cardiac output (CO) were measured, the latter by using a thermodilution technique. Blood pressure (BP) was recorded in the right arm during the procedure using a mercury sphygmomanometer. Systemic vascular resistance (SVR) was then calculated using the formula SVR = BP-RAP x 80/CO (dynes sec^{-1} cm^{-5}).

Family study

A 24h urine collection was obtained from each of G.B's parents, as well as his siblings and analysed as described above for steroid metabolites. In addition the metabolism of [11α^{3}H]-F was studied in both parents using the same protocol described above. Prior to this isotope study antihypertensive therapy had been discontinued for 4 weeks.

Results

Study 1 Baseline investigations and dexamethasone suppression

Table 2.3 shows that GB had a normal cortisol circadian rhythm, normal ACTH levels, plasma catecholamines, testosterone and androstenedione. Plasma dehydroepiandrosterone sulphate was slightly elevated.

When in balance on his fixed Na^{+}/K^{+} diet and treated with nifedipine retard 20 mg b.d., amiloride 5 mg b.d., and potassium supplements (48 mmol/24h), dexamethasone 0.5 mg 6 hourly was given for

Table 2.3 Plasma steroid, ACTH and catecholamine levels

Day	Cortisol $nmol\,l^{-1}$ 0900h (N range 180-700)	Cortisol $nmol\,l^{-1}$ 2400h (N range <180)	ACTH $ng\,l^{-1}$ 0900h (N range 10-80)
1	513	156	35
2	519	290	32
3	601	217	45

Noradrenaline	1.3 $nmol\,l^{-1}$	(normal <5)
Adrenaline	0.06 $nmol\,l^{-1}$	(normal <1)
Testosterone	24 $nmol\,l^{-1}$	(normal 10 - 30)
Androstenedione	3.5 $nmol\,l^{-1}$	(normal 2 - 11)
Dehydroepiandrosterone sulphate	11 $\mu mol\,l^{-1}$	(normal 2 - 9)

48 hours. As shown in Fig 2.3 urinary free cortisol suppressed from 628 nmol/24 hrs (reference range 80-450 $nmoll^{-1}$) to <30 nmol/24 hrs. There was a marked natriuresis with potassium retention, urinary Na^+/K^+ ratio rising from 1.2 to 3.3. Plasma electrolytes showed a corresponding change, Na^+ falling from 143 to 136 $mmoll^{-1}$, K^+ rising from 3.5 to 4.5 $mmoll^{-1}$. Table 2.4 shows the response of plasma cortisol, renin activity, deoxycorticosterone, aldosterone, corticosterone, weight and subtraction potential difference during this and subsequent studies. Baseline values were obtained from the day immediately prior to the first dose of dexamathasone. As shown, PRA, initially suppressed despite nifedipine and a low dose of amiloride became detectable. Plasma aldosterone over this short period of time remained low as expected following prolonged suppression of the zona glomerulosa. When the urinary free cortisol levels reverted to pre-dexamethasone values maintenance dexamethasone was re-commenced (1.0 mg 2300 h and 0.5 mg 0900 h). Once again this produced a natriuresis and potassium retention, indeed the combination of dexamethasone and amiloride resulted in marked hyperkalaemia (K^+ peak 6.8 $mmoll^{-1}$) which was treated by parenteral hydrocortisone (100 mg intramuscularly), frusemide (40 mg intravenously) and withdrawal of K^+ and amiloride (Fig 2.3). Nifedipine was also stopped. When re-established in balance on dexamethasone 1.5 mg daily and off all other drugs, GB maintained a normal plasma K^+, with only a slight rise in blood pressure. On dexamethasone alone, subtraction p.d. remained in the normal range at 3-10 mV, as did PRA (0.5-1.5 $ngml^{-1}h^{-1}$). The zona glomerulosa showed some signs of recovery immediately prior to Study 2 (plasma aldosterone 140 $pmoll^{-1}$).

Figure 2.3 Metabolic balance study 1. Urinary free cortisol, urine and plasma electrolytes, blood pressure, plasma aldosterone, renin activity, subtraction p.d. and weight are shown during acute (2 mg/day for 48 h) and chronic (1.5 mg/day) dexamethasone therapy. The reference ranges for plasma Na^+, K^+ and renin activity are indicated. The mean $\pm$ s.d. of 10 readings for systolic and diastolic blood pressure is charted.

(overleaf)

TABLE 2.4

Plasma cortisol (F), Renin activity (PRA), DOC, Corticosterone (B), Aldosterone (Aldo), Subtraction p.d. and weight during metabolic studies. Baseline values were obtained on the day immediately prior to the first dose of dexamethasone when in metabolic balance. Other values reported refer to the last day of the treatment option described.

	Reference Range	Baseline	Acute Dexa suppression (2 mg/day for 48 hrs)	Dexa 1.5 mg/day	Dexa 1.5 mg + Hydrocortisone 10 mg	No Treatment
09.00 F	300--700 nmol l^{-1}	601	39	30	239	367
09.00 B	1.4--15.6 nmol l^{-1}	1.2	1.0	0.4	0.5	1.1
09.00 DOC	150--450 pmol l^{-1}	214	223	181	172	220
09.00 PRA (supine)	0.5--1.5 ng AI ml^{-1} hr^{-1}	<0.05	0.39	0.50	<0.05	0.1
09.00 Aldo (supine)	135--500 pmol l^{-1}	120	< 100	140	<100	<100
Subtraction p.d.	-5 → +25mV	-	8	3	59	48
Weight kg		57.0	56.7	56.6	57.2	57.1

Study 2 - Hydrocortisone administration

Administration of hydrocortisone (10 mg/24 h for 4 days) when G.B. was in metabolic balance and on dexamethasone 1.5 mg/24 h produced similar urinary free cortisol levels to those on no treatment. As shown in Fig 2.4 this caused marked Na^+ retention and a kaliuresis with a fall in urinary Na^+/K^+ ratio from 1.2 to 0.15. Plasma electrolytes showed corresponding changes, Na^+ rising from 136 to 145 $mmoll^{-1}$, K^+ falling from 4.5 to 3.3 $mmoll^{-1}$. As expected there was weight gain and suppression of PRA and plasma aldosterone (Table 2.3). Subtraction p.d. rose to 59 mV, indicative of mineralocorticoid excess. In addition there was a highly significant rise in both systolic and diastolic blood pressure (Table 2.5).

Study 3 - Withdrawal of dexamethasone

When back in Na^+/K^+ balance on dexamethasone alone, dexamethasone was discontinued for 8 days (Fig 2.5). Urinary free cortisol, which on dexamethasone was <30 nmol/24 h rose to 450 nmol/24h. There was a kaliuresis and Na^+ retention with urinary Na^+/K^+ ratio falling to 0.15. Plasma K^+ fell to 2.9 $mmoll^{-1}$ and Na^+ rose to 146 $mmoll^{-1}$. PRA suppressed once more as did plasma aldosterone (which had risen to 210 $pmoll^{-1}$ prior to withdrawal of treatment). Similarly subtraction p.d. was elevated at 48 mV, a pattern identical to that seen following cortisol administration (Table 2.4). In addition there was a significant rise in diastolic blood pressure (Table 2.5).

These changes were then reversed on re-starting dexamethasone; 1.25 mg dexamethasone/day (0.75 mg 2300 h, 0.5 mg 0900 h) was

Figure 2.4 Metabolic balance data, blood pressure, renin activity and subtraction p.d. for study 2. With GB on dexamethasone 1.5 mg/day, the response to hydrocortisone 10 mg/day for 4 days is shown.

(overleaf)

Figure 2.5 Metabolic balance study 3. Dexamethasone was discontinued for 8 days.

(overleaf)

Table 2.5 Mean Blood Pressure Readings During Metabolic Studies

Treatment Option	Systolic B.P. mmHg (mean ± s.d.)	Diastolic B.P. mmHg (mean ± s.d.)
A. Dexamethasone (1.5 mg/day)	161 ± 7	105 ± 7
B. Dexamethasone (1.5 mg/day) Hydrocortisone (10 mg/day)	177 ± 8	114 ± 12
C. No Treatment	163 ± 6	112 ± 8

"p" value

	systolic	diastolic
A vs B	<0.001	<0.001
A vs C	NS	<0.001

sufficient to suppress urinary free cortisol to <30 nmol/24 h and prevent hypokalaemia.

Study 4 - Administration of cortisone acetate

This study was performed 12 months after Study 3. During this period blood pressure had been measured twice weekly by G.B. himself and averaged 110/70 mmHg. Blood pressure had been controlled in the interim on dexamethasone 1.25 mg/day, captopril 25 mg b.d., frusemide 40 mg/day. Frusemide and captopril were discontinued 11 days prior to the administration of cortisone acetate.

G.B. was was once more established on the same Na^+/K^+ diet, and dexamethasone 1.25 mg/day was continued. When in metabolic balance cortisone acetate 37.5 mg/day (25 mg 0900 h, 12.5 mg 1700 h) was given orally for three days. This resulted in high urinary free cortisol levels with associated Na^+ retention though only a moderate kaliuresis (Fig 2.6). Prior to giving cortisone there was activation of the renin-angiotensin-aldosterone system (PRA 13.4 $ngml^{-1}h^{-1}$, urinary aldosterone 74.3 nmol/24h) which we attributed to previous ACE/diuretic therapy and/or over treatment with dexamethasone. Following 3 days of cortisone acetate PRA fell to 3.4 $ngml^{-1}h^{-1}$, urinary aldosterone to 4 nmol/24h. Both systolic and diastolic blood pressure rose during this period as shown in Table 2.6.

During the first 24 hours of cortisone acetate administration, plasma cortisol, measured throughout the day showed two distinct peaks (720 $nmoll^{-1}$ at 10.00h and 680 $nmoll^{-1}$ at 18.00h) indicating that G.B. is able to convert cortisone to cortisol.

Figure 2.6 Metabolic balance study 4. Changes in metabolic parameters and blood pressure are shown following the administration of cortisone acetate 37.5 mg/day for 3 days.

(overleaf)

Table 2.6 Blood pressure during cortisone acetate administration

Treatment Option	Systolic BP mmHg (mean ± s.d.)	Diastolic BP mmHg (mean ± s.d.)
A. Dexamethasone 1.25 mg/day	142 ± 3	99.7 ± 6
B. Dexamethasone 1.25 mg/day		
Cortisone acetate 37.5 mg	153.8 ± 5	102.3 ± 6
P value A vs B (paired t test)	<0.001	NS

Study 5 Metabolism of [11α^{3}H]-cortisol

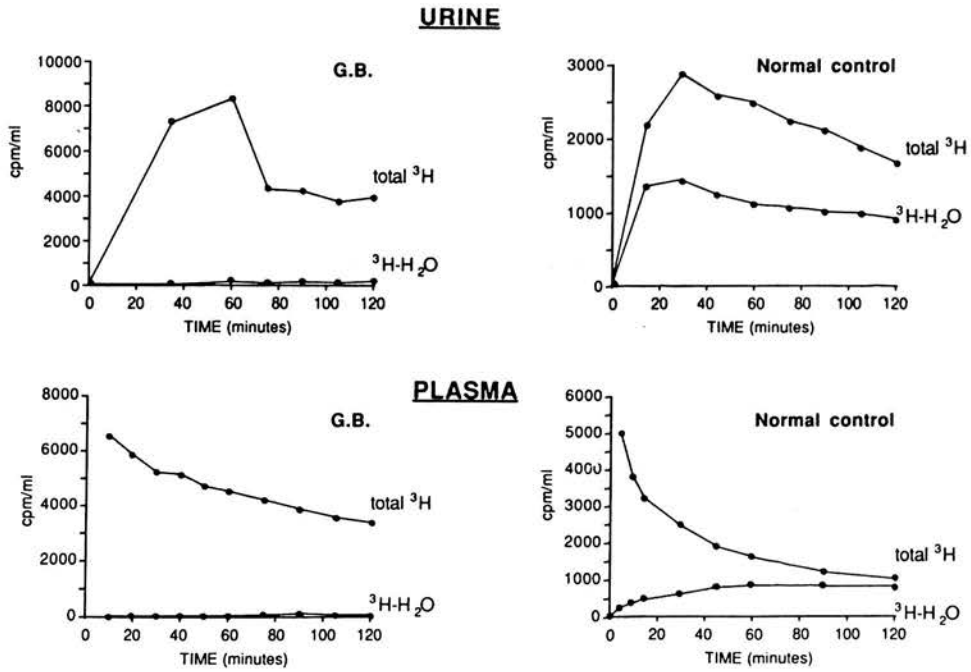
This was performed with G.B. taking dexamethasone 1.25 mg/day only, and off all other medication for over three weeks. A preliminary study in a normal volunteer showed that dexamethasone therapy did not alter plasma and urinary total ^3H and ^3H-H_2O levels (data not shown). Similarly in vitro dexamethasone was shown by Lugg et al to have no effect on 11β-OHSD activity (Lugg and Nicholas 1978).

The half life of [11α^{3}H]-cortisol in GB was prolonged at 131 min. In 8 normal volunteers the mean plasma half-life was 40.4 ± 5.3 min which is in agreement with that found by Hellman (Hellman et al 1971). When [11α^{3}H]-cortisol is oxidised by 11β-OHSD equimolar amounts of ^3H-H_2O and cortisone are produced. G.B. is unable to carry out this reaction as shown by the failure to produce ^3H-H_2O in both his plasma and urine (Fig 2.7).

Haemodynamic results

Heart rate, blood pressure, cardiac output (C.O.) and systemic vascular resistance (S.V.R.) were measured in the erect and supine position. The mean of three readings recorded at 10 min intervals was taken. In the supine position heart rate was 116 beats/min, mean arterial pressure 149 mmHg, C.O. elevated at 8.8 l/min (ref. range 4-6), and S.V.R. 1341 dynes $sec^{-1}cm^{-5}$ (ref. range 800-1400). In the erect posture, heart rate rose to 154 beats/min, M.A.P. was 136 mmHg, C.O. was 4.3 l/min and S.V.R. elevated at 2578 dynes sec^{-1} cm^{-5}.

Figure 2.7 Plasma and urine total ^{3}H and $^{3}H\text{-}H_2O$ in GB and age matched control on the same Na^+ intake following 1.19 MBq [11α ^{3}H]-cortisol intravenously at time = 0 min.



Family data

G.B's father, S.B., (aged 55) had a 20 year history of hypertension controlled for the last four years on hydralazine and a beta blocker/diuretic combination. P.B., his mother, (aged 57) had hypertension diagnosed at the age of 53 and had been treated with diuretics only. Under close supervision all medication was withdrawn for 4 weeks and S.B. and P.B. were investigated further. Blood pressure recordings were made using a Copal automatic sphygmomanometer and 24 hour urinary steroid metabolites measured (Table 2.2).

As indicated in Table 2.7, it seemed that G.B's mother, P.B. had a defect in cortisol metabolism, with a half-life for [11α ^{3}H]-F of 54.8 min which is clearly 2 s.d. above the mean value and higher than the half-life of 40.4 min seen in an age and sex matched control. In addition her supine plasma renin activity and urinary aldosterone was suppressed on two separate occassions, which together with a hypokalaemic alkalosis suggests mineralocorticoid excess. Despite this metabolic disturbance, urinary free cortisol was normal as were her urinary steroid metabolites. G.B's father had a normal half-life for [11α ^{3}H]-F at 43.9 min, and normal urinary steroid metabolites.

All the siblings had normal blood pressure but their steroid excretion patterns were not entirely normal. Both brothers in addition to G.B. showed high urinary levels of androsterone, etiocholanolone and its 11β and oxo metabolites. In the 16 control males (mean age 30.7yr), the mean androsterone and etiocholanolone excretions were 2292 $\pm$ 1131 (s.d.) and 2430 $\pm$ 744 μg/24h respectively. One brother, T.B., had a marginally elevated THF + allo THF:THE ratio (Table 2.2).

Table 2.7 Investigations on G.B.'s parents P.B and S.B. off all therapy for 4 weeks.

	ref range	P.B.	S.B.
Plasma Na^+ ($mmoll^{-1}$)	135-145	140	139
Plasma K^+ ($mmoll^{-1}$)	3.5-5.0	3.3	3.9
Bicarbonate ($mmoll^{-1}$)	21-26	33	27
Blood pressure (mmHg)		165/93	167/103
Supine plasma renin activity (ng/ml/hr)	0.5-1.5	0.3	0.7
Urinary aldosterone (nmol/24hr)	10-55	4	15
Urinary free cortisol (nmol/24hr)	80-450	272	155
09.00h plasma cortisol ($nmoll^{-1}$)	180-700	571	470
Plasma half-life of [$11\alpha^3$H]-F (min)	40.9 ± 4.8	54.8	43.9

DISCUSSION

It is not surprising that patients with 11β-hydroxysteroid dehydrogenase deficiency with hypertension, hypokalaemic alkalosis and suppressed plasma renin and aldosterone levels should have been described as having the syndrome of apparent mineralocorticoid excess. Extensive studies however, using a variety of mineralocorticoid bioassays have failed to demonstrate any evidence of a circulating mineralocorticoid excess (Ulick et al 1977). The cause of the syndrome, which has previously only been described in children, and which is often fatal, has been unclear despite the elucidation of the enzyme deficiency.

Marver reported that 5α-dihydrocortisol potentiated the action of aldosterone in an animal model (Marver et al 1978), but although 5α-dihydrocortisol levels were elevated in reported cases these fell at puberty despite continuation of the syndrome (Ulick et al 1979). Also when 5α-dihydrocortisol was infused in such patients the syndrome could not be reproduced (Oberfield et al 1983).

Detailed metabolic balance studies by New's group have indicated the important role of cortisol in 11β-OHSD deficiency. Their work has suggested that in these patients cortisol was acting as a mineralocorticoid and this effect could be blocked by spironolactone (New et al 1982, Oberfield et al 1983). They postulated a receptor defect with an abnormal receptor recognising cortisol as a mineralocorticoid. This syndrome is familial and this hypothesis would presumably require two genetic defects, one relating to the enzyme and the other to the receptor abnormality (assuming that these are not linked).

As I have discussed 11β-OHSD occurs in many tissues in man, almost certainly as at least two distinct enzymes, an hydroxy-dehydrogenase and reductase system. This evidence comes not only from fibroblast tissue cultures (Abramovitz et al 1982), but also from clinical cases. Like other cases described (Ulick et al 1979, Monder et al 1986) G.B. was unable to convert cortisol to cortisone, yet could carry out the reverse reaction normally. In the literature there now exists two documented cases of a failure of this reverse reaction, i.e. conversion of cortisone to cortisol. Both cases were females who presented with extensive hirsutism and amenorrhoea; in one case a familial defect was noted with a marked increase in the THE/THF ratio seen in a sibling (Phillipou et al 1985, Taylor et al 1984).

This chapter describes the first adult case of 11β-OHSD deficiency. The metabolic balance studies show that cortisol acted as a potent mineralocorticoid on the kidney causing Na^+ retention and hypertension with hypokalaemia. Dexamethasone by suppressing the hypothalamo-pituitary adrenal axis was able to reverse the hypokalaemia, cause a natriuresis and lower blood pressure, providing a new therapeutic approach in this patient. The gastro-intestinal tract appeared also to be exposed to excessive amounts of mineralocorticoid as judged by the marked elevation in subtraction p.d. on no treatment and during cortisol administration, suggesting a defect in 11β-OHSD activity locally in the GI tract.

The haemodynamic data indicate a "hyperdynamic circulation" with a high resting cardiac output and normal systemic vascular resistance. This has been described in other patients with "mineralocorticoid excess" states such as primary aldosteronism (Tarazi et al 1973). On

adopting the erect posture cardiac output fell by 50% and the heart rate increased, suggesting a non-compliant left ventricle as seen in ventricular hypertrophy.

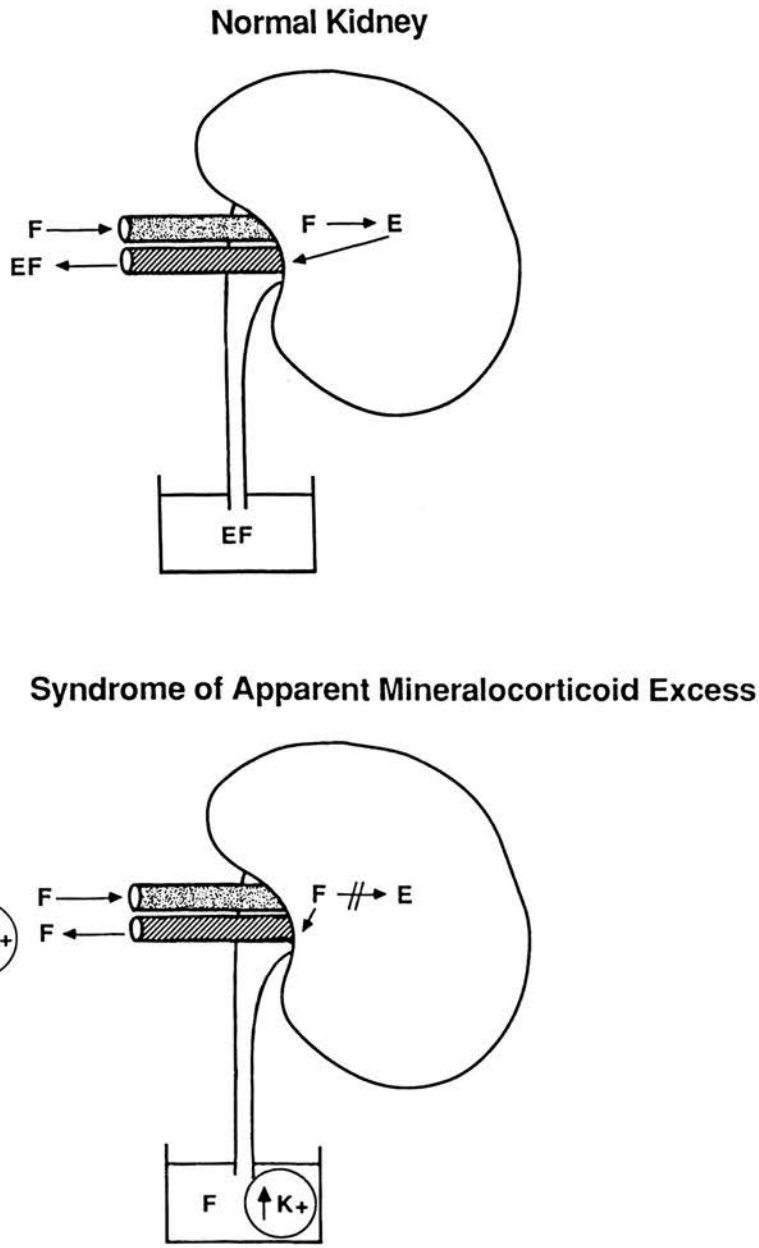
Several studies indicate that there is a genetic basis for this syndrome. Shackleton described two siblings from Chile with the disorder (Shackleton et al 1985), and Monder two black female children (Monder et al 1986). Shackleton has gone further to investigate the parents and other siblings of his two cases, and found normal urinary steroid metabolic profiles. In addition he was unable to demonstrate any abnormality after ACTH administration. However, until now, no one has studied the metabolism of [11α^3H]-cortisol in the relatives of reported cases. Although the metabolism of [11α^3H]-F was normal in G.B's father, this was clearly not the case in his mother, P.B., who had an abnormally long half-life for the isotope. This finding, together with mild hypertension, hypokalaemic alkalosis and suppression of the renin-aldosterone system, was of particular interest as, until this study was performed P.B. had been labelled as simply having essential hypertension. Whilst suggesting that that the inheritance of the enzyme defect arose from G.B.'s maternal family and for the first time providing evidence for a heterozygous state in the condition, it also raised the question whether such defects in cortisol metabolism were seen in other "essential hypertensives". The normal urinary steroid metabolites seen in P.B. may be telling us that this is a crude method for diagnosing subtle defects in enzyme activity.

