RENAL DOPAMINE AND SALT-RETAINING STATES

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Doctor of Philosophy

ASTON UNIVERSITY
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THESIS SUMMARY

Although generally regarded as a neurotransmitter, dopamine is also known to be secreted by the kidney whereby it promotes sodium excretion in its role as a natriuretic hormone. Peripheral dopamine may be formed by two alternative pathways; the decarboxylation of circulating L-Dopa by L-aromatic amino acid decarboxylase (LAAAD), and the desulphation of dopamine sulphate by arylsulphatase A (ASA), the latter being poorly represented in the literature. In many conditions and diseases with which sodium retention is associated, a reduced urinary excretion of dopamine has been noted implicating the involvement of dopamine in the maintenance of sodium homeostasis. This study investigates renal dopamine production via the desulphation of dopamine sulphate in a sample cohort during normal unregulated dietary sodium intake and following a low sodium regimen. After dietary salt restriction urinary dopamine sulphate levels were significantly increased, indicating that dopamine sulphate is indeed a physiological reservoir of active free dopamine, the necessity for which is reduced during salt depletion. This confirmed the dopamine/dopamine sulphate pathway as one which may be relevant to the maintenance of sodium homeostasis. The activity of urinary ASA was investigated in diabetes mellitus as an example of a sodium-retaining state, and compared with that in a matched normal control group. A decreased ASA activity was anticipated, given the blunted dopamine excretion observed in many sodium-retaining states, however an unexpected increase in activity in the diabetic group was observed. Enzyme kinetic analysis of ASA showed that this was not due to the existence of an isoform having an altered affinity for dopamine sulphate. This rather paradoxical situation, that urinary-dopamine is decreased while ASA activity is increased, may be explained by the sequestering of free dopamine by autoxidation to 6-hydroxydopamine as has been hypothesised recently to occur in diabetes mellitus. To confirm the homogeneity of ASA in the normal and diabetic groups, four amplicons spanning the 3637bp intronic and exonic regions of the gene were generated by PCR. These were sequenced utilising a fluorescent-dye terminator reaction using the forward PCR primer as sequencing primer. Although single nucleotide polymorphisms were observed between the two groups these occurred either in intronic regions or, when exonic, generated silent mutations, supporting the enzyme kinetic data. The expression of ASA was investigated to determine the basis of the increased activity observed in diabetes mellitus. Although a validated comparative RT-PCR assay was developed for amplification of *arsa* transcripts from fresh blood samples, expression analysis from archived paraffin-embedded renal tissue was complicated by the low yield and degradation of unprotected mRNA. Suggestions for the development of this work using renal cell-culture are discussed.

Keywords: Arylsulphatase A, Diabetes Mellitus, Housekeeping gene, Gene Expression, High Performance Liquid Chromatography, RT-PCR.

For My Family

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ABBREVIATIONS

6-OHDA	6-hydroxydopamine
A	Adrenaline
AC	Adenylate cyclase
ACE	Angiotensin converting enzyme
actb	β Actin gene
AGE	Advanced gylcated end-products
AMP	Adenosine monophosphate
AMV	Avian myeloblastosis virus
ANP	Atrial natriuretic peptide
arsa	Arylsulphatase A gene
ASA	Arylsulphatase A
AT	Annealing temperature
ATP	Adenosine triphosphate
BMI	Body mass index
bp	Nucleotide base pairs
C_{18}	Octadecyl-silica
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CCD	Cortical collecting duct
cDNA	Complementary DNA
COMT	Catechol-O-methyl transferase
CS	Cerebroside sulphate
DA	Dopamine
DAG	Diacylglycerol
DARPP-32	Dopamine-and-cAMP-regulated phosphoprotein
DBP	Diastolic blood pressure
DCT	Distal convoluted tubule
DEPC	Diethyl pyrocarbonate
DHBA	Dihydroxybenzylamine hydrobromide
DMPC	Dimethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DOMA	3,4-dihydroxymandelic acid