The urinary steroid excretion values for G.B.'s brothers were not normal. Like G.B. the brothers had evidence of high androgen

production through elevated excretions of androsterone and etiocholanolone. Most notable was the high excretion of 11β-hydroxyandrosterone which could reflect increased activity of side-chain cleavage in cortisol metabolism or substantial 11β-hydroxylation of testosterone and androstenedione. Apart from T.B. the THF + allo-THF : THE ratios were normal.

Funder has postulated that classical type I mineralocorticoid receptors have similar affinity for both aldosterone and cortisol, occupation by the latter being in part dependent upon concentrations of renal extravascular cortisol binding globulin (Funder 1985). (Plasma levels of cortisol binding globulin are normal in 11β-OHSD deficiency (Ulick et al 1979) and we have no reason to suspect alterations in tissue levels). We postulate from our observations that renal 11β-OHSD is critical in determining the intrarenal concentration of active glucocorticoid, deficiency of renal 11β-OHSD elevating active glucocorticoid levels in the kidney which spill over onto mineralocorticoid receptors causing hypertension and hypokalaemia. An indication of the abnormal renal handling of cortisol is the moderate elevation of urinary free cortisol levels in the condition despite normal plasma cortisol levels and a prolonged cortisol half life. This, through negative feedback control, lowers ACTH and hence cortisol production. If our hypothesis is true this would suggest that this unique biological experiment has disclosed a previously unknown physiological mechanism by which the kidney protects itself from excessive amounts of gluco and mineralocorticoid via 11β-OHSD. Fig 2.8 shows our working hypothesis more clearly, the renal defect in the cortisol → cortisone shuttle seen in AME producing sodium retention and hypokalaemia.

Figure 2.8 The role of renal 11β-OHSD in normal man and in the "syndrome of apparent mineralocorticoid excess."



This syndrome indicates the quite striking results of a gross disturbance of this cortisol → cortisone shuttle. Could it be possible that more subtle defects in this mechanism are important in other types of hypertension?

CHAPTER 3 ENZYMOLOGY AND METHODOLOGY: FURTHER EVALUATION OF 11β-HYDROXYSTEROID DEHYDROGENASE

The existence of 11β-hydroxysteroid dehydrogenase in tissues was first established in the 1950's using rat liver preparations. By 1959 Hurlock had obtained a fairly highly purified enzyme using acetone dried powders of microsomes from rat livers (Hurlock and Talalay, 1959). However, it was not until the 1960's that Bush continuing this theme characterised the enzyme further in liver preparations (Bush et al 1968, Bush 1969). Using whole homogenates, washed microsomes or acetone-dried powders of washed microsomes, he studied the kinetics of 11β-OHSD, establishing a Michaelis-Menten (Km) for the enzyme for cortisol of 2×10^{-5}M. The capacity for this conversion of cortisol to cortisone seemed to be extensive, with cortisol concentrations up to 8 times the Km undergoing metabolism at the same velocity. Several important conclusions were made from Bush's early in vitro studies on the hepatic enzyme. Firstly NADP(H) was a very efficient and NAD(H) a very poor co-enzyme for the reaction. Secondly, using a variety of substrates, he was able to summarise the critical groups within the substrate responsible for close apposition with the enzyme complex. In particular a flat A/B ring of the steroid was required. The insertion of a halogen derivative at the 9 position blocked the oxidation of the 11β hydroxy steroids but increased reduction of the 11 ketones. Thus he showed that 9α-fluorocortisol was a competitive inhibitor of the oxidation of cortisol → cortisone but 9β-fluorocortisone was reduced 5 → 10 times the velocity of cortisone.

Thirdly, he evaluated the effect of pH on both reductive and oxidative activity, and showed a 25 fold increase in F → E conversion with no change in E → F on raising the pH of the buffer from 6.5 to 9.5.

As well as studying steroid conversion in homogenate/microsomal preparations directly, spectrophotometric methods for 11β-OHSD activity have been devised relying upon generation of NADPH (Koerner, 1969). More recently, studies from Germany have shown higher activity of hepatic 11β-OHSD in the sexually mature male rat than in the corresponding female, this difference being dependant upon the presence of androgens in the male (Lax et al 1978).

In man thyroid function is known to affect the metabolism of cortisol to cortisone. In the early 1960's elevated thyroid hormone was shown to alter the metabolism of cortisol towards its 11-keto derivatives, with hypothyroidism having the opposite effect i.e. shifting cortisol metabolism towards its 11β-hydroxy derivatives (Hellman et al 1961). This has subsequently been evaluated by the same group using the [11α^{3}H]-cortisol and ^{14}C-cortisol isotopes. Hyperthyroidism increases both F → E and E → F, but the enzyme 'set point' is moved towards E, explaining the increase in the urinary THE/ THF ratio. Hypothyroidism appears to reduce F → E and E → F equally, with no change in the enzyme 'set point'. Interestingly many hypothyroid patients have an increase in the THF/ THE ratio and it is suggested that increased conversion of THE to cortoic acid may be responsible (Zumoff et al 1983).

In the rat however, thyroxine given in vivo, reduced hepatic 11β-OHSD activity in both directions but had no effect in vitro (Koerner and Hellman 1964). Curiously thyroxine given in vivo had no effect on renal conversion of F → E. This highlights the point that there are obviously species differences in 11β-OHSD, and that results from in vitro work may be misleading if not accompanied by in vivo experiments.

The purification of 11β-OHSD has largely been carried out by Monder's group in New York. Using detergents in the form of zwittergents and tritons to modify the membrane structure of rat liver microsomes, they have solubilized this microsomal enzyme (Lakshmi and Monder 1985a and 1985b). It is now clear from their work (and from studies on 11β-OHSD in the lung, see later) that 11β-OHSD exists as two distinct, though closely associated 11β-dehydrogenase and 11-keto reductase enzymes. Monder's group have gone further to suggest that both these enzymes are composed of at least two kinetically different forms. In particular there is now good evidence to suggest the existance of two distinct 11β-dehydrogenase enzymes, a high Km (Km 4.5μM) and a low Km enzyme (0.3μM). Using Triton DF-18 they have extracted the high Km enzyme from liver microsomes, revealing a protein with a molecular weight of 26000. The first 42 amino acid sequence from the N terminus of the enzyme protein have now been characterised and shown in fig 3.1. (Nandivada and Monder 1987, and personal communication).

Figure 3.1 The amino acid sequence of the NH_2 terminus of rat hepatic 11β-dehydrogenase (courtesy of Dr. Carl Monder).

```
1                5                   10                  15
MET-LYS-LYS-TYR-LEU-LEU-PRO-VAL-LEU-VAL-LEU-X-LEU-GLY-TYR-TYR-TYR-
        20                  25                  30
SER-THR-ASN-(GLU)-(GLU)-PHE-ARG-LEU-(GLU)-MET-(LEU)-GLN-(GLY)-(LYS)-
                  35                  40
LYS-(VAL)-(ILEU)-VAL-(ILEU)-(GLY)-(ALA)-(ASP)-(LYS)-X-(ILEU)-
```

X UNDETERMINED OR ATYPICAL AMINO ACID

( ) TENTATIVELY IDENTIFIED RESIDUE

In man the predominant direction of 11β-OHSD in the liver is towards cortisone, possibly as a result of the redox potential therein (i.e. NADPH:NADP ratio) (Bush 1968). Although the hepatic enzyme has received most attention, 11β-OHSD is present in many extrahepatic tissues. Activity was first shown in the placenta in 1960 (Osinski, 1960). This has been studied more recently by Bernal, who, using both conventional methods for steroid separation and a luminometric method for enzyme activity showed a predominantly oxidative reaction in both placenta and choriodecidua at term. Indeed despite addition of NADPH they were unable to demonstrate cortisone $\longrightarrow$ cortisol conversion (Bernal et al 1980, Bernal et al 1986).

Extensive work by Murphy's group in Montreal has looked at 11β-OHSD activity in the lung, particularly in the fetus at the time of birth. Clinically their studies are of considerable relevance as it is known that rising levels of glucocorticoids play a role in fetal lung maturation via surfactant production near term, if not the onset of parturition itself. Murphy has shown a decline in F $\rightarrow$ E metabolism in fetal lung as term approaches with an increase in E $\rightarrow$ F. The resulting raised intracellular F, generated through this change in 11β-OHSD activity may be the important factor in the final stages of lung maturation (Murphy 1978, 1981b). Using human fetal lung cultures, they have also indicated that 11β-OHSD exists as two distinct enzymes, the dehydrogenase component associated with epithelial cells, the reductase enzyme with fibroblast cells (Abramovitz et al 1982, Abramovitz et al 1984). In an earlier communication Koerner had demonstrated the dependance on NADP of the

conversion of F → E in the rat lung (Koerner 1966). In vivo, workers in Australia have established a Km for the enzyme in an isolated rat lung preparation (5×10^{-6}M for cortisol) (Nicholas and Lugg 1981). Dexamethasone, given parenterally had no effect on 11β-OHSD activity in the rabbit lung (Lugg and Nicholas 1978).

The renal enzyme has received little attention. Renal 11β-OHSD enzyme activity was first shown in 1960 in vitro in rat renal slices, minces and homogenates; conversion of F → E being dependant upon NADP (Mahesh and Ulrich 1960). The conversion of cortisol to cortisone is the predominant reaction in kidney tissue, indeed Jenkins was unable to show any metabolism of E → F in human renal slices (Jenkins 1966). Reach et al in Paris perfused cortisol in an isolated rat kidney model and showed complete metabolism of cortisol by 120 minutes, principally to cortisone, dihydrocortisol and dihydrocortisone (Reach et al 1977). More recently this pattern of metabolism has been confirmed using corticosterone in rat kidney slices (Hierholzer et al 1984, Hoyer et al 1984). However, with the exception of Hierholzer's data, very little in vitro or in vivo work has been done on the renal enzyme.

Following the impetus and observations from our index case in which it appeared that it was the renal 11β-OHSD that was deficient and responsible for the clinical manifestations, I aimed my initial in vitro work on the kidney enzyme in animal models and in man.

METHODOLOGY

CHEMICALS AND BUFFERS

Non-radioactive steroids used in this study were obtained from Sigma chemicals and were stored at -20°C as 10^{-3}M solutions in 100% ethanol. The [1,2,6,7-^{3}H]-cortisol (sp.act. 86 Ci/mmol), [1,2,6,7-^{3}H]-corticosterone (sp.act. 84 Ci/mmol) and [1,2-^{3}H]-cortisone (sp.act. 53 Ci/mmol) were purchased from Amersham International. The purity which was regularly checked by thin layer chromatography was >95%. Collagenase (CLS, Worthington) was purchased from Lorne Diagnostics, Ficoll (type 400) and hyaluronidase (type 1-5) from Sigma. Bovine serum albumin (Miles, fraction V, reagent grade) was purchased from ICN Biomedicals. Reagent grade salts, analytical grade solvents, Cocktail T 'Scintran' scintillation fluid and Merck (5553) thin-layer chromatography plates were all obtained from BDH Chemicals. Glass distilled deionized water was used for all aqueous solutions. Krebs-Ringer bicarbonate buffer had the following composition: 118mM NaCl, 3.8mM KCl, 1.19mM KH_2PO_4, 2.54mM $CaCl_2.2H_2O$, 1.19mM $MgSO_4.7H_2O$, 25mM $NaHCO_3$. This was gassed for 60min with 95%O_2: 5%CO_2. Glucose (11.1mM) was added prior to use.

Two methods were then used for tissue preparations, either an enzyme tubule dispersion or a cortical tissue homogenate. In addition cortical tubules were separated into proximal and distal tubules using a Ficoll gradient.

Method 1. PREPARATION OF DISPERSED RENAL CORTICAL TUBULES

Whole kidneys were obtained, usually from male Wistar rats (though also from sheep and occasionally from human transplant operations), and were immediately placed in 0.9% saline at 4°C. Following decapsulation, the kidney was halved longitudinally and a portion of the outer cortical tissue (approx. 2 g) was dissected away from the medulla and then diced and minced with surgical scissors. The cortical tissue was incubated for 60 min at 37°C in a shaking water bath in 10ml of 0.05% (w/v) collagenase and 0.1% (w/v) hyaluronidase (in KRB containing 0.2% glucose and 2% BSA). Tubules were mechanically dispersed using a broad tipped 5 ml pipette at 30 and 60 min, and the enzyme solution replenished at 30 min. The suspension containing the isolated dispersed tubules was filtered through a 100μm nylon gauze (Henry Simon) to retain glomeruli and tissue fragments. The filtered suspension was transfered to a plastic centrifuge tube and centrifuged at 600 g for 10 min at 4°C. The supernatant was withdrawn by suction, the tubule pellet gently resuspended in 10 ml of cold KRB and centrifuged again at 600 g for 10 min at 4°C. This washing procedure was repeated twice. The washed pellet was finally suspended in 10 ml KRB (Rasmussen 1975). Light microscopy revealed a preparation consisting mainly of short fragments of both proximal and distal tubular elements. The integrity of the tubules was evaluated by the dye exclusion method (Phillips 1973). Following a tubular count using a haemocytometer the final tubular preparation was diluted to yield approx. 10^5 tubules/400μl buffer.

Isolation of rat cortical tubules enriched in proximal and distal segments using a Ficoll gradient

Following preparation of the cortical tubule dispersion described above, the tubules were subjected to unit gravity sedimentation through a Ficoll gradient to separate proximal and distal tubules (Scholer and Edelman 1979). Ficoll (polysucrose; MW 400,000) was freshly dissolved in KRB (0.2% glucose) in concentrations of 1,2,6,8 and 12% (wt/wt), pH 7.4 corresponding to a density range of 1.013 - 1.053 g/ml. A discontinuous gradient consisting of 30 ml layers was generated in a 150 ml container (ID = 50 mm) by careful layering with a pipette. 5 ml of the kidney tubule dispersion was then loaded on the gradient. When the particle front reached the 8%/12% interphase fractions were collected from the top with a 5 ml pipette.

Three 20 ml and then nine 10 ml fractions were collected and analysed individually. Reproducibility of the gradient and of the collection was monitored by measuring the refractive index of each fraction with a refractometer and the density was also measured by accurately weighing a known volume.

Unstained, unfixed tubular suspensions from each fraction were inspected by light microscopy and differential counts of proximal and distal segments were made on paired independent preparations. Proximal tubules were distinguished by their yellowish colour and very broad structure whereas distal elements were observed to be more transparent and narrower. Fractions 2-4 (50 ml) were combined and

were designated as distal enriched fraction (approx. 85% distal tubules) while fractions 8-10 were combined and designated as proximal enriched fraction (approx. 70% proximal tubules). The integrity of the tubules was evaluated as previously described (Phillips 1973). The fractions were washed twice with cold KRB and following a tubular count the final tubular preparation was diluted to yield approximately 10^5 tubules/400µl buffer.

Method 2. PREPARATION OF A CORTICAL HOMOGENATE

Whole kidneys were obtained from male Wistar rats and immediately placed in 0.9% saline at 4°C. The kidneys were then decapsulated, halved longitudinally and 0.5 g of cortical tissue (wt/wt) retrieved from each rat. This was homogenised in 10 ml KRBG using a Dounce tissue grinder (Wheaton Scientific, New Jersey) for about 30 strokes. A 1 in 8 diluted homogenate was made using KRBG and a protein assay performed using the Bio-Rad protein assay kit. This assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Blue G-250 shifts from 465 nm to 595 nm when binding of protein occurs (Bio-Rad Laboratories 1981). The results were read on a double beam spectrophotometer (Shimadzu UV-210-A). Protein concentration in mg/g wet weight tissue was calculated and homogenates from each rat diluted as necessary with KRBG to yield a fixed protein concentration in each homogenate. When ^{3}H-corticosterone was used as the substrate a 1 in 4 homogenate dilution was used for incubation, whereas a 1 in 1 was used for ^{3}H-cortisol and

^{3}H-cortisone (see below).

Rat liver was processed where stated using the same protocol, enabling us to study the relative contribution of F → E interconversion by different organs from the same rat using a fixed tissue enzyme concentration.

EXPERIMENTAL PROTOCOL

10µl of the tritiated steroid (=1µCi) was then incubated with either 400µl of the tubule preparation (containing approx. 10^5 tubules) or 400µl of the homogenate dilution and 600µl of KRBG (0.2% BSA) in a shaking water bath at 37°C for 60 min. The final concentration of steroid in the incubate was 1.19×10^{-8}M for corticosterone and 1.25×10^{-8}M for cortisol and cortisone. Where stated other steroids were added to each incubate as 10µl volume, the final steroid concentration being recorded. All incubations were carried out in triplicate. Blank samples were run in each experiment and consisted of incubation media and steroid but no tissue preparation. At the end of incubation the tubes were transfered to ice and immediately centrifuged at 1500 g for 15 min. The supernatant was removed and stored at -70°C until steroid extraction.

IDENTIFICATION AND QUANTIFICATION OF METABOLITES

Each sample was extracted twice with 5 ml ethyl acetate by vigorous shaking for 10 min to extract the steroids. The combined

organic phase extracts were evaporated to dryness and redissolved in 100µl of ethanol for spotting on a silica gel thin layer plate in parallel with standards of unlabelled cortisol, cortisone, corticosterone or 11-dehydrocorticosterone, depending on the substrate used. The plate was developed using a solvent system containing chloroform:95% ethanol (92:8) to a distance of 15 cm. The steroids were detected under ultraviolet light and the zones corresponding to reference steroids were scraped directly into vials, eluted with ethanol, evaporated to dryness and redissolved in 4 ml scintillation fluid. The vials were counted using a Packard 4430 series Beta counter (68% counting efficiency). This procedure separated either cortisol and cortisone or corticosterone and 11-dehydrocorticosterone and the conversion could be calculated from the radioactivity of each fraction expressed as counts per min (c.p.m.). The recovery of radioactivity always exceeded 85%.

RESULTS

Using a collagenase dispersed rat renal tubular preparation (method 1), a simple time course experiment was performed. Fig 3.2 shows conversion of cortisol to cortisone and corticosterone to 11-dehydrocorticosterone by such a preparation.

Fig 3.3 reflects activity of 11β-OHSD in rat kidney proximal and distal tubules. Although activity was present in both sites, it was more marked in the distal tubule ($p<0.001$).

Figure 3.2 % conversion of ^{3}H-cortisol to ^{3}H-cortisone and ^{3}H-corticosterone to ^{3}H-11-dehydrocorticosterone by an enzyme dispersed rat renal tubular preparation.

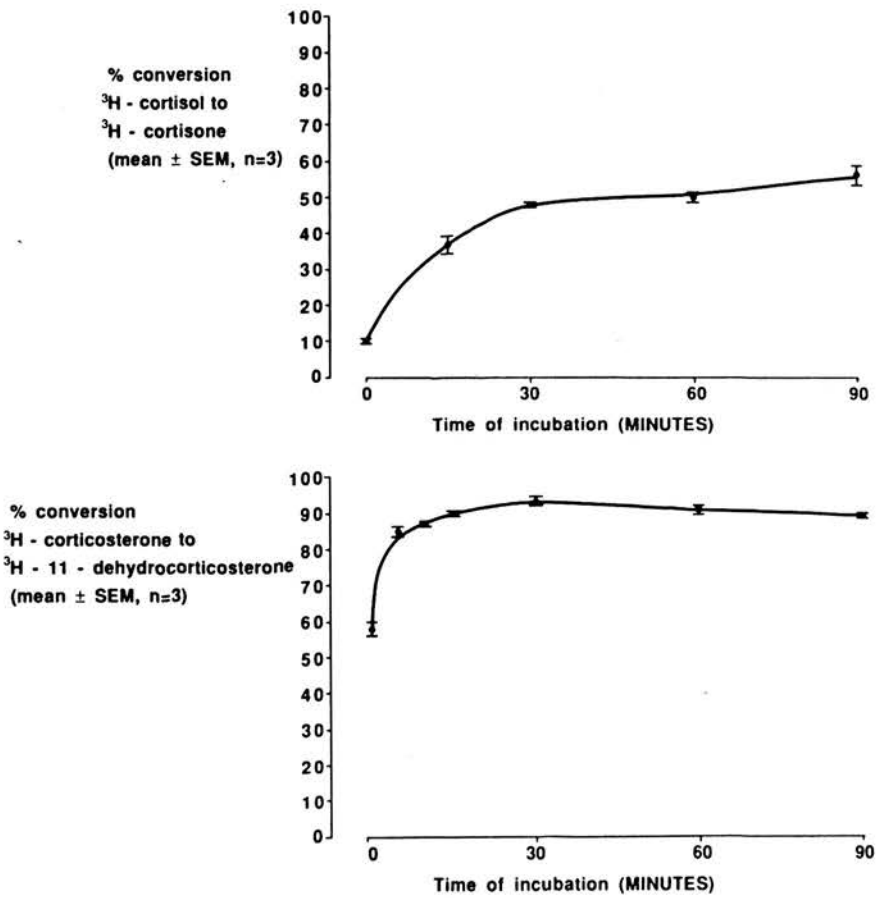
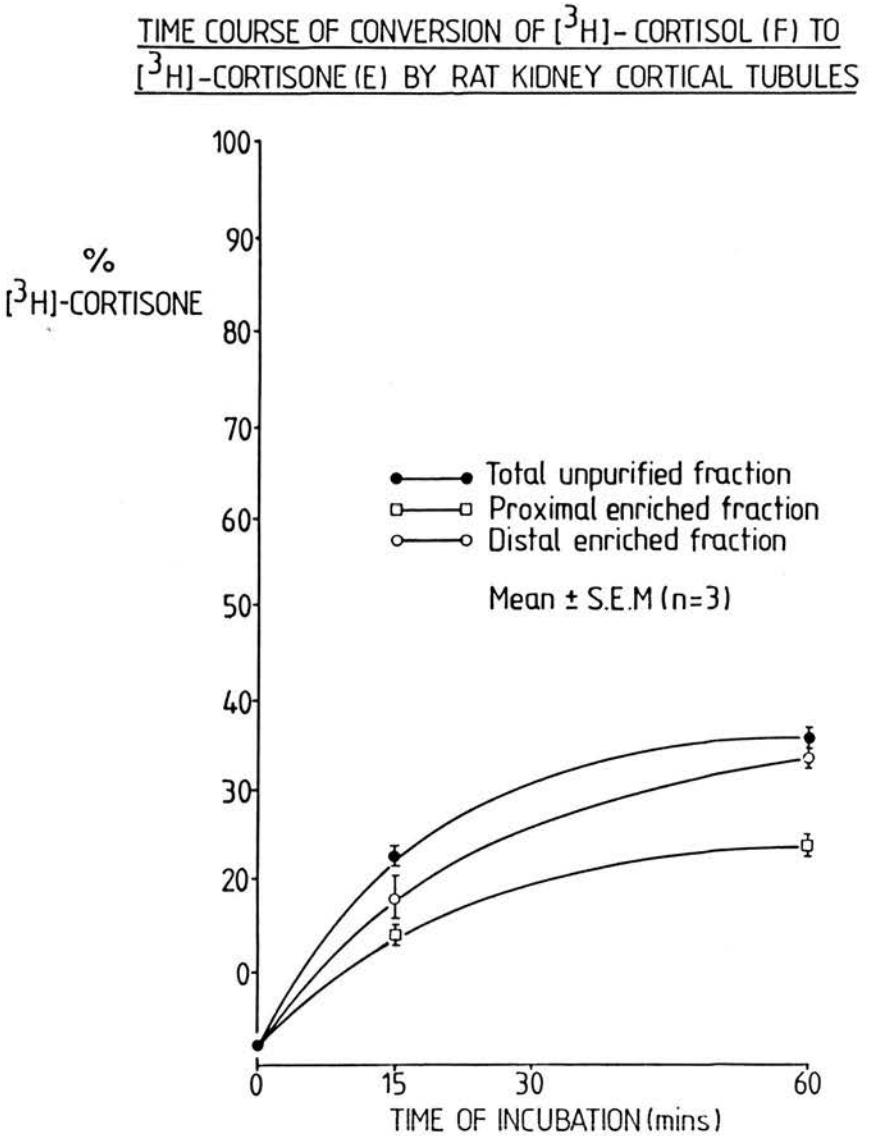


Figure 3.3 % conversion of ^{3}H-cortisol to ^{3}H-cortisone by an enzyme dispersed rat renal tubular preparation following separation of proximal and distal tubules using a Ficoll gradient.



Following this a series of in vitro experiments were performed using rat kidney tissue prepared by method 1:

1. Establishing a Michaelis-Menten (Km) for renal 11β-OHSD in the rat using corticosterone as a substrate

This had never been evaluated previously, and was done using a Lineweaver-Burk plot (Lineweaver and Burk 1934). A fixed amount of ^{3}H-corticosterone was added to each incubate along with increasing amounts of cold corticosterone. Incubation time was 7.5 min; at this time point a plot of %conversion vs time gave a linear reading. Table 3.1 shows the substrate concentration [S] in each incubate and the % conversion, from which the mass converted [V] could be calculated. A plot of 1/[V] vs 1/[S] was made using a linear regression (fig 3.4) and the Km and Vmax determined from this plot as indicated. These were 1.6×10^{-4}M and 4.4×10^{-5}M respectively.

2. The effect of NADP and progesterone derivatives on enzyme activity

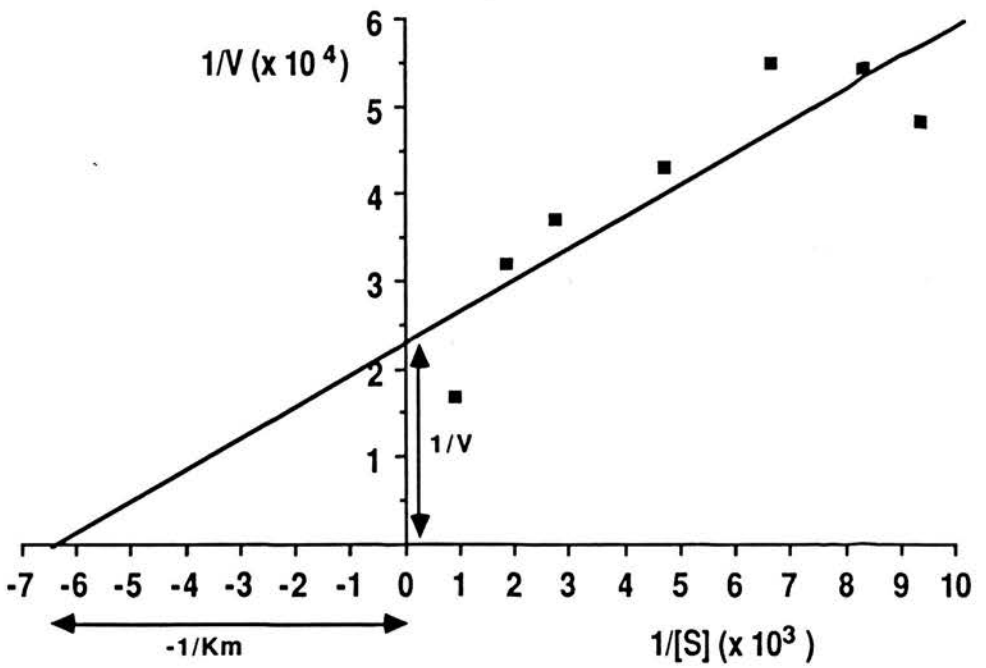
It had been reported that progesterone and some of its hydroxy derivatives, notably 11α-hydroxyprogesterone were potent inhibitors of placental 11β-OHSD activity (Murphy 1981) and we were keen to evaluate this in the kidney.

Figs 3.5, 3.6 and 3.7 show the effect of NADP, progesterone and some of its hydroxy derivatives on conversion of F → E by renal

Table 3.1. Substrate concentration, and % conversion of corticosterone by rat renal tubular preparation. Mass converted is equal to [S] x % conversion. % conversion is expressed as the mean of 3 readings.

[S] $x10^{-4}$mol/l	% conversion	[V] (mass converted) $x10^{-5}$mol/l
1.07	19.4	2.08
1.20	15.5	1.86
1.50	12.2	1.83
2.10	11.1	2.33
3.60	7.5	2.70
5.40	5.8	3.13
10.70	5.5	5.89

Figure 3.4 A Lineweaver-Burk plot for rat renal 11β-OHSD using corticosterone as a substrate. The Km and V_{max} are calculated from this plot as shown.



tubular preparations from the rat, sheep and man. Conversion of F → E was clearly dependant on NADP. Progesterone was inhibitory to 11β-OHSD in the rat, sheep and human in a dose dependant manner even when added in equimolar concentrations (10^{-8}M). We confirmed that 11α-hydroxyprogesterone was a potent enzyme inhibitor in all 3 species and have also documented a inhibitory action of 17α-hydroxyprogesterone (though this was not as marked as for progesterone itself).

Tubular counts whilst giving a rough indication of the amount of tissue, hence enzyme present, were unsuitable for comparing one animal to another, one experiment to another, or tissue to tissue within an animal. In order to improve both intra- and inter-experimental accuracy method 2 using a protein assay to quantify enzyme concentration was developed for tissue preparations. Using this method the following experiments were run:

1. Correlation of homogenate protein concentration with enzyme conversion

This was necessary to establish the correct tissue dilution needed for incubation for both corticosterone and cortisol. From the initial homogenate preparation doubling dilutions were made, 1 in 2, 1 in 4, 1 in 8, etc, assayed for protein as described and incubated with a fixed amount of either ^{3}H-corticosterone or ^{3}H-cortisol for 60 min. Fig 3.8 shows a typical plot of %conversion vs protein concentration (mg/g wt/wt), in this case for corticosterone and a renal homogenate. On the basis of this an homogenate dilution was used on the linear part of the curve (i.e. 1 in 4). Using cortisol or cortisone as

Figure 3.5 The effect of NADP, progesterone, and its hydroxy derivatives on the conversion of cortisol to cortisone by a rat renal tubular preparation.

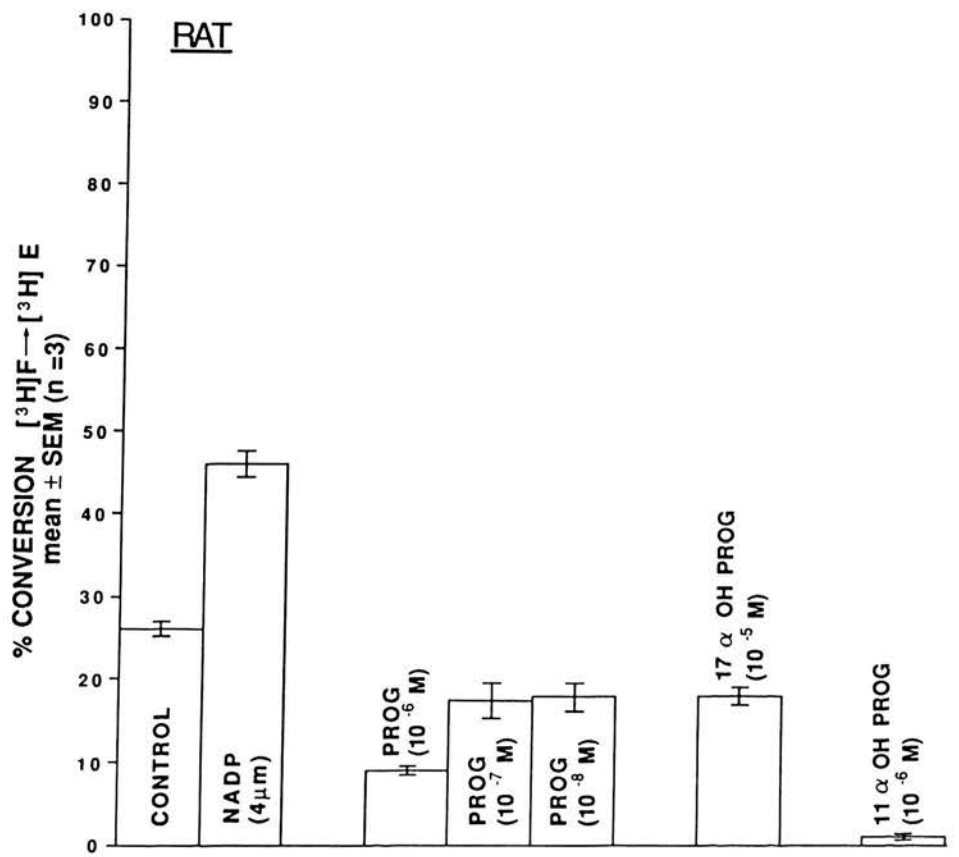


Figure 3.6 The effect of progesterone, and its hydroxy derivatives on the conversion of cortisol to cortisone by a sheep renal tubular preparation.

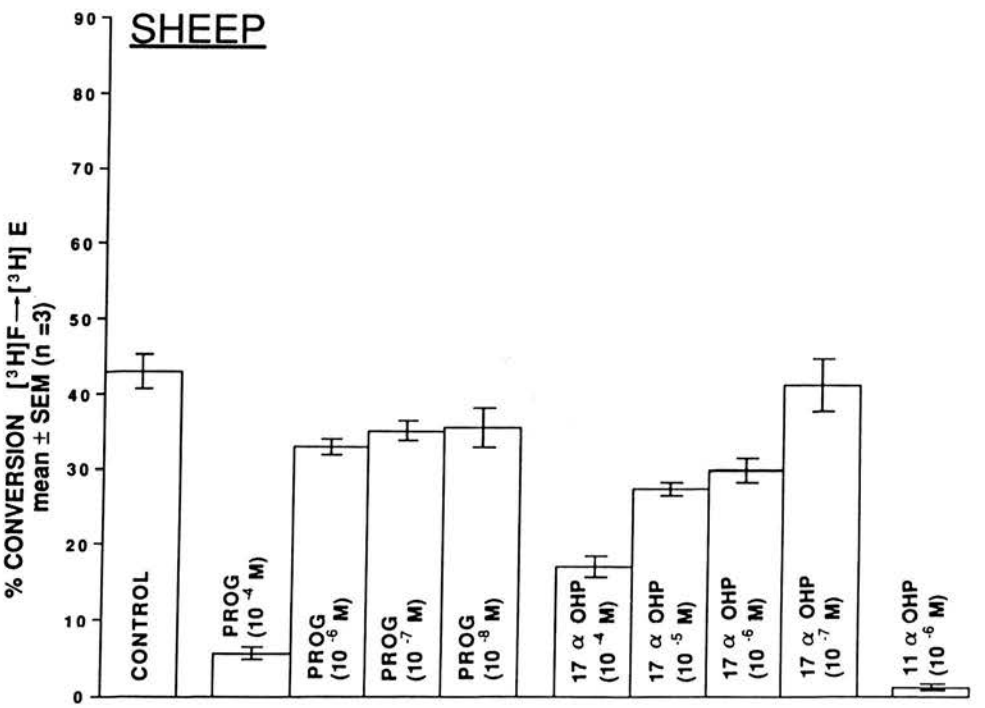


Figure 3.7 The effect of NADP, progesterone and its hydroxy derivatives on the conversion of cortisol to cortisone by a human renal tubular preparation.

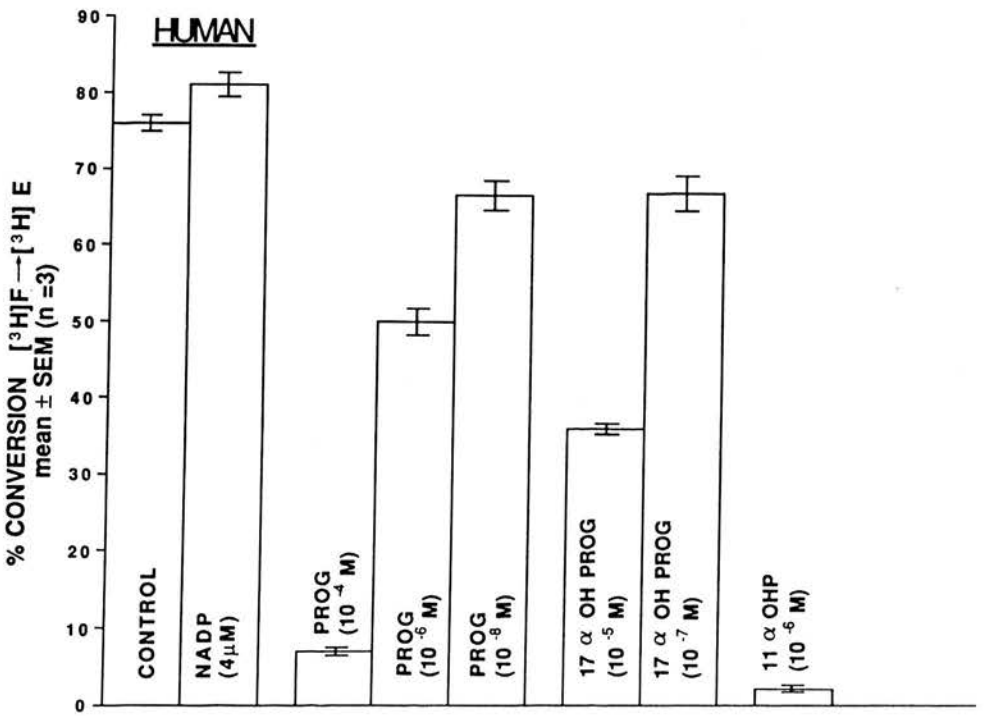
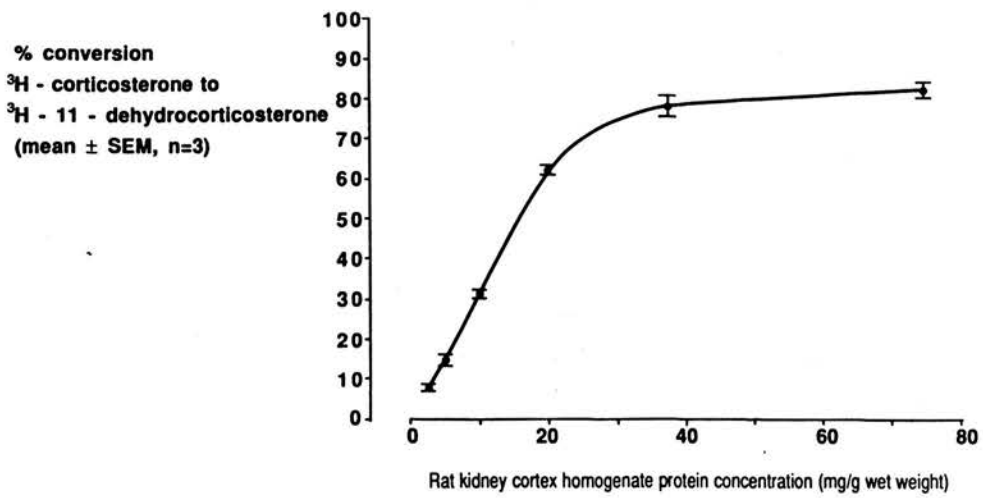


Figure 3.8 % conversion of corticosterone to 11-dehydrocorticosterone by a rat renal homogenate of varying protein concentration.



substrates a 1 in 1 homogenate dilution was used. These homogenate dilutions were also shown to be optimum when liver was used instead of kidney (i.e. 1 in 4 for corticosterone, 1 in 1 for cortisol/cortisone).

2. Conversion of cortisol to cortisone and vice-versa by renal and hepatic homogenates from the same animal

Kidneys and livers were taken from 6 male Wistar rats of the same age and processed as described above in method 2. An equal protein concentration (mg/g wt/wt) was used for each tissue preparation.

Corticosterone is the principal corticosteroid in the rat, but ^{3}H-11-dehydrocorticosterone is not commercially available. As we were keen to evaluate the reductase component of 11β-OHSD we elected to use F and E in addition to corticosterone, which, although not present in the rat will still undergo similar metabolism by 11β-OHSD. NADP and NADPH were added as shown in a final concentration of 4μM. Fig 3.9 shows the conversion of corticosterone, cortisol and cortisone by the rat kidney and liver homogenates. From this we concluded;

a) corticosterone has (not surprisingly) a higher affinity for rat renal and hepatic 11β-OHSD than cortisol.

b) NADP increases the metabolism of the dehydrogenase step, NADPH increasing activity of the reductase step.

c) in the kidney 11β-OHSD activity is set towards F, i.e. F → E metabolism occurs to a much greater extent than E → F, confirming the findings of Jenkins (Jenkins 1966). The ratio of conversion of F to E/ E to F is 4.7.

d) in the liver 11β-OHSD activity is set towards E, i.e E → F occurring more readily than F → E, the ratio of F to E/ E to F being 0.6.

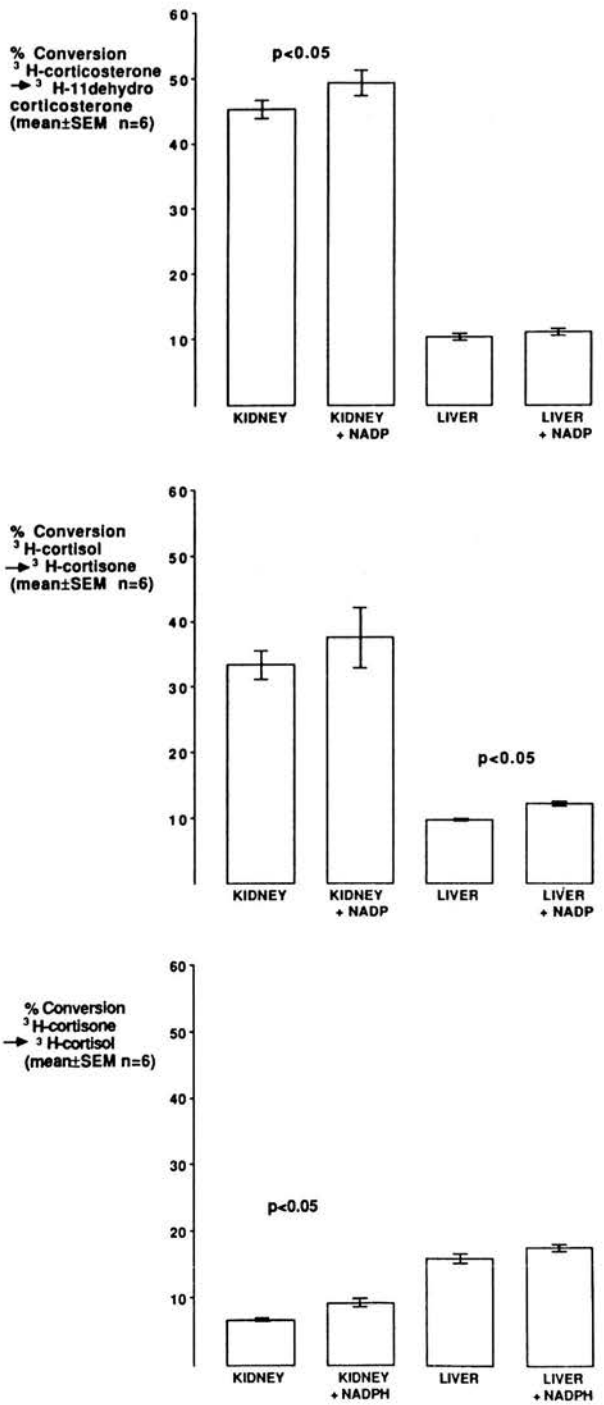
e) comparing 11β-OHSD activity in liver and kidney, the kidney converts more F → E than the liver on a weight for weight basis (i.e. for a given protein and hence enzyme concentration), and the liver more E → F than the kidney. Co-factors in the form of NADP and NADPH did not alter this difference, which suggests that something other than local re-dox potential is controlling this tissue discrepancy in enzyme activity. It is possible that 11β-OHSD exists as two structurally differing enzymes at these sites.

SUMMARY

Two separate methods for preparing in vitro renal tissue have been developed, and have been clearly shown to possess 11β-OHSD activity. These methods will be referred to in subsequent chapters of this thesis.

For the first time renal 11β-OHSD has been characterised in an animal model. Using a dispersed tubular preparation, we have demonstrated activity in both proximal and distal tubules (distal >

Figure 3.9 Conversion of corticosterone, cortisol and cortisone by rat renal and hepatic homogenates (n = 6). All the homogenates had a fixed mg/g. wet weight protein concentration.



proximal) and have proceeded to investigate the kinetics of 11β-OHSD, establishing a Km and V_{max} for the enzyme with corticosterone. Being the first group to study this, we have no means of validating our results. However Bush's findings of a Km of 2x 10^{-5}M for the liver enzyme using cortisol as a substrate suggests our results are comparable (Bush 1969). As noted by previous workers studying 11β-OHSD activity in other sites (Burton 1965, Murphy 1981), we have confirmed that physiological concentrations of progesterone and some of its hydroxy derivatives are inhibitory on renal 11β-OHSD.

Whilst validating a more reproducible and accurate homogenate tissue preparation, we have shown the effect of co-factors on enzyme activity, investigated the enzyme set point in the liver and kidney, and, perhaps most important of all, have some evidence to support our clinical hypothesis albeit in an animal model in which corticosterone is the major corticosteroid, that the kidney is one of the principal sites of conversion of cortisol to cortisone, exceeding that of the liver.