DOPAC	3,4-dihydroxyphenylacetic acid
DOPEG	3,4-dihydroxyphenylethylene glycol
DOPET	3,4-dihydroxyphenylethanol
ECF	Extracellular fluid
ED	Electrochemical detection
EDTA	Ethylenediaminetetraacetic acid
Fe^{2+}	Ferrous ion
gapd	Glyceraldehyde-3-phosphate dehydrogenase gene
GCG	Genetics computer group
GC-MS	Gas chromatography with mass spectrometry
gDNA	genomic DNA
G_p	PLC-coupled G-proteins
G_s	Stimulatory G-proteins
H_2O	Water
HbA1c	Glycated haemoglobin
Hema SB	Sulphobutylhydroxymethacrylate
HPLC	High performance liquid chromatography
hr(s)	Hour(s)
HVA	Homovanillic acid
IDDM	Insulin dependent diabetes mellitus
IFG	Impaired fasting glycaemia
IGT	Impaired glucose tolerance
IP_3	Inositol trisphosphate
KCl	Potassium chloride
K_m	Michaelis constant
L-AAAD (DDC)	L-aromatic amino acid decarboxylase
L-dopa	L-3,4-dihydroxyphenylalanine
LRH	Low Renin Essential Hypertension
MAO	Monoamine oxidase
MAP	Mean arterial pressure
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium chloride
min(s)	Minute(s)
MLD	Metachromatic leukodystrophy
M-MLV	Moloney murine leukaemia virus

MOPEG	3-methoxy-4-hydroxyphenylethylene glycol
MOPET	3-methoxy-4-hydroxyphenylethanol
mRNA	Messenger ribonucleic acid
MSD	Multiple sulphatase deficiency
MTAL	Medullary thick ascending limb
NA	Noradrenaline
Na^+/K^+-ATPase	Sodium-potassium adenosine triphosphatase
NaOH	Sodium hydroxide
NCBI	National center for biotechnology information
NCS	Nitrocatechol sulphate
NIDDM	Non-insulin dependent diabetes mellitus
OGTT	Oral glucose tolerance test
PAGE	Polyacrylamide gel electrophoresis
PBRIK	Paraffin block RNA isolation kit (Ambion, UK)
PCR	Polymerase chain reaction
PCT	Proximal convoluted tubule
PD-ASA	Pseudodeficiency of arylsulphatase A
P-E	Paraffin-embedded
PH	Peak Height
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PKA	Cyclic adenosine monophosphate dependent protein kinase
PKC	Protein kinase C
PLC	Phospholipase C
PNMT	Phenylethanolamine-N-methyl transferase
PP_1	Protein phosphatase 1
PRA	Plasma renin activity
RAA	Renin angiotensin aldosterone system
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
s	Second
SBP	Systolic blood pressure
SD	Standard deviation
SPE	Solid phase extraction
SPSS	Statistical Package for the Social Sciences
TAE	Tris-acetate buffer

Taq	DNA polymerase from *Thermus aquaticus*
TE	Tris-EDTA buffer
TEC	Trace enrichment cartridge
T_m	Theoretical melting temperature
$UDASO_4$	Urinary dopamine sulphate
UFDA	Urinary free dopamine
UK	Urinary potassium
UNa	Urinary sodium
UTDA	Urinary total dopamine
UV	Ultraviolet
VMA	3-methoxy-4-hydroxymandelic acid
WHO	World Health Organisation
yr(s)	Year(s)