CHAPTER 4 LIQUORICE INDUCED HYPERTENSION

Liquorice has been used medically for at least 5000 years (Shen 1975), but scientific interest was aroused in 1946 in the small Netherlands village, Heerenven. Here, Revers studied many patients suffering from peptic ulcer, who improved after taking a proprietary liquorice preparation (succus liquiritiae) from the local chemist (Revers 1946). Today Carbenoxolone, whose active principle is the liquorice derivative 18β-glycyrrhetinic acid, is still used in the treatment of peptic ulcers. However, as Revers noted only 2 years on from his initial observation, this therapy was not without side effects with 1 in 5 of his patients developing oedema and shortness of breath on exertion (Revers 1948).

Liquorice was first introduced as a confectionery in 1760 by Geo. Dunhill in Pontefract. Today it is a popular confectionery in Britain sold principally as liquorice allsorts and Pontefract cakes. Since Revers observations there have been numerous worldwide reports of liquorice induced hypertension with hypokalaemia (Groen et al 1952, Koster and David 1968, Conn et al 1968, Epstein et al 1977, Ibsen 1981, Toner and Ramsey 1985) complicated in some cases by myopathy (Gross et al 1966, Blachley and Knochel 1980, Sundaram and Swaminathan 1981) and even life threatening cardiac arrhythmias (Bannister et al 1977, Neilson and Pederson 1984).

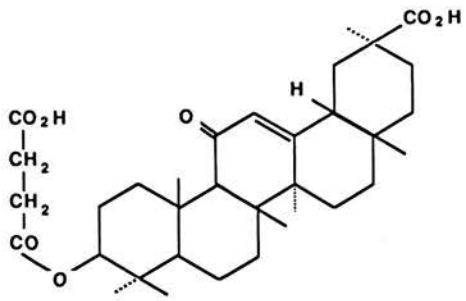
Patients present as a mineralocorticoid excess state with suppression of the renin-angiotensin system (Epstein et al 1977), hypokalaemia and Na^+ retention with elevation of exchangeable Na^+ (Card et al 1953, Beretta-Piccoli et al 1985). The condition is

reversible on stopping liquorice and responds to spironolactone administration (Salassa et al 1962). Initially doses of liquorice given to volunteers to study the pathogenesis of this mineralocorticoid excess state were large, but more recently smaller doses (approx 100 g/day) have been shown to suppress the renin-angiotensin system (Epstein et al 1977).

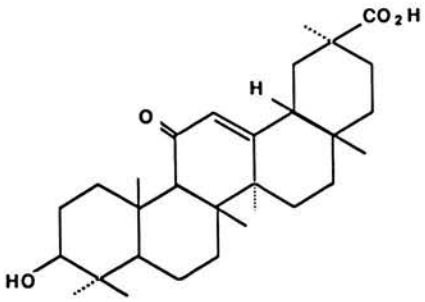
Although it is known that the active mineralocorticoids in liquorice are glycyrrhizic acid (GI) and its hydrolytic product glycyrrhetinic acid (GE) (fig4.1) (Groen et al 1951, Louis and Conn 1956) the mechanism of their action remains in doubt. It is generally believed that GI and GE act on aldosterone (type I) receptors causing Na^+ retention and hypokalaemia. This is based on only two receptor studies; the first from Corvol's group in Paris which showed that GE but not GI had an affinity of $1/15000^{th}$ that of aldosterone for the mineralocorticoid receptor (Ulmann et al 1975). In 1983 Funder's group reported that both GI and GE had affinity for the type I receptor with affinities of 1/50,000 and 1/10,000 the affinity of aldosterone for such sites respectively (Armanini et al 1983). They argued that although low, this affinity becomes significant because of the large amounts of GI/GE needed to reproduce the syndrome. However with smaller doses now reported to cause mineralocorticoid effects, this is no longer tenable. Consider also that the affinity of cortisol for the type I receptor is only $1/100^{th}$ the affinity of aldosterone.

There were several other important clues that suggested that inhibition of 11β-OHSD might explain the pathogenesis of liquorice induced hypertension:-

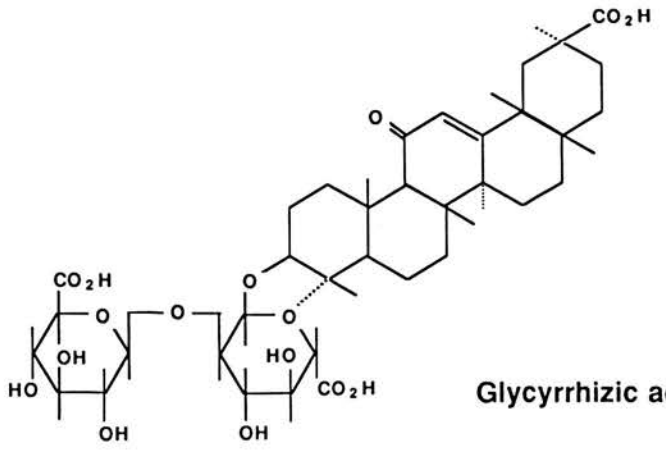
Figure 4.1 The structures of the active mineralocorticoids in liquorice, and their similarity to that of carbenoxolone.



Carbenoxolone



Glycyrrhetinic acid



Glycyrrhizic acid

1. Several early studies indicated that liquorice was ineffective in subjects with Addison's disease. Borst in 1953 concluded that the "deoxycortone-like action of liquorice, always present in people with intact suprarenal glands, was absent in three patients with Addison's disease". However 10 mg cortisone given daily to these patients restored the mineralocorticoid effect fully (Borst et al 1953). Similar conclusions were made by other workers in patients with both Addison's disease (Molhuysen et al 1950, Elmadjian et al 1956), and bilateral adrenalectomies (Hudson et al 1954).

Two patients have been reported with Addison's disease who could be maintained on liquorice extracts without becoming mineralocorticoid deficient (Groen et al 1952, Cotterill et al 1973). However such patients may have persisting adrenocortical cortisol production, and it is of interest to note that such a phenomenon has never been reported in patients with bilateral adrenalectomies (who have no residual cortisol production).

This human work has been backed up by animal data; liquorice having no biological effect in adrenalectomised rats (Card et al 1953, Girerd et al 1960).

Although early studies, these point strongly to the requirement of functional adrenal tissue and/or the presence of glucocorticoids for liquorice to possess mineralocorticoid activity, and argue against affinity for the type I receptor (which would presumably be unaltered in Addison's disease or adrenalectomised patients) being the predominant mechanism.

2. An apparent change in the renal handling of cortisol occurs in subjects consuming liquorice. It was shown in 1978 in 10 of 13 volunteers who consumed 100-200 g of liquorice/day, that, although there was no change in plasma cortisol on liquorice, urinary free cortisol doubled (Epstein et al 1978). The urinary metabolites of cortisol and cortisone were measured (tetrahydrocortisol and tetrahydrocortisone) and although there was no significant change in them per se, the THF:THE ratio (not mentioned by the authors) doubled during liquorice administration, which suggests 11β-OHSD inhibition (Table 4.1).

3. Finally, dexamethasone has been shown to have antimineralocorticoid activity in subjects given GE (Hoefnagels and Kloppenborg 1983). They gave 500 mg of GE/day to 4 normal volunteers and 2 patients with adrenal insufficiency for a total of 27 days whilst on a fixed Na^+/K^+ intake. For the last 10 days dexamethasone 2 mg/day was given. All subjects had a marked rise in urinary Na^+/K^+ ratio within 3 - 4 days of taking dexamethasone. However, in the two patients with adrenal insufficiency cortisone was given in an equipotent dose to 2 mg dexamethasone and this had no antimineralocorticoid activity. They concluded that the antimineralocorticoid activity of dexamethasone in this condition was achieved by suppression of cortisol production.

Thus as shown in Table 4.2 there are many similarities between 11β-OHSD deficiency and liquorice induced mineralocorticoid excess. The following experiments were designed to evaluate the effect of liquorice on 11β-OHSD activity, and took the form of <u>in vitro</u> and <u>in vivo</u> studies in both rat and man.

Table 4.1. Urinary free cortisol, THF and THE in 7 volunteers consuming liquorice (≈700 mg glycyrrhizic acid/day) for one week

	Unconjugated cortisol ug/24 h	THE mg/24 h	THF mg/24 h	THF:THE ratio
Control week	95 ± 46	1.4 ± 0.5	1.9 ± 0.6	1.3
1 week of liquorice	304 ± 166	1.1 ± 0.4	2.7 ± 1.4	2.5

n = 7 (mean ± s.d) From Epstein et al 1978.

Table 4.2

Similarities Between 11ß-OHSD Deficiency and Liquorice Excess			
		11ß-OHSD deficiency	Liquorice excess
"Mineralocorticoid" hypertension	Na^+ retention	✓	✓
	Hypokalaemia	✓	✓
	↓ Plasma renin activity	✓	✓
	↓ Plasma aldosterone	✓	✓
Response to Spironolactone		✓	✓
↑ urinary free cortisol		✓	✓
↑ THF: THE ratio*		✓	✓
"Antimineralocorticoid" action of Dexamethasone		✓	✓
* THF – tetrahydrocortisol, THE – tetrahydrocortisone } Principal urinary steroid metabolites of cortisol and cortisone			

In vitro experiments

A human kidney was obtained from a cadaveric donor, the transplant not proceeding for technical reasons. A collagenase/hyaluronidase cortical tubular preparation was used as described in method 1 in chapter 3. As shown in fig 4.2 conversion of F to E in the control incubates (n=3) was 76.1 ± 1.0% (mean ± SEM), increasing to 81.0 ± 0.4% when NADP (4μM) was added. GE when added in a final concentration of 10^{-4}M, 10^{-6}M and 10^{-8}M reduced conversion to 43.3 ± 1.3, 66.4 ± 1.8% and 74.6 ± 1.9% respectively. Carbenoxolone produced a similar though not as marked effect. Similar findings in vitro were seen when collagenase dispersed renal tubules from the rat were incubated with corticosterone (fig 4.3).

In vivo experiments

18 male Wistar rats of the same age and weighing between 150-180 g were divided into 3 groups:

Group A received 0.5 ml distilled water b.d. by gavage (control)

Group B received 50 mg/kg glycyrrhizic acid in dist. water b.d.

Group C received the same as group B plus dexamethasone 10μg b.d.

The rats were given a normal chow diet (0.3% Na^+) with unrestricted fluids, the experiment running for 11 days. Blood pressure was measured in all rats using the tail cuff method on days -1 and 0 and on days 10,11 of the experiment (tail-cuff plethysmography and oscilloscope purchased from Harvard apparatus). These measurements were carried out in triplicate between 09.00h - 11.00h by the same observer. Weight (g), fluid intake (ml) and food consumption were

Figure 4.2 Conversion of cortisol to cortisone by a human renal tubular preparation incubated with NADP, Glycyrrhetinic acid and Carbenoxolone.

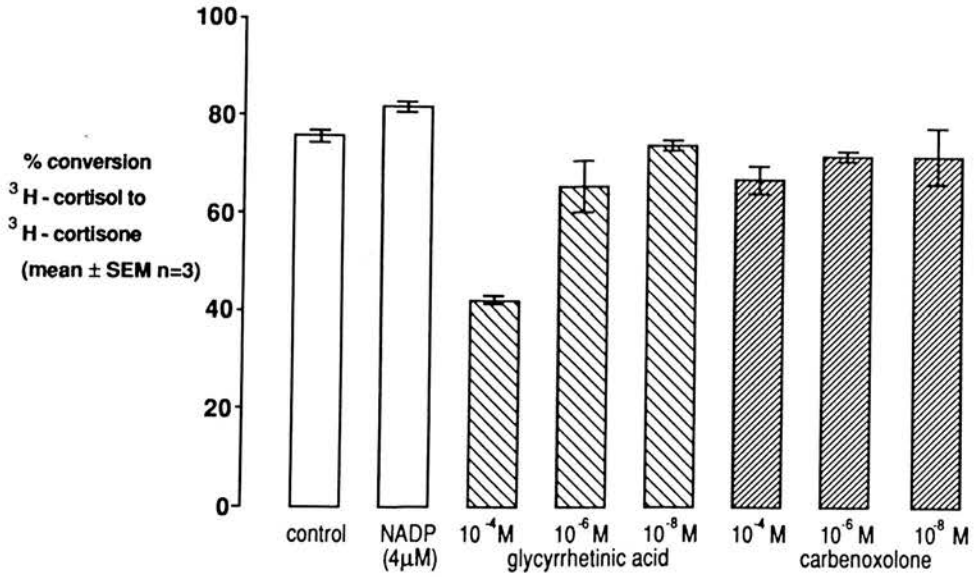
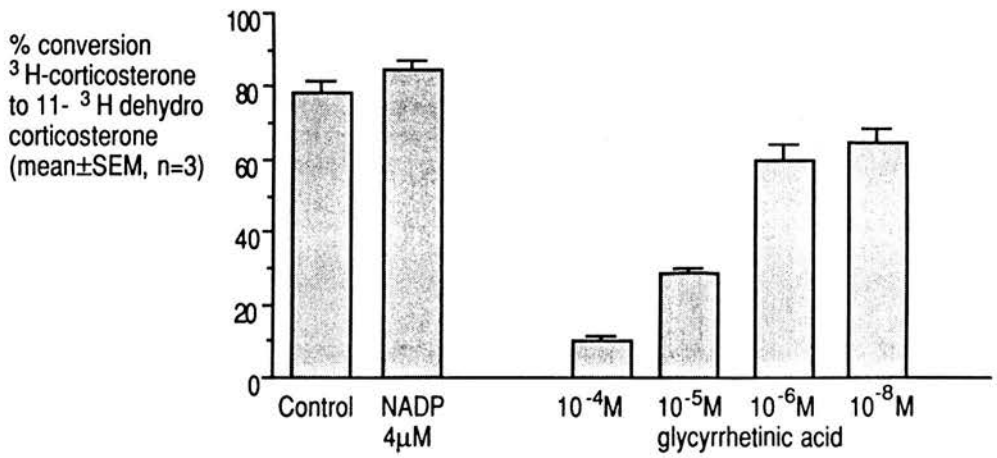


Figure 4.3 **Conversion of corticosterone to 11-dehydrocorticosterone by a rat renal tubular preparation incubated with NADP and Glycyrrhetinic acid.**



recorded daily in each rat. On day 12 at 09.00h the rats were killed by decapitation at which time blood was taken for estimation of corticosterone (Al-Dujaili et al 1981) and plasma renin activity (Haber et al 1969, assay procedure described in chapter 2). The renal cortices were immediately removed and processed according to method 2 in chapter 3 (i.e. using a protein assay (Bio-rad) on an homogenate preparation). Using a 1 in 4 homogenate dilution from each rat, incubation was carried out with 10^{-8}M ^{3}H-corticosterone (Amersham International 84 Ci/mmol) for 60 min at 37°C with and without the addition of NADP in a final concentration of 4μM (as used previously in similar experiments (Bernal 1980)). The steroids were extracted from the incubates as described in chapter 3 and the conversion of ^{3}H-corticosterone to ^{3}H-11 dehydrocorticosterone expressed as a percentage.

Results

a) With no NADP added conversion in the three groups was as follows;

Group A: 35.5 ± 2.9% (mean ± s.d. of conversion in 6 rats performed in triplicate i.e. 18 values).

Group B: 29.4 ± 2.8%

Group C: 29.6 ± 3.5%

Statistical analysis using the unpaired Student's "t" test showed significant inhibition of renal 11β-OHSD activity in the rats receiving GI (A vs B, $p<0.005$. A vs C, $p<0.01$).

b) With NADP (4μM) added to each incubate conversion was as follows;

Group A: 67.9 ± 1.8%

Group B: 67.4 ± 2.1%

Group C: 69.3 ± 1.7%

There was no significant difference in conversion between these three groups.

Weight change, plasma renin activity (PRA) and corticosterone (B) in groups A,B and C (mean ± s.d.)

	Weight change (g)	PRA (ng/ml/h)	B (ng/ml)
Group A	+ 50.7 ± 6.9	9.1 ± 2.7	102.5 ± 90.6
Group B	+ 48.3 ± 2.9	11.0 ± 3.0	63 ± 105.6
Group C	- 34.1 ± 8.9	19.4 ± 12.2	10.4 ± 8.2
		n.s.	A vs C $p<0.05$

Systolic blood pressure did not alter in any of the groups across the 12 day study period (139 ± 8 to 142 ± 6 mmHg, Group A, 147 ± 8 to 144 ± 6, Group B, 141 ± 7 to 143 ± 7, Group C). Water consumption was similar in all groups, but the rats treated with dexamethasone ate less food and failed to grow.

Conclusions

1. Glycyrrhetinic acid when added in vitro in concentrations similar to those measured in the plasma after liquorice ingestion to a renal cortical tubular preparation produced a marked inhibition of 11β-OHSD activity in a dose-dependant manner.

2. Glycyrrhizic acid, an active "mineralocorticoid" in liquorice inhibited renal 11β-OHSD activity in vivo in the rat. There was no increase in blood pressure or suppression of PRA in the rats given GI, which may not be surprising in view of the short duration of this experiment and the small doses of GI used. Previous workers in this area have used doses of 100 mg/kg (Girerd et al 1960).

3. Dexamethasone, given in doses sufficient to suppress corticosterone levels, had no direct action on enzyme activity. Thus its known antimineralocorticoid action in liquorice induced mineralocorticoid excess (Hoefnagels 1983) does not appear to be mediated through a direct effect on 11β-OHSD action (It has previously been demonstrated that dexamethasone has no direct action on 11β-OHSD activity in the rabbit (Lugg et al 1978)).

4. NADP when added in in a final concentration of 4μM; a) produced a doubling in conversion in the control group, confirming the dependancy of the 11β-dehydrogenase enzyme on this co-factor and, b) abolished any inhibitory effect seen with GI. This may suggest that GI inhibits 11β-OHSD by inhibiting NADP generation. However, inhibition of enzyme activity was still seen in our in vitro tubular preparation, which, unlike the homogenate preparation has an intact redox potential and normal co-factor concentrations. Although this concentration of NADP

(i.e. 4μM) has been used previously in similar homogenate incubates (Bernal 1980), it seems likely that it was added in excess.

The effect of liquorice ingestion on 11β-OHSD activity in man

Materials and Methods

Local Ethical Committee approval was obtained for this study. Seven normal male volunteers (mean age 30.1 ± 1.9 yr) on no medication were established on a fixed Na^+/K^+ diet (130 mmol Na^+, 80 mmol K^+) with a caloric intake similar to their estimated normal diet. Following a run-in period to achieve metabolic balance, confectionery liquorice 200 g/day (containing 580 mg glycyrrhizic acid) was given in divided doses for a total of 10 days (courtesy of Bassetts, Sheffield). 24 hour urine collections were made daily and analysed for Na^+, K^+ and creatinine, aldosterone and urinary free cortisol. On days -1 (the last day of the run-in balance), 4 and 10 of liquorice ingestion, the principal urinary cortisol and cortisone metabolites were measured by capillary column gas liquid chromatography (GLC) (Wallace et al 1987) i.e. (THF), allo-tetrahydrocortisol (allo-THF) and tetrahydrocortisone (THE). On days -1, 3, 5, 7 and 11 of the study, the volunteers lay supine for at least 30 minutes at 08.30 hours. Three blood pressures recordings were made using an automatic Copal sphygmomanometer, following which blood was taken for Na^+, K^+, plasma renin activity and cortisol estimation.

Plasma glycyrrhetinic acid was measured in all subjects before liquorice (day -1) and on day 11 with the subject having taken no liquorice for the preceeding 10 hours. The method used was the GLC assay described for carbenoxolone sodium (the hemisuccinate ester of

GE) (Rhodes 1974), except that standards of 18β-GE (0, 10, 20, 30, 40, 50, 60 μg/ml) were used in place of β-carbenoxolone standards. Similarly 18α-GE (25μg/ml) replaced α-carbenoxolone as an internal standard. The detection limit of the assay was 1 μg/ml.

Plasma renin activity and urinary aldosterone were measured by existing radioimmunoassays (Haber et al 1969, Al-Dujaili and Edwards 1981b, assay procedure described in chapter 2), plasma and urinary cortisol by a recognised radioimmunoassay proceedure adapted from that described by McConway (McConway and Chapman 1986).

In 3 subjects the metabolism of [11α^{3}H]-cortisol was studied before and 7 days after liquorice 200 g/day. [11α^{3}H]-cortisol was synthesised according to the method of Hellman (Hellman et al 1971 and see chapter 2) (specific activity 14.6 Ci/mmol). With the subject fasted overnight 500 ml water was given orally at 8.30 am. 0.7 mg [11α^{3}H]-cortisol was given intravenously as a bolus in 15 ml sterile water. Plasma was collected at regular intervals for 120 min, urine at time 0, 60 and 120 min. Each sample was counted for ^{3}H on a Packard Tricarb 4330 series beta counter (after correction for quench) to a counting error of <2%. Cocktail T (BDH Chemicals) was used as a scintillant. Samples were counted for total ^{3}H ([11α-^{3}H]-cortisol and ^{3}H-$H_2$0) and, following sublimation for ^{3}H-$H_2$0. By subtracting the counts of ^{3}H-$H_2$0 from total ^{3}H a half-life for [11α^{3}H]-cortisol could be established. This was seen to consist of a distribution and elimination phase but only the latter, representing cortisol metabolism was expressed i.e. sampling times 45 to 120 mins. The isotope study repeated on liquorice was performed with the subject having consumed no liquorice since the previous day (approx. 10 hr).

100 g of liquorice was analysed for Na^+/K^+ after prolonged digestion and found to contain 3.5 mmol and 15 mmol/100 g respectively. This was subtracted from the urinary Na^+ and K^+ results when plotting Na^+ and K^+ balance. Faecal electrolytes were not measured; however, no subject had a change in bowel habit during the study.

Statistical analysis was performed using Student's paired 't' test. All results are expressed as mean $\pm$ SEM.

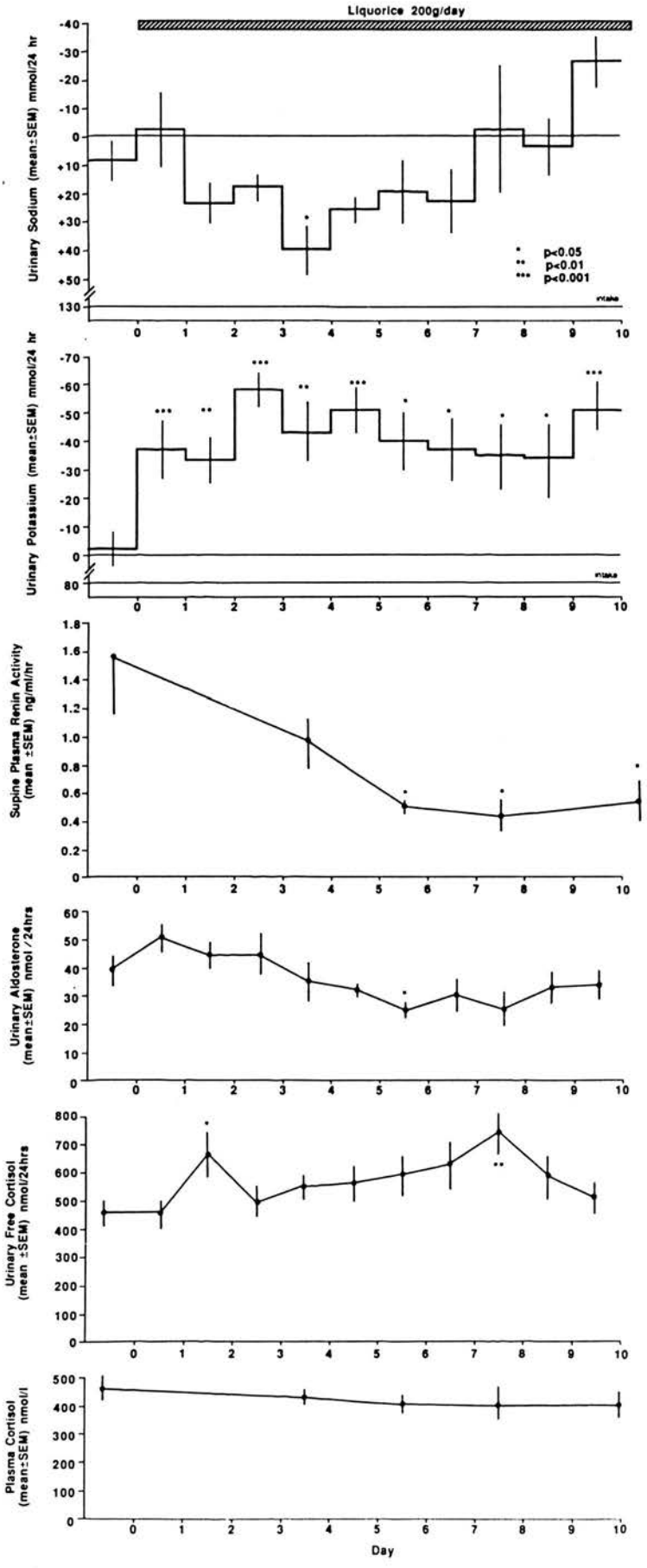
Results

Metabolic Balance Data

All subjects achieved metabolic balance after a 5 day run-in period apart from one subject who came into balance after 6 days. Subsequent liquorice administration from day 0 for 10 days (fig 4.4) produced sodium retention in all subjects. In the group as a whole significant sodium retention occurred by day 4 (urinary Na^+ 90.4 ± 8.3 vs 121.4 ± 6.7 on day -1, $p<0.05$), with a positive sodium balance maintained until day 7. However by days 8-10, 6 out of the 7 volunteers appeared to be in mineralocorticoid escape. All subjects had a marked kaliuresis producing negative potassium balance. When analysed as a group this was highly significant on each day of liquorice ingestion. Plasma potassium fell in all subjects; in the group as a whole it fell from 4.11 ± 0.2 to 3.69 ± 0.1 (day 11) $mmol\,l^{-1}$ ($p=0.06$).

Associated with these changes we demonstrated significant suppression of the renin-angiotensin-aldosterone system by day 6 of

Figure 4.4 The effect of liquorice on urinary Na^+, K^+, the renin-angiotensin-aldosterone system and cortisol levels in seven normal volunteers on a fixed Na^+/K^+ diet.



liquorice ingestion. Supine plasma renin activity (ref. range 0.5-1.5 ng/ml/h) fell from 1.57±0.4 (day -1) to 0.51±0.06 (day 6) ($p<0.05$), urinary aldosterone (ref. range 15-55 nmol/24 h) from 40.1±4.7 to 25.9±2.9 nmol/24 h ($p<0.05$). There was no significant change in either the volunteers' weight or blood pressure during this short period of liquorice administration.

Plasma glycyrrhetinic acid levels were less than 1 μg/ml (limit of detection for the GLC method) in all volunteers when measured on the last day of the run-in balance and after 10 days of liquorice ingestion.

Steroid Metabolites

Urinary free cortisol increased in all subjects whilst taking liquorice. This reached significance on day 2 ($p<0.05$) and day 8 ($p<0.01$) of liquorice ingestion. Despite this, 0900 h plasma cortisol showed no change throughout the study.

The results of the urinary steroid metabolites are shown in Table 4.3. The ratio of allo-THF + THF: THE increased in all subjects from basal values (day -1), reaching significance when measured on day 4 ($p<0.05$) and day 10 ($p<0.01$) of liquorice ingestion. In addition the allo-THF: THF ratio increased during liquorice ingestion. Although this did not reach significance, this altered ratio with a preference to form allo-THF as opposed to THF, is well described in 11β-OHSD deficiency states (Monder et al 1986).

Table 4.3. Urinary steroid ratios during liquorice ingestion.

THF + allo THF/THE ratio

Subject	Day -1	Day 4	Day 10
1	0.96	0.97	1.30
2	0.60	0.71	0.70
3	0.95	1.03	1.08
4	1.17	1.50	1.47
5	0.91	1.05	1.04
6	0.84	0.91	1.05
7	1.04	1.52	1.62
Mean ± SEM	0.92 ± 0.07	1.10 ± 0.11	1.18 ± 0.12
		$p<0.05$	
			$p<0.01$

allo THF/THF ratio

Subject	Day -1	Day 4	Day 10
1	0.51	0.57	0.53
2	0.26	0.32	0.28
3	0.76	0.77	0.98
4	0.89	1.24	1.20
5	0.72	0.80	0.57
6	0.62	0.71	0.86
7	0.97	0.99	1.47
Mean ± SEM	0.67 ± 0.09	0.77 ± 0.11	0.84 ± 0.16
	n.s		n.s

Isotope Study

As shown in fig 4.5. the plasma half-life of [11α^{3}H]-cortisol was doubled whilst taking liquorice in all 3 volunteers studied (84.3 ± 5.0 min vs 40.7 ± 0.7 min). In addition, the percentage of total ^{3}H excreted in the urine as ^{3}H-H_2O fell from 27.7 ± 0.3% to 12.3 ± 0.9% one week after liquorice.

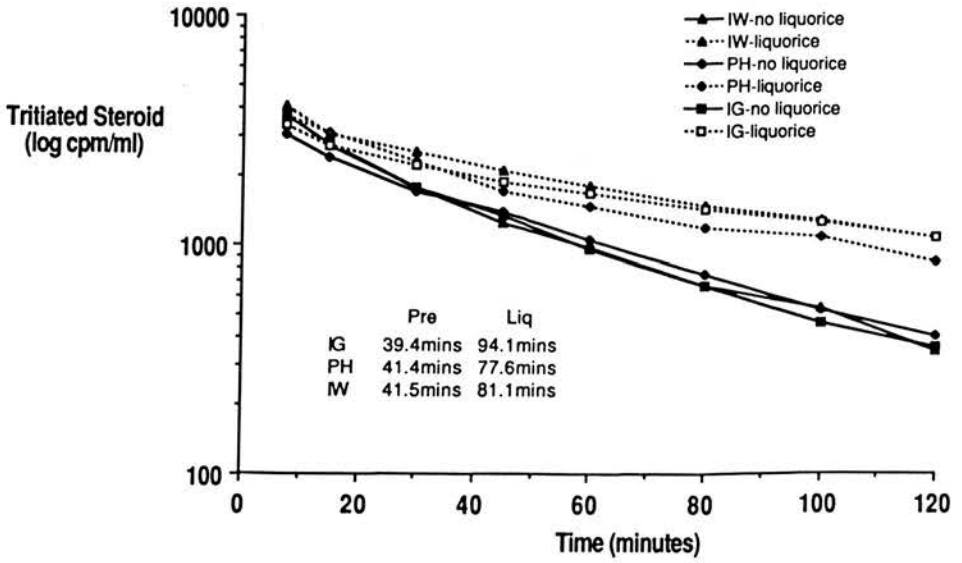
Discussion

This study confirms the well known mineralocorticoid effect of liquorice. With 7 normal volunteers on a fixed Na^+/K^+ intake it produced significant Na^+ retention and a marked kaliuresis with suppression of the renin-angiotensin-aldosterone axis.

Plasma glycyrrhetinic acid levels were less than 1 μg/ml 10 hours after consuming liquorice. It has previously been reported using more sensitive methodology that subjects consuming 100-200 g/day of liquorice had GE levels of 80-480 ng/ml (Hughes 1977). These levels of GE, the active principal of liquorice, are much lower than would be required to displace aldosterone as calculated from the *in vitro* mineralocorticoid receptor studies (Ulmann et al 1975, Armanini et al 1983), and in the face of obvious mineralocorticoid activity, suggests that a direct action on the type I receptor cannot be responsible for its effect.

The administration of liquorice was associated with a marked disturbance in the metabolism of cortisol and a significant increase in urinary free cortisol despite no change in plasma levels; the latter has previously been shown by Espiner's group in New Zealand

Figure 4.5 The metabolism of [11α^{3}H]-cortisol in three volunteers before and 7 days after liquorice (200 g/day). The half-lives are shown in minutes.



(Epstein et al 1978). In our study the urinary THF + allo THF: THE ratio increased in all subjects when measured on day 3 and 10 of the study, this pattern being diagnostic of 11β-OHSD deficiency. Similarly there was an increase in the 5α urinary cortisol metabolite (allo THF) compared to the 5β (THF) cortisol metabolite, indicated by a rise in the allo-THF: THF ratio. Impaired activity of 5β reductase is known to co-exist with 11β-OHSD deficiency and was observed with our index case described in chapter 2 (Monder et al 1986).

This inhibitory effect was confirmed further in the three volunteers in whom the metabolism of [11α^{3}H]-cortisol was studied. When acted on by 11β-OHSD, cortisone and ^3H-H_2O are produced; thus the half life of [11α^{3}H]-cortisol reflects the action of the 11β-dehydrogenase component of the 11β-OHSD enzyme complex responsible for cortisol - cortisone conversion. Although it is known that insertion of ^3H at the C_{11} position of cortisol produces an isotope effect (Hellman et al 1971), we can see no reason why this should change between the two studies in our 3 volunteers. An increase in the plasma half life of [11α^{3}H]-cortisol from a mean of 41 min to 83 min is indicative of 11β-OHSD inhibition. This is in keeping with our in vitro and in vivo animal studies showing inhibition of renal 11β-OHSD by glycyrrhetinic and glycyrrhizic acid described above.

11β-OHSD deficiency has previously only been described in its congenital form, resulting in often fatal low renin, low aldosterone hypertension with severe hypokalaemia. Our observations from investigating the first adult case (chapter 2) have lead us to postulate that renal 11β-OHSD is a critical physiological mechanism determining the distal tubule exposure to cortisol. The enzyme can be

seen as a protective shuttle mechanism converting active cortisol to the inactive cortisone. Inhibition or deficiency of this shuttle results in raised intrarenal glucocorticoid concentrations which saturate renal extravascular cortisol binding globulin thus increasing free cortisol levels which act on the type I mineralocorticoid receptor. The human studies presented here of the mineralocorticoid excess state seen with liquorice ingestion have added further weight to our hypothesis regarding this mechanism, and show for the first time, that 11β-OHSD deficiency may be relevant in mineralocorticoid excess states other than that occurring in a small number of hypertensive children.

CHAPTER 5 11βETA HYDROXYSTEROID DEHYDROGENASE AND 'ESSENTIAL' HYPERTENSION

In 95% of cases no cause is found to account for a patient's hypertension, and they are then said to suffer from 'essential' hypertension. Despite a vast amount of research in the field of hypertension, probably more than any other disease process, the pathophysiology of the condition remains unclear.

Following on from the results found in liquorice induced hypertension and the finding of a possible deficiency of 11β-OHSD in GB's mother PB (who until then had been labelled as having essential hypertension (see chapter 2)), there was a clear need to explore the role of 11β-OHSD, more specifically, renal 11β-OHSD in essential hypertension. In addition impressive evidence was emerging implicating the primacy of the kidney in the pathogenesis of hypertension, which could possibly be mediated through a partial defect in renal 11β-OHSD.

If a defect in renal 11β-OHSD was to be important in the pathogenesis of hypertension then one might expect to see the following documented observations in an early or pre-hypertensive population;

a) Evidence suggesting a primary role for the kidney in the development of hypertension
b) Suppression of the renin-angiotensin system
c) An expanded extracellular and/or blood volume consequent upon sodium retention
d) Hypokalaemia

e) A disturbance in the peripheral metabolism of cortisol, with evidence to suggest an aetiological role for cortisol.

What evidence is there, if any, to support these observations?

a) The "primacy of the kidney" in the pathogenesis of essential hypertension.

In 1919 Fahr first suggested that a primary kidney defect might be a cause of essential hypertension (Fahr 1919). For many years this 'defect' was argued to be renal ischaemia, expanded by the now classical experiments of Goldblatt (Goldblatt 1958). This became less popular as studies repeatedly failed to show changes in renal blood flow when patients were compared with controls.

More recently the central role of the kidney in the development of hypertension has been argued through the lucid hypotheses put forward by many cardiovascular physiologists, notably Borst and Guyton.

Borst has applied Starling's laws of fluid balance and circulatory homeostasis (Patterson and Starling 1914) to essential hypertension, and has hypothesised that hypertension is an integral part of of a homeostatic reaction to deficient sodium excretion (Borst and Borst-de-Geus 1963). When sodium output is insufficient at a normal arterial pressure, accumulation of extracellular fluid raises blood pressure to an abnormally high level to re-establish sodium balance. Thus deficient sodium excretion must be a pre-requisite in essential hypertension, "normal" sodium balance being maintained at the expense of hypertension.

Guyton has expanded this argument in the light of experiments performed by himself on isolated kidney preparations. As arterial pressure rises the amount of urine and electrolytes excreted by the kidney increases markedly, a phenomenon called pressure diuresis (Thompson and Pitts 1952). Thus when a patient's arterial pressure is too high, excess salt and water is excreted until the pressure falls back to a level to stop the pressure diuresis. This 'steady state' pressure will vary from individual to individual depending upon the state of the kidney, but in normals is set to maintain a mean arterial pressure of say 100 mmHg. Guyton argues that the usual factors of paramount importance in the acute regulation of blood pressure i.e. peripheral resistance, cardiac output and venous compliance cannot be applicable to the long-term control or genesis of hypertension since these would merely acutely elevate blood pressure which would then normalise via the renal-fluid volume mechanism described above. Similarly an acute increase in extracellular fluid will not elevate blood pressure because the baroreceptor system and the renin-angiotensin vasoconstrictor system will prevent this. However over a long time period the renal-fluid volume mechanism becomes overridingly dominant in controlling blood pressure (Guyton et al 1972). Hence if hypertension is to occur it must be due to a defect in this renal fluid mechanism caused by an intrinsic defect within the kidney. Furthermore, invoking autoregulatory cascade mechanisms, Guyton has calculated that this chronic increase in extracellular fluid need only be in the order of 150 ml to cause a resultant marked increase in peripheral resistance (Guyton 1977). These two hypotheses have formed the autoregulatory theory of essential hypertension.

Many workers in the field do not agree with this hypothesis, arguing that pressure diuresis, whilst occurring in an isolated kidney preparation, does not occur in an intact innervated kidney. Without expanding on this in too much detail, the most current opinion, following studies in patients with autonomic neuropathy (Schalekamp et al 1985), suggests not only that pressure diuresis is present in man, but also confirms that the basis of the autoregulatory theory is correct (i.e. the increased vascular resistance in hypertension is consequent upon abnormal renal sodium handling).

More conclusive studies have emerged since the early 1970's suggesting a primary role for the kidney in hypertension. In 1973-1974 Giuseppe Bianchi's group in Milan clearly showed that hypertension 'followed the kidney'. Using the genetically hypertensive Bianchi-Milan rat and its genetic normotensive control this phenomenon was shown in a series of cross transplantation experiments i.e. transplantation of a kidney from an hypertensive animal caused a higher blood pressure in the recipient than transplantation of a control kidney. The converse was also true with a fall in blood pressure when an 'hypertensive' rat received a kidney from the genetic normotensive strain (Bianchi et al 1973, 1974 and 1979). Moreover this effect was present even when the kidney was removed from the rat in the pre-hypertensive stage (<26 days) excluding secondary changes in the kidney. This is also the case in at least two other rat models of genetic hypertension, the Dahl hypertensive rat (Dahl and Heine 1974), and the Wistar spontaneously hypertensive rat (SHR) (Kawabe et al 1978).

Similar though more indirect evidence is now available from human studies. In a 2 year retrospective study 50 cadaveric kidney recipients were analysed by Bianchi's group, with respect to the presence or absence of hypertension in the family of both donors and recipients. Recipients from normotensive families had higher blood pressures and required more antihypertensive therapy ($p<0.01$) if they received a kidney from a donor with a hypertensive family than those receiving a kidney from a donor from a normotensive family (Guidi et al 1985). This work is supported by another recent retrospective Danish study. Thirty seven cadaveric kidney donors who died from subarachnoid haemorrhage had a higher blood pressure than found in 41 donors dying from cerebral tumour or head injury. In 23 recipients with normal graft function, blood pressure and antihypertensive requirement was significantly greater in recipients receiving kidneys from donors dying of subarachnoid haemorrhage than cerebral tumour/head injury ($p<0.004$) (Strandgaard and Hansen 1986). Both of these retrospective studies support Bianchi's animal work that a cadaveric kidney obtained from either an hypertensive or from a donor with hypertensive parents has a defect which may cause hypertension in the recipient. As with the genetic experimental models the reverse also appears to be true in man. Curtis, studying albeit only 6 patients, in whom essential hypertension had resulted in nephrosclerosis and renal failure, followed these subjects for a mean of 5 years post transplantation from normotensive donors. All 6 subjects remained normotensive (Curtis et al 1983).

b) Suppression of the renin-angiotensin system.

Research in hypertensive patients is always plagued by the contribution made by primary changes in physiological processes and

those arising from secondary hypertensive changes. Bianchi's group have to some extent overcome this by studying a pre-hypertensive population i.e. siblings from hypertensive parents, compared with siblings from normotensive parents. Comparing 65 of the former with 56 of the latter, they showed a significant reduction in supine PRA in the siblings from hypertensive parents (1.56 $\pm$ 0.03 (SEM) vs 0.79 $\pm$ 0.07 ng/ml/h, $p<0.01$) (Bianchi et al 1983, Bianchi and Barlassina 1983). Other smaller studies (n=15) have shown similar findings without reaching significance (Hollenberg et al 1981, Wiggins et al 1978). Similar findings are reported in early hypertensives who have never received medication (Thomas et al 1978).

Whilst as a group therefore it would appear that there is suppression of the renin angiotensin system, not all hypertensives have suppressed renins. Although not evaluated in either early or pre-hypertensives, established hypertensives can be divided into 3 groups (when compared with normals); low renin (25-30%) (LRH), high renin (20-25%) and normal renin (45-55%) (Helmer 1965, Ganguly and Weinberger 1979).

c) Increased extracellular/blood volume and exchangeable sodium.

In the majority of studies, no increase in body fluids has been documented in hypertension (Hollander et al 1961, Schalekamp et al 1977, London et al 1977); indeed some studies report an inverse relation between blood pressure and blood volume (Tarazi 1976), suggesting that patients with hypertension have contracted extracellular fluid volumes. In the young hypertensive exchangeable sodium tends to be below normal (Beretta-Piccoli et al 1982). However

if Guyton's hypothesis described above is true, then the changes required may be so small as to be missed by our relatively insensitive methods. Moreover there is evidence to suggest that the hypertensive sub-group with low renins do have evidence of expanded extracellular volumes with significant elevation of total exchangeable sodium (Jose et al 1970, Woods et al 1969). Even when these measurements have not reached statisical significance, they still appear to be higher in this sub-group than normals (Bauer and Brooks 1979, Distler et al 1975, Padfield et al 1975, Davies et al 1979) (Table 5.1). Such a sub-group of hypertensives also appear to be more sensitive to the hypotensive effect of diuretics (Ganguly and Weinberger 1979), suggesting a marked dependency upon sodium.

d) Hypokalaemia

Only 0.4% of the total body potassium of 3500 mmol exists as plasma potassium (Edelman and Leibman 1959). As plasma potassium is therefore a poor reflection of potassium status workers have assessed total body potassium using isotopic dilution techniques. In two such studies there was no significant difference between hypertensives and controls (Schalekamp et al 1977, Davies et al 1979). The data from Davies et al is depicted in table 5.1. As shown, although the changes were not significant, hypertensives did have a lower total body potassium. Also in a large study looking at a heterogenous group of over 90 hypertensives, plasma and total body potassium was found to correlate inversely with arterial blood pressure especially in the younger hypertensive ($r=-0.51$, $p<0.001$, $n=69$) (Beretta-Piccoli et al 1982).

Table 5.1 Plasma electrolytes, exchangeable sodium and potassium in hypertensives and controls (Davies et al 1979).

Results expressed as mean ± SEM. Number of observations in parentheses.

	NORMALS	ESSENTIAL HYPERTENSIVES	
		normal renin	low renin
Mean age (yr)	32.6	43.8	50.9
Plasma Na^+ (mmol/l)	138.9 ± .2 (98)	140.0 ± .3 (91)	140.0 ± .4 (38)
Exchangeable Na^+ (% normal)	98.6 ± 1.1 (23)	100.4 ± 1.3 (47)	100.1 ± 2.5 (14)
Plasma K^+ (mmol/l)	4.15 ± .04 (96)	4.10 ± .04 (91)	4.00 ± .06 (39)
Exchangeable K^+ (% normal)	109.2 ± 1.3 (21)	106.6 ± 1.5 (43)	100.6 ± 1.6 (12)

e) Cortisol metabolism in hypertension

Cortisol metabolism in hypertension has received little attention. Kornel in the early 1960's showed that urinary 17-OH corticosteroids were unchanged in hypertensives (Kornel 1960). Plasma levels of the principal adrenocortical hormones are also normal basally, though some workers, following stimulation of the system with ACTH, have shown a disproportionate rise in 11-deoxycortisol and deoxycorticosterone in hypertensives suggesting a partial 11β-hydroxylase deficiency (Honda et al 1977, Simone et al 1985). As we have shown, "normal" circulating levels of corticosteroids do not imply that the metabolism of such steroids is normal and hence do not exclude the possibility that the steroid might be implicated in hypertension. Evidence for an aetiological role of cortisol or another ACTH dependant steroid in essential hypertension comes from a study by Hamilton (Hamilton et al 1979). In a controlled study dexamethasone given to 34 male hypertensives (0.5 mg t.i.d. for 8 weeks) produced a significant fall in blood pressure, this being associated with a marked suppression of plasma cortisol, but no change in PRA or plasma aldosterone. Similar findings were reported from an earlier study (Melby et al 1976).