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CHAPTER 1

1.INTRODUCTION

1.1 The Kidney

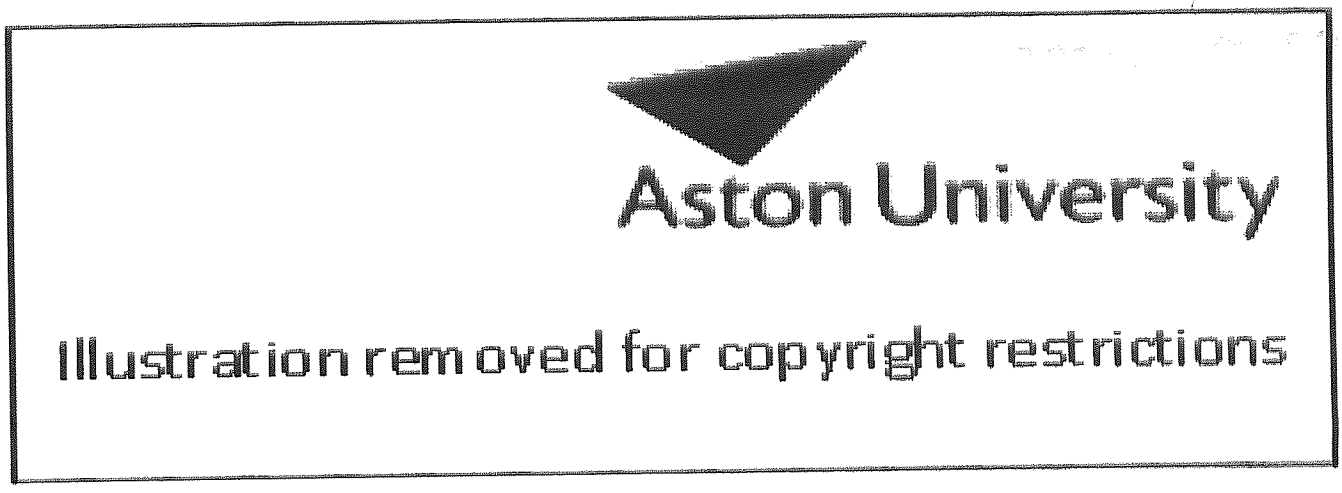
The kidneys play a pivotal role in the maintenance of electrolyte and water homeostasis. Here the structure of the kidney will be discussed with particular reference to the maintenance of sodium balance.

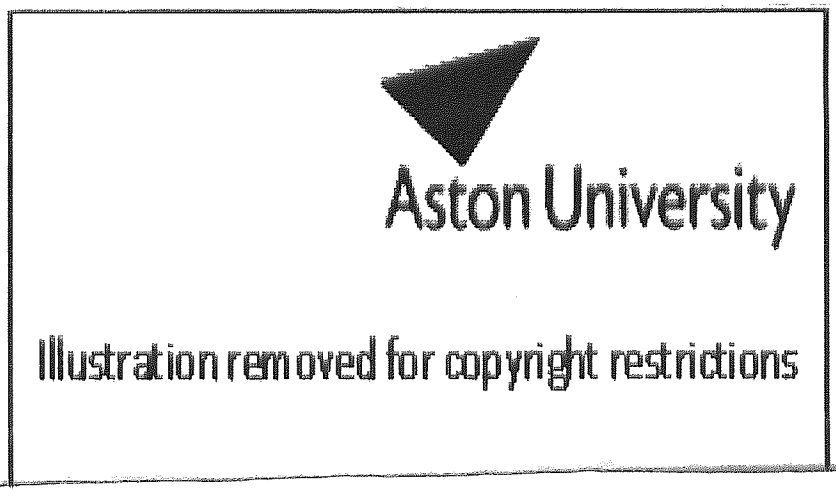
1.1.1 Structure of the Kidney

The kidney consists of two distinct regions, the cortex and medulla. The functional unit of the kidney is the nephron, of which there are approximately 1.3 million within each kidney. The bulk of each nephron is located in the renal cortex. Blood reaches each nephron via the afferent arteriole, is partially filtered via glomerular capillaries, and leaves the glomerulus via the efferent arteriole. It then passes through the peritubular capillaries, at which the majority of electrolyte exchange occurs, and leaves the kidney via the renal vein. The Bowmans capsule encapsulates the glomerulus of the renal cortex and collects fluid filtered by the glomerular capillaries (glomerular filtrate). Separating the blood from the glomerular filtrate are two layers of cells, the capillary endothelium and a layer of specialised epithelial cells which are separated by the non-porous basal lamina. Between the endothelium and the basal lamina are the mesangial cells, which, among other functions, are implicated in the development of glomerular disease. The glomerular filtrate is then passed through the proximal convoluted tubule (PCT) where electrolyte exchange occurs. It then proceeds