From our discussion above, if 11β-OHSD was to be relevant in essential hypertension, one might expect this to be more obvious in the low-renin group (LRH). The idea of a mineralocorticoid excess state being important in LRH is not new, with aldosterone (Grim 1973), deoxycorticosterone (Tan et al 1975), 18 hydroxydeoxycorticosterone (Melby et al 1971), and 16β-OH dehydroepiandrosterone (Liddle and Sennet 1974) being just some of the steroids implicated. However none of these steroids have consistently been shown to be relevant to the

pathogenesis of the condition (Ulick 1978). Other authors have argued that LRH is not a distinct entity, but merely an age related evolution of the hypertensive process, LRH being found more commonly in older hypertensives (Tuck et al 1973, Padfield et al 1975).

To further evaluate 11β-OHSD in this area I firstly used the genetically hypertensive Bianchi-Milan rat. This is the best hypertensive animal model available, having its own genetically identical normotensive strain (unlike the Wistar SHR). There are many similarities in the development of hypertension between this model and man (Bianchi and Barlassina 1983), and, as discussed above there seems little doubt that the hypertension is mediated through a renal mechanism. Isotopic studies in hypertensive patients and normal controls were performed in addition to the animal experiments.

A. STEROID METABOLISM IN THE BIANCHI-MILAN RAT

Hypertension in this genetic animal model develops in a well defined fashion: a) the pre-hypertensive phase until about 26 days old, b) the development of hypertension in the hypertensive strain occurring between 26-40 days and c) established hypertension after 40 days. I studied both rats with established hypertension, and to exclude secondary hypertensive changes, pre-hypertensive rats.

Materials and methods

All the rats used in these experiments were purchased from the department of Zoology, University of Sheffield (courtesy of Prof. I. Henderson). To my knowledge Sheffield is one of only two centres in

the UK with its own colonies of Bianchi-Milan rats originating from Bianchi's own strain. Two separate groups of rats were purchased: Group a) 16 rats all aged 42 days, half of which had established hypertension and Group b) 18 rats all aged 22 days, half of which had parents with established hypertension. All rats used were male.

Rats in group b) were settled in an animal unit for 4 days and then killed. Rats in group a) were divided into 4 cages (Cage 1 and 2 were hypertensive, 3 and 4 normotensive) and studied for 3 weeks prior to decapitation. Food and water consumption was recorded daily from each cage (n=4 rats), rats were weighed daily, and blood pressure measured using a tail cuff method (Harvard apparatus) on at least 2 occasions during this period. This method uses an oscilloscope to detect blood flow, picking up systolic pressure. All recordings were made between 09.00h and 11.00h. On two occasions all rats were placed in metabolic cages for 24h and urine collected. Urinary aldosterone was measured (Al-Dujaili and Edwards 1981b, assay methodology in Chapter 2).

Rats were killed by decapitation using a guillotine and processed as described in Chapter 3 using Method 2 i.e. an homogenate preparation. In addition to a renal homogenate, liver tissue was also used from each rat. Blood was recovered from the older rats at decapitation and analysed for plasma renin activity (Haber et al 1969) and corticosterone (Al-Dujaili et al 1981). Due to the size of the pre-hypertensive rats it was not possible to obtain sufficient blood for these analyses.

Results

All results are expressed as mean ± s.d. Statistical analysis was performed using Student's unpaired t test.

As shown in table 5.2 there was a significant increase in weight gain, fluid intake, aldosterone and corticosterone in the hypertensive rats. PRA was not suppressed, suggesting that the weight gain observed was not secondary to an expansion of body fluids as may occur in mineralocorticoid excess.

Fig.5.1. shows conversion of ^{3}H-corticosterone to ^{3}H-11-dehydrocorticosterone in renal homogenates from rats in group a) and b). There was no difference in renal 11β-OHSD activity between either the hypertensive or pre-hypertensive rats and their respective controls (established hypertensives 63.8 ± 4.5% vs 62.1 ± 4.4%, pre-hypertensives 36.7 ± 9.3% vs 30.7 ± 4.2%). However there did appear to be a significant reduction in hepatic 11β-OHSD activity in both the hypertensive (37.7 ± 3.9 vs 49.9 ± 6.5%, $p<0.001$) and pre-hypertensive (46.7 ± 3.1 vs 52.5 ± 5.2%, $p<0.01$) rats.

Summary

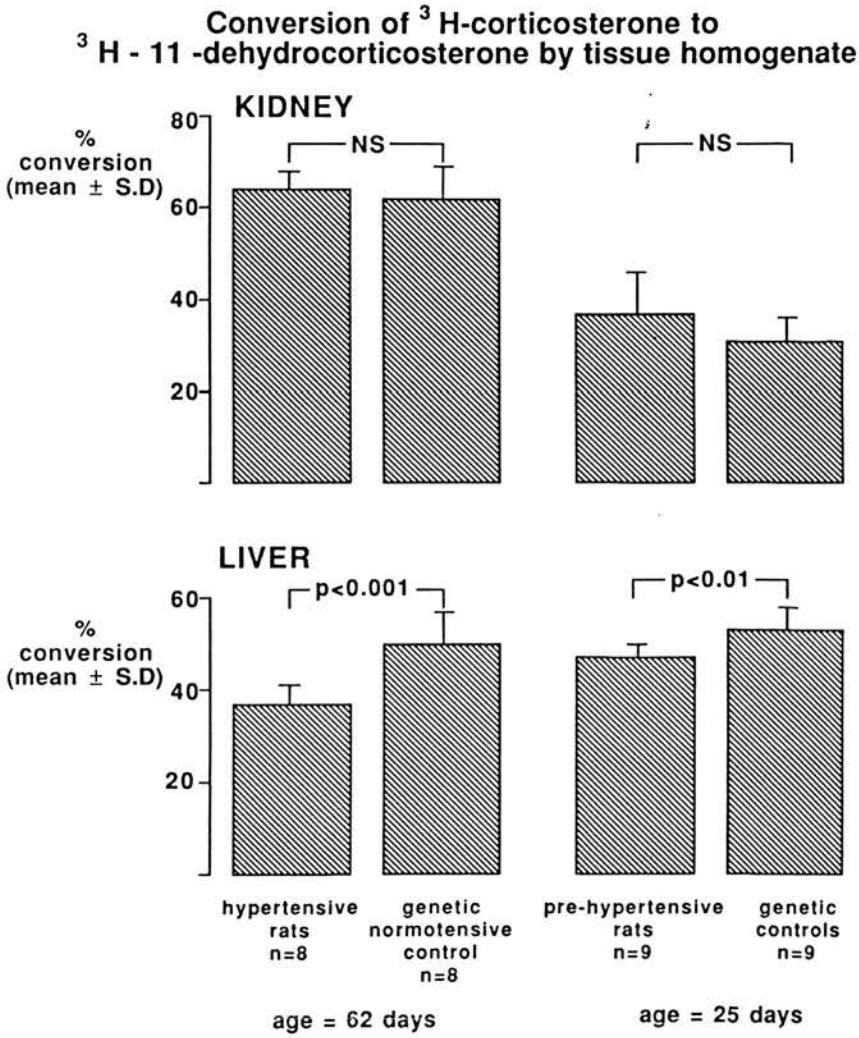
It was rather disappointing to observe such similarities in renal 11β-OHSD activity between the hypertensive rats and their genetic controls. However the finding of reduced hepatic 11β-OHSD activity in both the pre-hypertensive and established hypertensive rat is of considerable interest. Whilst this would not fit with our observations and hypotheses raised from our index case (chapter 2), it is of interest to speculate that such a defect in hepatic 11β-OHSD

Table 5.2 Metabolic data on the Bianchi-Milan rats.

Results are expressed as mean ± s.d.

	HYPERTENSIVE	NORMOTENSIVE	'p' value
Water consumption (ml/24h/cage of 4 rats)	154.1 ± 59.6	116.1 ± 41.9	<0.01
Food consumption (g/24h/cage of 4 rats)	129.2 ± 75.4	103.3 ± 57.9	ns
Urine volume (ml/24h)	7.6 ± 1.2	4.9 ± 1.8	<0.01
Systolic blood pressure (mean of 2 readings mmHg)	152.7 ± 16.7	114.9 ± 7.7	<0.001
Total weight gain (g)	99.2 ± 19.4	64.5 ±10.8	<0.001
Urine aldosterone (pmol/24h)	106.1 ± 27.1	73.8 ± 21.5	<0.01
Plasma renin activity (ng/ml/h)	0.76 ± 0.5	0.63 ± 0.5	ns
Corticosterone (ng/ml)	356.6 ± 122.5	47.0 ± 61.8	<0.001

Figure 5.1 The conversion of ^{3}H-corticosterone to ^{3}H-11-dehydrocorticosterone by kidney/liver tissue homogenate in hypertensive, prehypertensive and genetic control Bianchi-Milan rats. Each homogenate was performed in triplicate, results expressed as mean ± s.d.



might be responsible for the higher levels of plasma corticosterone seen in these rats. However, this would suggest that there was also an abnormality in the negative feedback control of ACTH secretion.

B. 11β-OHSD ACTIVITY IN HUMAN HYPERTENSION.

Methods

A total of 14 hypertensive males and 3 hypertensive females were studied. None of these patients had received antihypertensive medication, and they had no clinical or biochemical evidence to suggest a secondary cause (e.g. renal artery stenosis, phaeochromocytoma etc) to account for their hypertension. All the patients denied excessive alcohol consumption (i.e. >2 units/day), and had normal liver function and thyroid function tests. Hypertension was diagnosed according to the WHO criteria of clinic pressures greater than 160/90 mmHg. Patients attended the Metabolic Unit on the study day at 08.30h fasted overnight, and lay supine for a minimum of 30 min. Three blood pressures were measured during this period using an automatic Copal sphygmomanometer (which was checked against a Hawksley random zero sphygmomanometer at weekly intervals), and the mean of these 3 readings recorded. Age and presence or absence of a family history of hypertension was also noted. Blood was taken in the supine position for electrolytes and creatinine, plasma renin activity (ref. range 0.5-1.5 ng/ml/h), aldosterone (ref. range 150-500 pmol/l) and cortisol (ref. range 180-650 nmol/l) and analysed using methods already discussed (see Chapter 2 and Haber et al 1969, Al-Dujaili and Edwards 1981a, McConway and Chapman 1986). The 14 male hypertensives then made a complete 24h urine collection which was analysed for

cortisol (ref. range 80-450 nmol/24h) (Gough and Ellis 1981) and steroid metabolite profile performed by Dr.C. Shackleton, San Francisco (Shackleton 1986).

Six of the above males and 3 females consented to the isotopic study, and these subjects were commenced on a fixed Na^+/K^+ intake (130 mmol/80 mmol) for 4 days prior to the study day. In the females where applicable the study was performed in the follicular phase of the menstrual cycle. [11α^3H]-cortisol was synthesised as described in Chapter 2 by Dr.J. Corrie (specific activity 14.6 Ci/mmol) and aliquoted into vials containing 0.7 mg of cortisol. After the baseline bloods described above, subjects emptied their bladders, and the [11α^3H]-cortisol injected as a bolus over 20 seconds. Plasma was taken at 7.5, 15, 30, 45, 60, 80, 100 and 120 min, and the patient asked to empty their bladder at +60 and +120 min. Using a Packard Tricarb 4330 counter these samples (plasma and urine) were counted for both total ^3H and ^3H-H_2O following sublimation. All samples were corrected for quench and counted to an error of <2% (approx 20-30 min counting time). Cocktail T (Scintran, BDH Chemicals) was used as a scintillant. Counts of ^3H-H_2O were subtracted from total ^3H giving counts for the steroid [11α^3H]-cortisol. Plots of c.p.m. [11α^3H]-cortisol vs. time were made, its metabolism consisting of a distribution and elimination phase. Using the time points representing the elimination phase (45-120 min) a slope was determined using a linear regression programme and the half-life then established (half-life = 0.693/ slope x 2.303).

Nineteen normotensive controls (17 male) with no family history of hypertension were also studied using a protocol identical to that

described above. Again all subjects denied excessive alcohol intake. Isotopic studies were performed in all these subjects, 6 of them being established on the fixed Na^+/K^+ intake. However subsequent experiments showed no change in cortisol metabolism in the same subject taking variable sodium dietary intakes and remaining volunteers were studied on their normal diet.

Results

Statistical analysis was performed using the non-parametric Wilcoxon rank sum test.

Table 5.3 summarises the findings in the 14 male essential hypertensives and 17 male controls. As a group the hypertensives were slighty older than the controls, but there was no difference in plasma K^+ (ref. range 3.5-5.5 mmol/l) renin activity or aldosterone in the groups. Plasma cortisol was higher in the hypertensives, though curiously urinary free cortisol was lower. The urinary steroid metabolic profiles were also similar. Elevation of the androgens androsterone and etiocholanolone, seen in our index case, was not observed in the hypertensives, and the THF + allo-THF/THE ratio, reflecting 11β-OHSD activity was "normal". The only significant steroid abnormality was an increase in the THF/allo-THF ratio, suggesting reduced 5α reductase activity in the hypertensives. The cortisol steroid profiles were similar in the 3 hypertensive females and the 2 controls.

In the 17 male controls, the half-life for [$11\alpha^3$H]-cortisol was 40.9 $\pm$ 4.6min (mean $\pm$ s.d). In the 2 females values of 40.4 and 39.7 min were obtained. As mentioned there was no correlation between

Table 5.3 Metabolic parameters and steroid levels in normals and patients with untreated essential hypertension.

Results expressed as mean ± s.d.

(* $p<0.05$, *** $p<0.001$, Wilcoxon rank sum test)

Parameter	Normals (n=17)		Hypertensives (n=14)
Age (yr)	37 ± 9	*	47 ± 11
Blood pressure (mmHg)	122 ± 12	***	170 ± 24
	74 ± 9	***	109 ± 13
Plasma K^+ (mmol/l)	4.06 ± 0.25		3.86 ± 0.25
Plasma renin activity (ng/ml/h)	0.80 ± 0.47		0.90 ± 0.56
Plasma aldo (pmol/l)	574 ± 221		533 ± 170
Plasma cortisol (nmol/l)	383 ± 113	*	476 ± 92
24h urinary free cortisol (nmol/24h)	349 ± 112	*	268 ± 69
Androsterone (µg/24h)	2292 ± 1106		1745 ± 823
Etiocholanolone (µg/24h)	2534 ± 859		2537 ± 1560
THF (µg/24h)	2134 ± 722		2478 ± 1196
allo-THF (µg/24h)	1499 ± 725		1109 ± 529
THE (µg/24h)	3491 ± 987		3351 ± 1403
THF + allo-THF/ THE	1.05 ± 0.27		1.06 ± 0.21
THF/ allo-THF	1.74 ± 1.13	*	2.54 ± 1.30

either the THF + alloTHF/THE ratio and the plasma [11α^{3}H]-cortisol half-life and sodium intake. Table 5.4 shows the half-lives for [11α^{3}H]-cortisol in the 9 hypertensives receiving the isotope alongside their PRA, aldo and steroid profiles (see earlier for ref. ranges). A half-life of over 50min (mean + 2s.d.) is abnormal. Both patient 1 and 7 have a prolonged half-life, though on the basis of their other findings it would be hard to implicate 11β-OHSD deficiency as being relevant in the pathogenesis of their hypertension. Neither patient has hypokalaemia, suppressed PRA or aldo or an abnormal THF + allo-THF/THE ratio. There is a well known isotope effect for the [11α ^{3}H]-cortisol (Hellman et al 1971) and our studies performed on patients receiving replacement hydrocortisone therapy have confirmed this. However when measured throughout the isotope study plasma cortisol in subjects 1 and 7 did not differ from other subjects. There was no change in the isotope half-life in subjects 3,4 and 9 who had a family history of hypertension.

Summary

1. In 14 patients with established essential hypertension who had never received antihypertensive medication, there was no evidence of hypokalaemia or suppression of the renin-angiotensin-aldosterone axis when compared to normotensive controls.

2. Plasma cortisol was significantly elevated in the hypertensive group ($p<0.05$), with a reduced urinary free cortisol. The cause of this remains obscure, though it is of interest to note that similar plasma findings were found in our animal hypertensive model.

Table 5.4 Details of the nine hypertensives given the cortisol isotope

Subject	1	2	3	4	5	6	7	8	9
Sex	M	M	M	M	M	M	F	F	F
Age (yr)	32	53	33	50	45	50	43	53	26
B.P. (mmHg)	160/95	154/104	158/107	155/100	186/98	147/111	205/110	181/112	155/99
Plasma K^+ (mmol/l)	4.3	3.7	3.8	3.7	3.9	3.8	4.2	3.3	4.0
Plasma renin activity (ng/ml/h)	0.9	0.6	0.5	0.5	0.5	1.5	1.8	0.4	0.7
Plasma aldo (pmol/l)	350	590	440	460	930	700	1200	720	750
09.00h plasma cortisol (nmol/l)	527	426	397	555	429	372	599	406	489
Urinary free cortisol (nmol/24h)	133	254	272	233	203	269	498	170	123
$\frac{\text{THF + allo-THF}}{\text{THE}}$	0.67	0.79	1.19	1.17	1.58	1.06	1.18	1.05	1.14
Half-life [11α^3H]-F (min)	69.6	38.4	35.2	35.3	45.0	38.6	81.6	41.7	42.7

3. Apart from an increased THF/allo-THF ratio in the hypertensive males suggesting a possible deficiency of 5α-reductase, the steroid metabolic profiles were remarkably similar. A pre-hypertensive population was not studied, but it seems unlikely from what we know from the reported cases of 11β-OHSD deficiency, that hypertension per se should alter this ratio. However as we saw with the liquorice experiment, changes in this ratio may be extremely small and larger numbers of patients may be required.

4. Apart from two cases the plasma half-life for [11α^{3}H]-cortisol was normal in the hypertensive population. In the two cases with an abnormal half-life, suggesting a 11β-OHSD defect, it does not seem likely that this could be of relevance in the development of their hypertension.

Chapter 6 ALCOHOL AND HYPERTENSION

Epidemiology

A link between alcohol and hypertension was first noted in 1915 from the observations of Lian who showed higher blood pressures in French sailors consuming over 2.5 litres of wine/day (Lian 1915). This provoked little attention until the 1960's when the Framingham group reported that very heavy drinkers had higher blood pressures than non-drinkers (Dawber et al 1967). Further support for the association has come from world-wide epidemiological studies (table 6.1). The largest of these was the Kaiser Permanente Health Examination Programme in 1977 which studied blood pressure and drinking habits in 84,000 men and women in California. Subjects taking 3 drinks or more/day (>30g ethanol/ day) had significantly higher systolic and diastolic pressures than controls, independent of age, sex, race, smoking, coffee intake and adiposity (Klatsky et al 1977). All the other reported studies come to similar conclusions with regard to heavy drinkers. It is however of interest that several of these studies suggest that subjects drinking one or two drinks per day had lower blood pressure and mortality than teetotallers.

The importance of alcohol as a cause of elevated blood pressure in the hypertensive population has been variably assessed. From mortality data Matthews has estimated that as much as 30% of hypertension in developed countries (his figures are from England and Wales) may be attributed to alcohol intake (Matthews 1976). This is probably an over-estimate. Nevertheless from the Kaiser study and an Australian

Table 6.1

Studies of the Cross-sectional Association of Blood Pressure with Alcohol Consumption

Reference	Year	Study	No. of subjects	Male subjects (%)	Age (yr)
North America					
Dyer et al	1977	Chicago W. Electric	1,899	100	40-55
Klatsky et al	1977	Kaiser-Permanente I	83,947	45	15-79
Criqui et al	1981	Lipid Research Clinics	5,783	52	20+
Kagan et al	1981	Honolulu Heart	8,006	100	46-68
Gordon & Kannel	1983	Framingham	5,209	42	29-62
Grunchow et al	1986	NHANES	9,553	45	18-74
European					
Gyntelberg & Meyer	1974	Copenhagen	5,249	100	40-59
Kozararevic et al	1980	Yugoslavia	11,121	100	35-62
Salonen et al	1983	North Karelia/Kuopio	8,479	50	30-64
Kornhuber et al	1985	Wurttemberg	3,351	88	20-65
Australian & New Zealand					
Cooke et al	1982	Sydney Hospital	20,920	65	18-70
Savdie et al	1984	Medicheck	11,000	75	43*
MacMahon et al	1984	Australia RFPS	5,550	50	25-64
Japanese					
Kondo & Ebihara	1984	Minamikawachi	3,083	37	53*

NHANES = National Health & Nutrition Examination Survey; RFPS = Risk Factor Prevalence Study
* Mean age

study (MacMahon et al 1984) alcohol consumption probably accounts for 5-10% of the prevalence of hypertension.

In summary therefore there appears to be a direct relationship between alcohol consumption and blood pressure which may account for up to 10% of the prevalence of hypertension.

Mechanisms of alcohol related hypertension

Whilst much data has been generated establishing a causal link between alcohol and hypertension, little has been done to elucidate the mechanism of the association. It seems likely that there are at least two different mechanisms underlying alcohol-induced hypertension (Potter et al 1984). The first of these relates to the acute pressor effect of alcohol which is probably less well understood than the second, hypertension associated with alcohol withdrawal in the alcohol dependant patient. These will be discussed together under a series of proposed mechanisms.

1. Catecholamines and increased sympathetic activity

There is good evidence suggesting that increased sympathetic activity is important in alcohol related hypertension. Acute alcohol administration increases both urinary noradrenaline and adrenaline excretion in man (Perman, 1961 & Olgata et al 1971). Plasma noradrenaline levels also seem to be raised acutely but not chronically (Arkwright et al, 1982).

In alcohol withdrawal there is activation of noradrenergic mechanisms in some studies (Carlsson & Haggendal, 1967) but not all

(Bannan et al 1984). Saunders, studying 96 alcoholics admitted for detoxification found a close correlation between the severity of alcohol-withdrawal symptoms and hypertension and postulated that alcohol related hypertension was simply the result of repeated withdrawal events (Saunders et al 1979). Although increased noradrenergic mechanisms were suggested no evidence was produced to support this.

2. The renin-angiotensin-aldosterone system

Acute alcohol administration stimulates both plasma renin activity (PRA) and plasma aldosterone levels. Levels are also raised during the hangover period (Linkola et al, 1979) probably consequent upon sodium and water depletion (Puddey et al 1985), (Nieminen 1983). PRA and aldosterone levels are also elevated in chronic alcoholics (Bannan et al 1984) though these do not correlate with blood pressure. By day 4 of alcohol withdrawal in this study PRA and aldosterone had reverted to normal.

3. Plasma vasopressin (AVP)

Following acute ethanol consumption, AVP is suppressed and this is responsible for the ensuing diuresis (Linkola 1978). Chronically however, and in hangover situation AVP are elevated secondary to volume depletion (Linkola 1978, Potter et al 1983). The concentrations of AVP achieved in alcoholics however are not sufficient to elevate blood pressure (Padfield et al 1981).

4. Calcium Metabolism

Some studies have suggested a positive correlation between plasma

ionised calcium levels and blood pressure in drinkers but not teetotallers (Arkwright et al 1984). The question of plasma calcium levels in hypertension per se is currently a controversial and expanding area, but further larger studies are needed to study the alcohol sub-group.

5. Cortisol

Acute ethanol intake raises plasma cortisol in man, and this is probably mediated by ACTH (Jenkins & Connolly 1968). Levels are also elevated during chronic intake and on withdrawal (Arkwright et al 1984, Bannan et al 1984). Again this is likely to be ACTH mediated either directly or indirectly by an effect of AVP on CRF release (Gillies et al 1982). Cortisol has been postulated to contribute to the hypertension by a number of mechanisms (e.g. raising plasma renin substrate; acting as mineralocorticoid as well as a glucocorticoid; increasing sensitivity to catecholamines).

Two recent observations suggested to me that 11β-OHSD inhibition may have a role to play in alcohol-related hypertension. Firstly workers in Italy, confirming Bannan's finding of hypercortisolaemia in 280 alcoholics admitted for detoxification, showed significant sodium retention in the hypertensive group when compared with the normotensive group and postulated a renal abnormality (de Marchi & Cecchin 1985). Secondly, from Stockholm it has been reported that steroid metabolic profiles in alcoholics during withdrawal showed an increase in the THF:THE ratio (Cronholm et al 1985). The possible implication of this altered steroid metabolic profile was not appreciated by the authors and no details of blood pressure, plasma renin activities or electrolytes were given. Furthermore the study was

not controlled. However alcohol is known to produce changes in the redox potential (NADP:NADPH and NAD:NADH ratio) (Lieber et al 1975) and this will affect the 'set-point' of 11β-OHSD, which as we have seen is clearly dependent upon the NADPH:NADP ratio.

With this background in mind we set out to evaluate 11β-OHSD activity in patients admitted for alcohol withdrawal.

Materials and methods

Twenty four alcoholic patients (20 male) admitted for alcohol withdrawal were studied. On admission blood pressure was recorded in the supine position using the same mercury sphygmomanometer, the mean of 3 being readings recorded. All patients (who had been drinking up until admission) were studied on day 5 of hospitalisation, by which time any severe withdrawal symptoms had subsided. It was at this time that Cronholm et al had observed their marked change in the THF/ THE ratio. On the 5^{th} morning blood was taken for the following investigations with the patient supine;

a) Plasma renin activity (radioimmunoassay method described in Chapter 2 (Haber et al 1969))

b) Plasma aldosterone (radioimmunoassay method described in Chapter 2 (Al-Dujaili and Edwards 1981a))

c) Plasma sodium, potassium.

d) Plasma cortisol (radioimmunoassay: Amerlex kit, Amersham International)

e) Starting at 09.00h a 24h urine collection was made. This was performed under close supervision by the nursing staff to ensure a complete collection. Urinary free cortisol and cortisone were

quantified using gas chromatography/mass spectrometry (Shackleton 1986), in addition to the principal steroid metabolites (THF, allo-THF, THE, cortols and cortolones).

Results

Of the 24 patients 6 were hypertensive on admission (i.e. blood pressure >160/90 mmHg). No subject had a past history of hypertension, and with the exception of one subject who was lost to follow-up, all have been shown to be normotensive off alcohol. The results of the above investigations are shown in tables 6.2, 6.3 and fig. 6.1. Statistical analysis was made using Student's unpaired 't' test. As shown the hypertensives were significantly older than the normotensives (55.5 $\pm$5.6 vs 41.3 $\pm$10.6 yr). There was evidence to suggest "mineralocorticoid excess" as the cause of the hypertension in this group, with significant suppression of plasma renin activity (PRA) and hypokalaemia in the hypertensive group. Plasma aldosterone was also reduced in the hypertensives, though not significantly so.

This possible "mineralocorticoid excess" did not appear to be secondary to 11β-OHSD inhibition, in that urinary free cortisol was not elevated in the hypertensive group, and the THF + allo-THF/THE ratios were similar in the two groups. However there was reduced excretion of the principal urinary cortisol metabolites (i.e. THF, allo-THF, THE and cortols) in the alcoholics when compared to the normal controls previously referred to in Chapter 5 (table 6.3). This effect was not related to the level of blood pressure. The most striking abnormality was an increase in the THF/ allo-THF ratio in all the alcoholics independant of blood pressure, indicating 5α-reductase

Table 6.2 Results from alcohol withdrawal in 24 patients, expressed as mean ±s.d. (* p<0.05, ** p<0.01, *** p<0.001).

	Normotensives (n=18,16 male)		Hypertensives (n=6, 4 male)
Age (yr)	41.3 ±10.6	**	55.5 ±5.6
B.P. (mmHg)	130.6 ±10.6	***	184.2 ±17.4
	84.6 ±6.3	**	115.2 ±19.3
PRA (ng/ml/h)	1.27 ±1.10	**	0.30 ±0.22
Plasma aldo (pmol/l)	684.2 ±538.2		433.3 ±193.9
Plasma Na^+ (mmol/l)	140.3 ±1.6		139.2 ±1.6
Plasma K^+ (mmol/l)	3.9 ±0.4	*	3.5 ±0.3
09.00h Plasma cortisol (nmol/l)	379.8 ±136.9		337.8 ±58.6
24h urinary free cortisol (µg/24h)	88.7 ±41.6		56.3 ±40.3
24h urinary free cortisone (µg/24h)	70.0 ±27.7		66.8 ±30.8

Table 6.3 Urinary steroid metabolic profile as measured by gas chromatography/mass spectrometry.
Results expressed as mean ± s.d. μg/24 hours

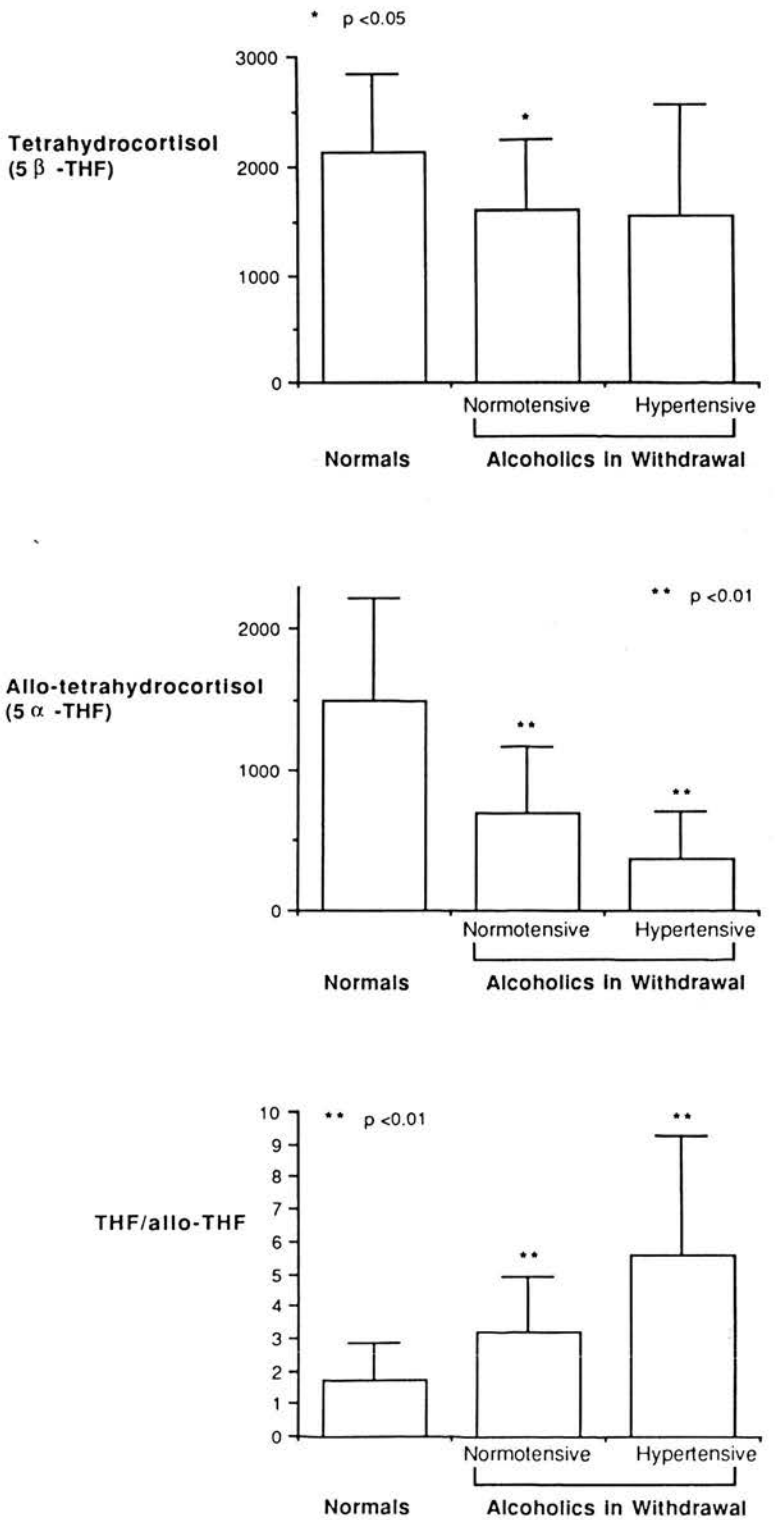
	Normals	Alcoholics in Withdrawal	
	(n=17)	Normotensive (n=18)	Hypertensive (n=6)
Tetrahydrocortisol (5β-THF)	2134 ± 722	1609 ± 641*	1550 ± 1017
Allo-tetrahydrocortisol (5α-THF)	1499 ± 724	689 ± 489**	364 ± 337**
Tetrahydrocortisone (THE)	3491 ± 987	2251 ± 902***	2037 ± 954**
Cortols	608 ± 111	487 ± 249	383 ± 243*
Cortolones	1602 ± 216	1518 ± 711	1312 ± 366
THF/allo THF	1.74 ± 1.13	3.19 ± 1.74**	5.56 ± 3.69**
THF + allo THF/THE	1.05 ± 0.27	1.03 ± 0.25	0.97 ± 0.37

* p = <0.05

** p = <0.01 Wilcoxon Rank Sum Test

*** p = <0.001

Figure 6.1 Impairment of 5α-reductase activity in 24 alcoholics in withdrawal. Results expressed as mean ±s.d. in μg/24h.



inhibition. In 17 non-alcoholic normotensives, and 14 essential hypertensives studied in chapter 5, the THF/ allo-THF ratios were 1.73 $\pm$1.12, and 2.54 $\pm$1.3 respectively (table 5.3 and fig. 6.1).

Discussion

These results are of considerable interest. We have clearly shown suppression of the renin-angiotensin system in hypertensive alcoholics when compared with their normotensive controls. Although the hypertensives were slightly older than the normotensives, the fall in PRA was too marked to be explained by age alone. Also PRA was elevated in the normotensive alcoholics when compared to our control population (1.27 $\pm$1.10 vs 0.80 $\pm$0.47 ng/ml/hr respectively, $p<0.05$), in keeping with previous studies showing activation of the renin-angiotensin system in alcoholics (Bannan et al 1984). Our results would support the hypothesis of De Marchi's group, who have postulated sodium retention as the mechanism of alcohol withdrawal hypertension (De Marchi and Cecchin 1985).

The mechanism of this volume mediated "mineralocorticoid" hypertension remains obscure. From our studies we cannot implicate cortisol acting as a mineralocorticoid via inhibition of 11β-OHSD, the THF +allo-THF/ THE ratio being the same in the two groups. We were unable to confirm Cronholm's finding of a high ratio in alcoholics (Cronholm et al 1985). However the reduction in excretion of the principal cortisol metabolites suggests a reduced daily cortisol production rate, seen in patients with a prolonged plasma cortisol half-life, as occurs in 11β-OHSD deficiency. Recently Dr.'s Mantero

and Ulick have described a patient with the syndrome of apparent mineralocorticoid excess, having a reduced daily cortisol production rate in the face of a normal THF + allo-THF /THE ratio (Ulick et al 1988), the so-called type 2 variant of AME. The role of cortisol in this variant condition remains to be elucidated.

The most striking finding in this study was an alteration in the steroid metabolic profile suggesting 5α-reductase inhibition (fig. 6.1). 5α-reductase is also responsible for the conversion of testosterone to the more potent dihydrotestosterone, and its activity has been studied mainly in animals fed alcohol. The results are conflicting, though it would appear that acute ethanol administration increases 5α-reductase activity in the liver (Rubin et al 1976, Vittek et al 1981), but chronic consumption (>1yr) reduces activity (Gordon et al 1979). Cortisol metabolism by 5α-reductase in rats fed alcohol has been studied by only one group (Bode et al 1978). They showed an increase in hepatic enzyme activity in rats given ethanol (40% of total calories) for 9 days. However this has not been evaluated in man, and to my knowledge the observation of an increase in the THF/ allo-THF ratio in this setting is a new one. It is interesting to note that a similar urinary steroid profile (i.e. an increase in the THF/ allo-THF ratio suggesting 5α-reductase deficiency) has been observed in Cushing's syndrome, with values up to 5-10 reported (normal range 1.0-2.4) (Donaldson et al 1981, Shackleton 1986). The full significance of this finding is not yet known.

Unfortunately ethical approval was not given to study the metabolism of [11α^{3}H]-cortisol in the alcoholics. We have clearly shown that quite small changes in the THF +allo-THF /THE ratio can be

associated with a profound mineralocorticoid effect. Further evaluation of the type 2 variant of AME may reveal cortisol acting as a mineralocorticoid despite a normal THF + allo-THF/ THE ratio. I feel therefore it would be important to confirm similar metabolic clearance of the cortisol isotope ([11α ^{3}H]-cortisol) in hypertensive and normotensive alcoholics before excluding a role for 11β-OHSD inhibition in alcohol withdrawal hypertension.

CHAPTER 7 11β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN RENAL IMPAIRMENT

Introduction

The role of the kidney in the metabolism of cortisol to cortisone is obviously key to our hypothesis for a paracrine role of renal 11β-OHSD. In Chapter 3, I demonstrated that for a fixed mg protein/ g wet weight tissue, the kidney was far more effective in converting F → E than the liver in the rat. Obviously this does not take into account the relative mass of these two organs *in vivo*. Without catheter studies across these organs in man the absolute contribution to the net production of cortisone cannot be established. Nevertheless the physiological importance of the kidney in this respect was indicated by Hellman's study on the renal capture and oxidation of cortisol in man (Hellman et al 1971) and by results of experiments in the isolated perfused rat kidney (Reach et al 1977). These complemented earlier *in vitro* work showing that cortisol was most active in converting cortisol to cortisone but did not reduce the 11-oxo group (Jenkins 1966).

In a small clinical study, plasma cortisone was reduced in 5 patients with renal impairment (Srivastava et al 1973), and we were keen to evaluate this in a larger group of such patients.

Methods

Eighty-eight patients with a variety of renal diseases (34 glomerulonephritis, 18 IgA nephropathy, 12 pyelonephritis, 20 miscellaneous including vasculitides, polycystic kidneys, analgesic

nephropathy and diabetes (n=4)) were compared with 47 controls. All these subjects were recruited by Dr. Judy Whitworth, consultant Nephrologist in Melbourne, Australia. Patients taking corticosteroid therapy were excluded. The following measurements were made between 08.30 and 09.30h in the supine position:

Supine blood pressure (mercury sphygmomanometer, mean of 3 readings in mmHg)

Plasma creatinine (Technicon Autoanalyser, mmol/l)

Age and drug history

10ml of blood was taken into a lithium heparin, plasma separated and frozen at -20°C. This was transported on dry ice to the Western General Hospital, Edinburgh for cortisol and cortisone assay.

Cortisol assay.

Cortisol was assayed directly by myself with the help of Dan Burt (senior technician, Department of Medicine) using Scottish Antibody Production Unit (SAPU) 1051F sheep anti-cortisol antiserum and ^{125}I-cortisol. 10µl plasma was diluted to 300µl with citrate/ phosphate buffer, pH 4, containing 0.1% BSA (assay diluent). Antiserum (giving a final concentration of 1:130,000) and 10,000 cpm ^{125}I-cortisol in 50µl assay diluent was added to this, and incubated at room temperature for 4h. Bound and unbound cortisol was separated by the addition of 50µl donkey anti-sheep antiserum (SAPU) at a concentration of 1:15 and normal sheep serum (SAPU) 1:200 in 1mM EDTA. The mixture stood overnight at 4°C, tubes were then centrifuged, supernatant decanted and residue counted.

Cortisone assay.

This was performed principally by Sheila Atherden, with assistance from myself. An anti-cortisone antiserum was raised in rabbits by Dr's Corrie, Stewart and Sheila Atherden (Department of Medicine, WGH) in New Zealand white rabbits. As cortisol cross-reacted to an extent of 3-4% with this antiserum, it was necessary to separate cortisol and cortisone prior to assay. Plasma was pre-extracted with hexane to remove lipid material and then extracted with 5 volumes of ethyl acetate. This extract was washed in turn with 0.1M NaOH and with water and aliquots were taken to dryness under nitrogen. Separation of cortisol to cortisone was carried out using HPLC (Waters associates, Cheshire, U.K.) with μbondapak column and methanol:water (1:1) mobile phase at a flow rate of 1ml/min. Each aliquot of extracted plasma was dissolved in mobile phase and injected onto the column. The appropriate fraction was collected, methanol evaporated off under nitrogen and the aqueous residue back extracted with ethyl acetate. Aliquots of this extract were evaporated to dryness and residues dissolved in 300μl assay diluent. Antiserum to give a final concentration of 1:140,000 and ^{125}I-cortisone (10,000 cpm), each in 50μl assay diluent were added and incubated at room temperature for 4h. Separation of bound and unbound cortisone was achieved by adding 50μl of donkey anti-rabbit antiserum (SAPU) at a concentration of 1:10 and normal rabbit serum (SAPU) 1:150 in 1mM EDTA. The remainder of the procedure was as for cortisol.

Assays were carried out on final extracts equivalent to 20μl plasma. Overall recovery was determined using ^{3}H-cortisone and values determined by radioimmunoassay were corrected by this factor (92.7%).

Renal patients were divided into 4 groups based on severity of

renal function as assessed by plasma creatinine:

Group A: normal renal function (plasma creat. <0.12mmol/l)

Group B: mild renal impairment (plasma creat. 0.12-0.20mmol/l)

Group C: moderate renal impairment (plasma creat. 0.21-0.45mmol/l)

Group D: severe renal impairment (plasma creat. >0.45mmol/l)

Statistical analysis was made using the Wilcoxon Rank Sum test and correlations using the Spearman Rank correlation test.

Results

As shown in table 7.1 and fig. 7.1 there was a marked fall in plasma cortisone with increasing renal impairment. Plasma cortisol was unchanged. Plasma cortisone was also significantly reduced in patients with renal disease but normal function (Group A). Fig. 7.2 shows a significant negative correlation between plasma cortisone and plasma creatinine ($r= -0.55$, $n=60$, $p<0.01$), and a positive correlation between the plasma F/E ratio and plasma creatinine ($r= 0.49$, $n=60$, $p<0.01$) in the 60 renal patients with an abnormal plasma creatinine (i.e. groups B, C, and D). There was no correlation between plasma cortisol and creatinine and no correlation between plasma cortisone or the F/E ratio and age, sex, blood pressure or drug therapy.

Discussion.

A prolonged plasma cortisol half-life in patients with uraemia was inferred from the early study of Englert et al (Englert, Jr et al 1958), and shown convincingly in more recent studies by Bacon and Kawai (Bacon et al 1973, Kawai et al 1985). Kawai et al studying the metabolism of cortisol, prednisolone and dexamethasone in 16 patients with chronic renal failure, showed a prolonged plasma half-life for cortisol (2.9 $\pm$1.0h (mean $\pm$s.d) vs 2.1 $\pm$0.2h controls, $p<0.01$) and

Table 7.1 Plasma creatinine, cortisone and cortisol in 88 patients wtih renal disease and 47 controls. Results shown as mean ± s.d.

	Normals	N Function Creatinine <0.12	Mild Creatinine 0.12-0.20	Moderate Creatinine 0.21-0.45	Severe Creatinine >0.45
Number	47 (30 female)	28 (16 female)	18 (5 female)	19 (6 female)	23 (11 female)
Age (yr)	35.8 ± 10.9	33.5 ± 16	*50.3 ± 16.6	*44.8 ± 17.1	*43 ± 12.6
Creatinine ($mmol^{-1}$)	0.09 ± 0.01	0.09 ± 0.01	***0.15 ± 0.02	***0.28 ± 0.07	***0.90 ± 0.2
E (cortisone) ($nmol\ l^{-1}$)	61.6 ± 20	**47.9 ± 20.5	*44.4 ± 15.7	**36.8 ± 15.1	***20.7 ± 15.7
F (cortisol) ($nmol\ l^{-1}$)	512 ± 250	644 ± 536	566 ± 234	648 ± 237	474 ± 276
F/E	8.7 ± 3.9	***14.6 ± 9.7	*14.6 ± 7.8	***19.9 ± 9.4	***26.9 ± 13

* p = <0.05

** p = <0.01 Wilcoxon Rank Sum Test

*** p = <0.001

Figure 7.1 Plasma cortisone and creatinine in 88 patients with renal disease and 47 normal controls. Results expressed as mean ±s.d.