through the descending and ascending limbs of the loop of Henle (located in the renal medulla), through the juxtaglomerular apparatus from which renin is secreted (located in the renal cortex) and along the distal convoluted tubule (DCT) to the cortical collecting duct (CCD, figure 1a). From here the filtrate passes to the renal pelvis from which micturition occurs.

Fig. 1a. The Structure of The Human Nephron





(Reproduced from www.cf.ac.uk/biosi/staff/jacob/ teaching/nephpic.html)

This scheme depicts a short-looped and a long-looped nephron together with the collecting system. Not drawn to scale. Within the cortex a medullary ray is delineated by a dashed line.

The maintenance of sodium homeostasis occurs at all three stages of urine formation: glomerular filtration, tubular reabsorption and tubular secretion. Sodium is filtered from the plasma via glomerular filtration and becomes part of the glomerular filtrate. At the PCT, DCT and CCD sodium moves by co-transport or exchange from the tubular lumen into the epithelial cells. From here, it is actively pumped into the interstitium by the sodium-potassium ATPase pump (Na^+/K^+-ATPase) (Sherwood, 1993). Sodium reabsorption is under the control of aldosterone, atrial natriuretic peptide (ANP) and it has been postulated, dopamine (Aperia *et al.*, 1994). Aldosterone promotes sodium reabsorption via the renin-angiotensin-aldosterone (RAA) system in the DCT and CCD. Renin is secreted in response to a decrease in extracellular fluid (ECF) volume, arterial blood pressure, or sodium concentration. This stimulates the formation of angiotensin I from hepatic angiotensinogen. Angiotensin-converting enzyme (ACE), derived from vascular endothelium, catalyses the conversion of angiotensin I to angiotensin II stimulating the adrenal cortex to secrete aldosterone. Aldosterone promotes sodium reabsorption in the DCT and CCD. Dopamine promotes natriuresis in the PCT , DCT and CCD via inhibition of Na^+/K^+-ATPase. Furthermore, dopamine receptors have been localised to all three nephron segments in the rat (Takemoto *et al.*, 1991).

1.2 Catecholamines

Catecholamines are secreted by the adrenal medulla of the adrenal glands which are located above each kidney. They function as both neurotransmitters and hormones. Structurally catecholamines consist of a catechol group (an aromatic structure with two hydroxyl groups) linked to an amine. Noradrenaline, adrenaline and dopamine are formed by the hydroxylation and decarboxylation of dietary phenylalanine and tyrosine (figure. 1b) and are secreted by both autonomic neurones and adrenal medullary cells. In addition, dopamine is secreted by the kidney (Kagedal & Goldstein, 1988). Noradrenaline and adrenaline act upon α and β G-protein-coupled adrenergic receptors located throughout the body, and dopamine acts upon D_1 and D_2, and DA_1 and DA_2 receptors to either inhibit or stimulate adenylate cyclase and / or phospholipase C. The direct biochemical effects of each catecholamine following stimulation of their respective receptors are summarised in table 1a.

Table 1a. Adrenergic and Dopaminergic Receptors

Receptor Type	Response to Catecholamines	Mechanism of Action
α_1	NA > A	Ca^{2+} Release stimulated
α_2	NA > A	AC Inhibited
β_1	NA = A	AC Stimulated
β_2	A > NA	AC Stimulated
D_1	DA > A = NA	AC Stimulated
D_2	DA > A = NA	AC Inhibited
DA_1	DA	AC and PLC stimulated
DA_2	DA	PLC stimulated