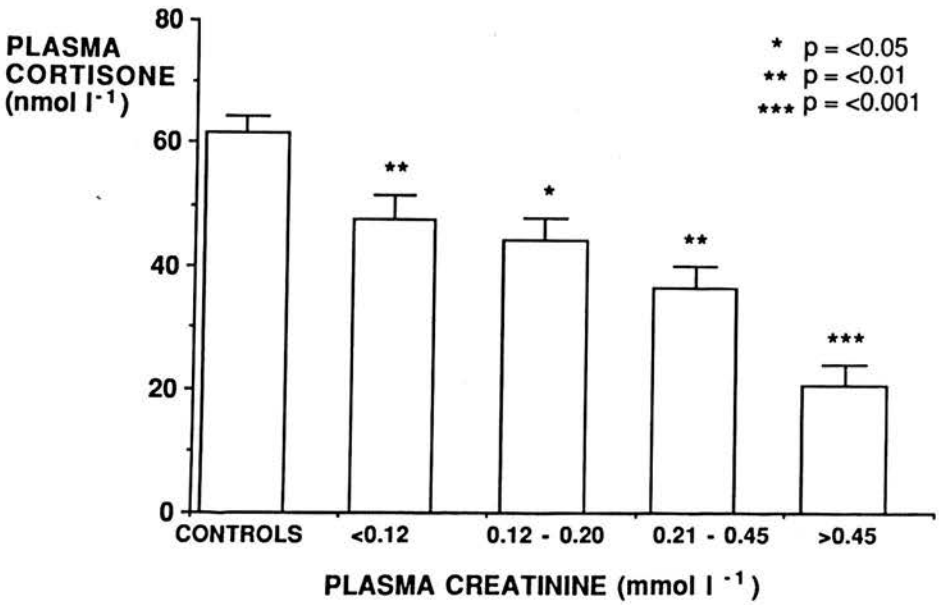
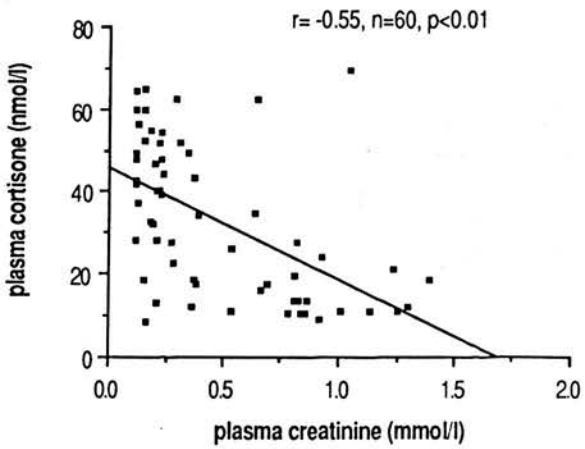
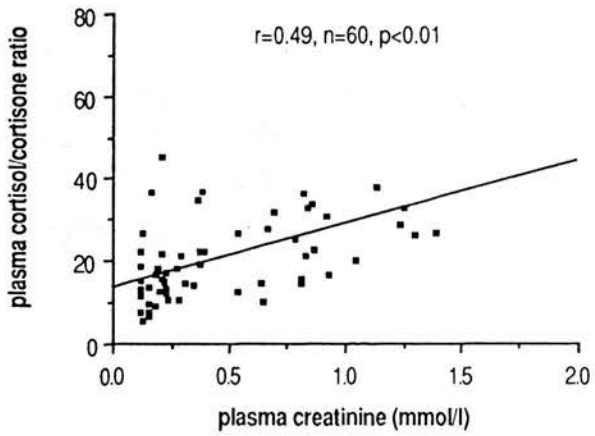


Figure 7.2 Correlation of plasma cortisone and the plasma cortisol/cortisone ratio vs plasma creatinine in 60 patients with renal disease and abnormal plasma creatinines (i.e. Groups B, C, and D)





prednisolone but a reduced half-life for dexamethasone. In 6 of these patients with severe renal failure treated with haemodialysis, there was a markedly reduced metabolic clearance rate (MCR) of cortisol (67 $\pm$33 litres/day/m^2 vs 114 litres/day/m^2 controls, $p<0.02$).

The cause for this prolonged cortisol half-life has not been explained. Emphasising the importance workers have placed on the major role of the liver in cortisol metabolism, it was suggested that conjugated cortisol metabolites accumulate in uraemia causing a decrease in the rate of hepatic removal of cortisol (Englert, Jr et al 1958). This has never been substantiated. The prolonged half-life seems unlikely to be secondary to changes in cortisol binding globulin, which if anything is on the low normal side in patients with renal disease (Doe et al 1964).

Srivastava documented reduced plasma cortisone in 5 patients with renal failure (Srivastava et al 1973), and we have expanded her findings. Despite no change in plasma cortisol, plasma cortisone was significantly reduced in 88 patients with renal disease when compared to 47 ethnic controls. There was an inverse relationship between plasma E and renal function as measured by plasma creatinine.

Measuring plasma F and E is, at best, a crude assessment of 11β-OHSD activity. Other factors such as an enhanced clearance of E may be operating to explain these findings. Twenty-four hour urinary steroid profiles have been taken from some patients with renal disease to resolve this issue further, and we are currently applying for ARSAC and Ethical Committee approval to study the metabolism of [11α^3H]-cortisol in such patients.

However the finding of a marked reduction in plasma cortisone in patients with renal failure is strongly suggestive that the elevated

plasma half-life and reduced MCR of cortisol seen in this condition is secondary to reduced renal conversion of cortisol to cortisone by 11β-OHSD. The physiological significance of this finding remains unknown. It is possible that such a defect may be relevant in the sodium retention and hypertension commonly seen in renal failure (Kincaid-Smith and Whitworth 1988), or even as a novel protective mechanism for maintaining potassium excretion in the face of a low glomerular filtration rate.

CHAPTER 8 CONCLUSIONS AND FUTURE DEVELOPMENTS

11βeta hydroxysteroid dehydrogenase activity was first shown in the late 1950's in placental and hepatic tissue, and is responsible for the interconversion of the hydroxyl and oxo group at the C_{11} position on the steroid nucleus. From Bush's early work on the hepatic enzyme, it was felt that 11β-OHSD was not a critical pathway in cortisol metabolism, although the reverse reaction (i.e. conversion of E → F) was obviously of considerable relevance to the efficacy of cortisone acetate when it was the principal glucocorticoid used for replacement therapy (Bush 1969).

Like so many enzyme systems, the full importance of 11β-OHSD has only become apparent through our understanding of the enzyme deficiency state. Deficiency of 11β-OHSD presents as severe mineralocorticoid hypertension with hypokalaemia in children. Although the first documented case of 11β-OHSD deficiency was the 3 year old girl described by Werder (Werder et al 1974), it was Stanley Ulick some 5 years later who first showed that this "syndrome of apparent mineralocorticoid excess" was associated with an inability to convert cortisol to cortisone (Ulick et al 1979). The mineralocorticoid responsible for the condition has remained in doubt; even the most recent publications in this area still refer to the syndrome of apparent mineralocorticoid excess (Monder et al 1986).

The case described in Chapter 2 of this thesis is particularly interesting. Firstly this is the only adult case of 11β-OHSD reported. Secondly, from our observations during the detailed metabolic balance studies described, it was clear that cortisol was

acting as a potent mineralocorticoid on both the kidney and rectum. The syndrome of apparent mineralocorticoid excess should perhaps now be renamed "a defect in the cortisol → cortisone shuttle." Perhaps more importantly though, we thought that this finding was telling us something very important about the control of the tissue response to corticosteroids, and hypothesised that 11β-OHSD was a critical paracrine mechanism in the kidney preventing high intra-renal glucocorticoid levels which could then act as mineralocorticoid. In normal individuals the "shuttling" of active cortisol to cortisone by 11β-OHSD prevents this from occurring.

Subjects with 11β-OHSD deficiency are not cushingoid and this has caused confusion with some people. However, the defect in cortisol metabolism results in a prolonged cortisol half-life. This, by operating through the negative feedback mechanism at the hypothalamus /pituitary reduces ACTH secretion and hence the daily cortisol production rate, resulting in normal plasma cortisol levels.

It was important to extend this hypothesis in other areas rather than just this single case of 11β-OHSD deficiency. In this respect the liquorice studies were particularly important. For many years it had been known that liquorice ingestion produced mineralocorticoid hypertension with hypokalaemia; indeed much of that work was performed in this hospital by Professor J. Strong and his co-workers (Card et al 1953). It was widely believed that the mineralocorticoid activity of liquorice was due to a direct action of its metabolites on the mineralocorticoid receptor, although as I discussed in Chapter 4, my reading on the subject suggested this might not be the case. In addition there were many similarities between 11β-OHSD deficiency and

liquorice excess. It was therefore particularly satisfying to be able to show inhibition of 11β-OHSD by liquorice in an animal model *in vitro* and *in vivo*, prior to demonstrating in man that the mineralocorticoid activity of liquorice was associated with inhibition of 11β-OHSD.

In essential hypertension and alcohol withdrawal hypertension the results are of interest but are, as might be expected, less clear cut. Two of the untreated hypertensives studied had a prolonged half-life for our cortisol isotope, but they had a normal THF + allo-THF/ THE ratio, normokalaemia and no suppression of the renin-angiotensin-aldosterone axis. With our current state of knowledge this finding remains unexplained. The further evaluation of the type 2 variant of AME, with the normal THF/ THE ratio may help resolve this issue (Ulick et al 1988, personal communication). Larger numbers of hypertensives will have to be studied if we are to show what must be a mild defect in cortisol metabolism. One area to be explored is the use of dexamethasone similar to the study by Hamilton and co-workers (Hamilton et al 1979) in hypertensives on a fixed Na^+/K^+ diet.

In our study, alcohol withdrawal hypertension appeared to be associated with hypokalaemia and suppression of the renin-angiotensin system, suggesting a mineralocorticoid excess state, but more patients are required in addition to cortisol isotopic studies. The characteristic urinary steroid metabolic profile (i.e. inhibition of 5α -reductase) is notable and may be useful clinically in alcoholic subjects.

Plasma cortisone was significantly depressed in 88 patients with renal disease despite no change in plasma cortisol (Chapter 7). Taken

in context with the prolonged plasma cortisol half-life in renal failure (Bacon et al 1973, Kawai et al 1985), we believe that this reflects a major contribution by the kidney to the the metabolism of cortisol. Although there are limitations with this pilot study, these findings are in keeping with our own in vitro studies (Chapter 3), previous in vitro (Jenkins 1966, Hoyer et al 1984) and in vivo (Reach et al 1977) studies, and isotopic studies in man (Hellman et al 1971). The kidney therefore appears to be a major site for the conversion of cortisol to cortisone in man.

I have discussed the concept of 11β-OHSD exerting a paracrine role in preventing cortisol acting as a mineralocorticoid in the kidney, but how this is achieved at the cellular level remains unknown. Several recent developments may have helped us to resolve this issue.

Does 11β-OHSD convey specificity for the mineralocorticoid receptor?

Receptors for corticosteroids have been classically divided into Type 1 (mineralocorticoid) and Type 2 (glucocorticoid) receptors (Funder 1985). Type 1 receptors are found in the kidney, parotid, colon and hippocampus. The Type 1 hippocampal receptor is of interest in that it binds corticosterone, cortisol and aldosterone with equal affinity (Beaumont et al 1983) and was initially referred to as a glucocorticoid receptor (de Kloet et al 1975). It has susequently been shown, both from stimulation studies (Nestler et al 1981), and following the recent cloning of the human mineralocorticoid receptor (Arriza et al 1987), that this receptor is identical to the type 1 renal mineralocorticoid receptor. Indeed the type 1 receptor isolated by Arriza in Evan's laboratory binds cortisol, aldosterone and

deoxycorticosterone with equal affinity *in vitro*. In the kidney however, although corticosterone (cortisol in man) is present in much higher concentrations, aldosterone appears to bind preferentially to the type 1 receptor *in vivo* (Funder 1985, Sheppard and Funder 1987a). The kidney along with the parotid is therefore referred to as an 'aldosterone selective' tissue, the hippocampus (and heart), 'aldosterone non-selective'. Hence there must be something other than receptor structure conferring receptor specificity. Funder suggested that this might be extravascular cortisol binding globulin (CBG), found in high concentrations in the kidney, but absent in the hippocampus. CBG, having negligible affinity for aldosterone, could act to 'mop' up any excess cortisol, preventing cortisol acting on the type 1 receptor (Funder 1985). I suggested in Chapter 2 that when this localised tissue CBG became saturated, as might occur in 11β-OHSD deficiency, then free cortisol could be available to act on the type 1 receptor.

It would now appear that this is not the case. In a recent publication Funder's group have shown that the specificity for the receptor is retained even in the 10 day old rat which is deficient of CBG (Sheppard and Funder 1987b). Our studies infusing cortisol into our index case indicated that he had lost the 'normal' specificity for the type 1 renal receptor, i.e. cortisol was acting in preference to aldosterone on this receptor, and this raises the question as to whether it is 11β-OHSD itself which is responsible for conveying specificity to the type 1 receptor. In normal subjects 11β-OHSD by shuttling cortisol to cortisone enables aldosterone (which, possessing a 11, 18 hemiacetal group is not metabolised by 11β-OHSD) to act on the type 1 receptor.

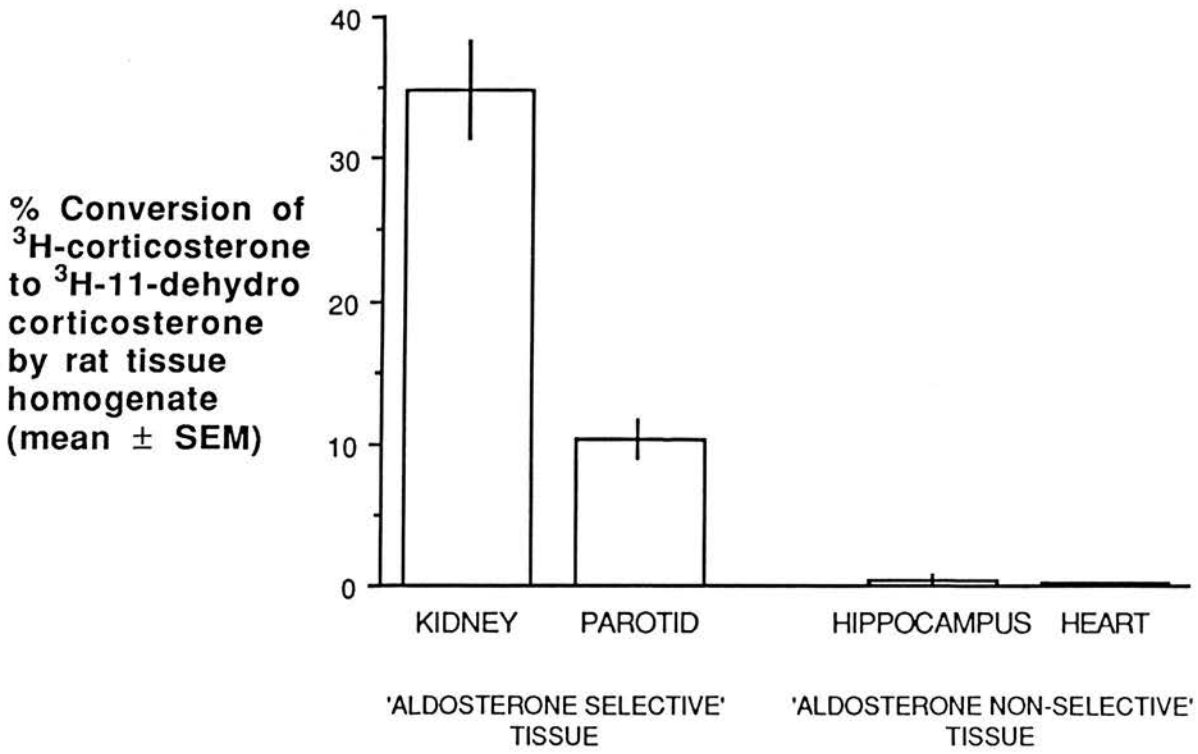
The first step was to show that 11β-OHSD activity was much lower or absent in the aldosterone non-selective tissues, i.e. the hippocampus and heart, as compared to the kidney and parotid, and this has recently been done. Using a fixed mg protein/g wet weight tissue, conversion of ^{3}H-corticosterone to ^{3}H-11 dehydrocorticosterone by a homogenate preparation prepared as described in Chapter 3 was assessed in these organs from 6 male Sprague Dawley rats. The results are depicted in Fig. 8.1.

Collaborative studies are now underway with Funder's group in Melbourne. Preliminary binding studies in adrenalectomised rats have suggested that our hypothesis may be correct. The normal binding of ^{3}H-aldosterone and ^{3}H-corticosterone (i.e. aldo > B), to the kidney and parotid type 1 receptors was altered to the binding seen in the hippocampus (i.e. aldo= B) when an inhibitor of 11β-OHSD such as glcyrrhetinic acid was added (Funder, Edwards and Stewart, unpublished data). It looks likely therefore that the conclusions drawn from our unique index case could have uncovered a novel mechanism dictating specificity of the mineralocorticoid receptor.

Molecular biology of 11β-OHSD

The purification of 11β-OHSD from hepatic microsomes in the rat has been carried out by Carl Monder's team in New York (Lakshmi and Monder 1985a, 1985b). He has recently given me the NH_2 terminal 42 amino acid sequence of the protein (fig.3.1). In collaboration with the MRC Cytogenetics Unit here at the Western General Hospital (thanks to Bob Hill and Andy Gill), an oligonucleotide has been synthesised

Figure 8.1 11β-OHSD activity in the aldosterone selective and aldosterone non-selective tissues in the male Sprague Dawley rat. Results expressed as a mean ± SEM, n=6.



for the highly specific tyrosine groups (amino acids 14-18 incl., i.e. Gly-Tyr-Tyr-Tyr-Ser). Rat liver and kidney cDNA libraries are currently being screened using this oligonucleotide.

Carl Monder has also raised a specific antibody to the purified enzyme protein in the rabbit, and has kindly donated this to me. Immunofluorescence studies to determine the specific tissue localisation of the enzyme will be performed in the near future.

Clinical implications for 11β-OHSD

In addition to the topics discussed in this thesis (i.e. 'essential' hypertension, alcohol related hypertension and renal disease), the following clinical areas may involve 11β-OHSD in their pathogenesis.

1. The mineralocorticoid activity of Carbenoxolone

Carbenoxolone is the synthetic derivative of glycyrrhetinic acid first introduced in the 1960's for the treatment of peptic ulceration (Doll et al 1962). The drug is used little today, partly as result of the efficacy of the newer H_2 receptor antagonists (Cimetidine, Ranitidine), but also because of the side-effects of carbenoxolone. These are principally sodium retention, hypertension and hypokalaemia, i.e. mineralocorticoid related, and occur in up to 50% patients taking the drug (Turpie and Thomson 1965). Again receptor studies from Funder's group suggest that these may be secondary to a direct effect of carbenoxolone on the type 1 receptor (affinity 1/10,000 that of aldosterone) (Armanini et al 1982), but in the light of our liquorice study, this may not be the case. The effect of

carbenoxolone administration to 6 normal subjects on sodium and potassium balance and 11β-OHSD activity is near completion.

2. Hypertension in diabetic nephropathy

One area where a defect in renal 11β-OHSD could be important is in diabetic nephropathy. With the development of sensitive methodology for detecting early diabetic renal disease (i.e. microalbuminuria - 24h urinary protein 30 - 300mg) (Viberti et al 1982), it now seems clear that diabetics with microalbuminuria have higher blood pressures than those with no microalbuminuria (Wiseman et al 1984). Although tight glycaemic control at this early stage does not alter the degree of proteinuria or the rate of decline in GFR, antihypertensive therapy does reduce proteinuria and the rate of fall in GFR (Mogensen 1982, Viberti et al 1983). The mechanism of this 'nephropathy associated hypertension' remains unknown. However the common consensus is that it is a volume-mediated hypertension with expansion of extracellular sodium (Beretta-Piccoli et al 1982, Feldt-Rasmussen et al 1987). Despite having nephropathy, which might activate the renin-angiotensin-aldosterone axis, these patients have normal or suppressed levels of renin, aldosterone and AII (Christlieb et al 1976, Feldt-Rasmussen et al 1987). Insulin is thought to act on the distal renal tubule via an as yet unknown mechanism to cause Na^+ retention (De Fronzo et al 1976), and it has been suggested that insulin administered peripherally into the systemic rather than the portal circulation may be responsible for hypertension in type 1 diabetics (Drury 1985). This would not explain its close link with nephropathy however. From our findings in the Australian patients with non-

diabetic renal disease, I have suggested that a defect in renal 11β-OHSD occuring in diabetic nephropathy may account for the observed volume-mediated hypertension. A research programme looking at urinary steroid metabolic profiles and cortisol isotopic studies in a carefully controlled insulin-dependant diabetic population with and without nephropathy has just been submitted for ethical approval.

In conclusion therefore I hope that this thesis has highlighted a previously unsuspected and novel mechanism whereby the action of the principal corticosteroid in man, cortisol is controlled locally by the action of an enzyme 11βeta hydroxysteroid dehydrogenase. I have shown the result of a defect in this "cortisol → cortisone" shuttle, resulting from both a congenital deficiency of the enzyme (i.e. our index case), and acquired inhibition (i.e. following liquorice ingestion).

I have evaluated 11β-OHSD activity in other hypertensive conditions where it may be of clinical relevance, and have discussed in this Chapter my future avenues of research.

I have derived immense pleasure both from performing the enclosed experiments and writing this text. I hope that this thesis will continue to provide an impetus for myself, the Department of Medicine at the Western General Hospital and for other workers in what I'm sure will be an expanding and exciting field of research.

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Publications arising from this Thesis

1. Stewart PM, Shackleton CHL, Edwards CRW. (1987) "The cortisol→ cortisone shuttle and the genesis of hypertension." In: Corticosteroids and peptide hormones in hypertension, F. Mantero, P. Vecsei, eds. Serono publications from Raven Press, Vol 39, pp 163-177.

2. Shackleton CHL, Gutkin M, Stewart PM. (1987) "Disorders of terminal steroid metabolism in hypertension." In: Corticosteroids and peptide hormones in hypertension, F. Mantero, P. Vecsei, eds. Serono publications from Raven Press, Vol 39, pp243-259.

3. Stewart PM, Wallace AM, Valentino R, Burt D, Shackleton CHL, Edwards CRW. (1987) "Mineralocorticoid activity of liquorice: 11βeta hydroxysteroid dehydrogenase deficiency comes of age." Lancet ii: 821-824.

4. Stewart PM, Corrie JET, Shackleton CHL, Edwards CRW. (1988) "The syndrome of apparent mineralocorticoid excess: a defect in the cortisol → cortisone shuttle." J. Clin Invest 82: 340-349.

Abstracts and oral communications

1. Edwards CRW, Stewart PM, Nairn IM, Grieve J, Shackleton CHL. (1985) "Cushing's disease of the kidney." J. Endocrinol 104S: 53.

2. Stewart PM, Edwards CRW. (1986) "11βeta hydroxysteroid dehydrogenase deficiency: the first adult case." Scot Med J. 31: 265. Oral communication to the Scottish Society for Experimental Medicine, Dundee 1986.

3. Stewart PM, Valentino R, Edwards CRW. (1987) "Inhibition of 11βeta hydroxysteroid:NADP dehydrogenase (EC 1.1.1.146) by liquorice." Endocrine Society 1987, abstract 647.
Oral communication to the 69th meeting of the American Endocrine Society, Indianapolis 1987.

4. Valentino R, Stewart PM, Burt D, Edwards CRW. (1987) "Liquorice inhibits 11βeta hydroxysteroid dehydrogenase in the rat." J. Endocrinol 112S: 260.
Communication to the British Endocrine Societies, Warwick 1987.

5. Edwards CRW, Stewart PM. (1987) "Defective cortisol → cortisone shuttle: a novel mechanism in hypertension." J. Steroid Biochem 28: 85.

6. Stewart PM, Wallace AM, Edwards CRW. "The mineralocorticoid activity of liquorice." Presented to the Scottish Society of Physicians, Paisley 1987. Winner of the Fitzgerald Peel Prize.

7. Stewart PM, Burt D, Whitworth J, Valentino R, Atherden SM, Edwards CRW. (1988) "The kidney is a major site of cortisol metabolism." 8th International Congress of Endocrinology, Kyoto Japan, 1988.

8. Stewart PM, Whitworth JA, Burt D, Atherden SM, Edwards CRW. (1988) "Cortisol metabolism in alcohol withdrawal." 8th International Congress of Endocrinology, Kyoto, Japan, 1988.

Grants

1. £17,105 for the investigation of the role of 11βeta hydroxysteroid dehydrogenase in hypertension. Scottish Home and Health Department, January 1986.