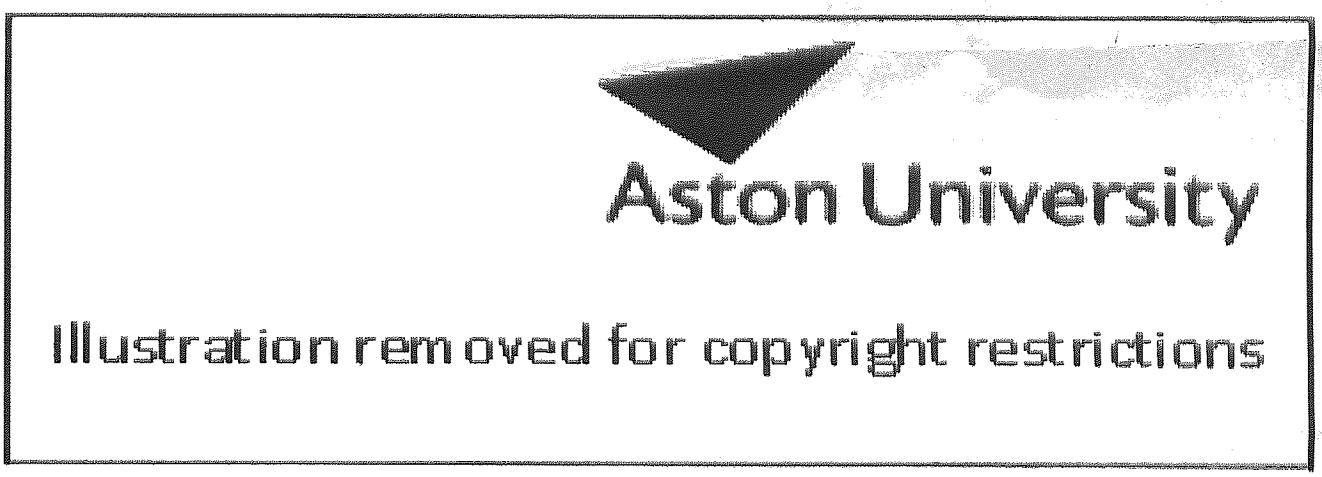
NA = noradrenaline, A = Adrenaline, DA = Dopamine, AC = Adenylate Cyclase, PLC = Phospholipase C (adapted from Ganong, 1991).

In plasma, approximately 98% of dopamine (Kuchel *et al.*, 1984[a]), and 70% of both adrenaline and noradrenaline are conjugated to their chemically inert sulphate or glucuronide conjugates, with sulphation being the major conjugate of dopamine in humans (Abenhaim *et al.*, 1980; Demassieux *et al.*, 1986).

Catecholamines have a half-life of approximately 2 minutes in the circulation. They are methoxylated and then oxidised to 3-methoxy-4-hydroxymandelic acid (VMA, figure 1b). Thus, only small amounts of free noradrenaline and adrenaline are excreted. However, dopamine is found in the plasma and urine in appreciable quantities, half of which is secreted by the adrenal medulla with the remainder having alternative origins including secretion by the kidney (Kagedal & Goldstein, 1988).

Despite their short half-life and low levels in the circulation, the development of increasingly sensitive laboratory techniques allows the detection of catecholamines, their precursors and metabolites in urine (Wu & Gornet, 1985; Green *et al.*, 1989; Cummings *et al.*, 1990; Pastoris *et al.*, 1995; Bayly *et al.*, 1999), plasma (Shum *et al.*, 1982; Smedes *et al.*, 1982; Goldstein *et al.*, 1984; Ito *et al.*, 1984; Eisenhofer *et al.*, 1986; Weiker, 1988; Dutton *et al.*, 1999), and other biological samples including cerebrospinal fluid (Boomsma *et al.*, 1988), brain tissue (Mayer & Shoup, 1983) and human cell lines (Cosentino *et al.*, 2000). Catecholamines can be measured using gas-chromatography with subsequent mass spectrometry (GC-MS), or high performance liquid chromatography (HPLC) with UV / fluorescence detection (Bergquist *et al.*, 2002). However, measurement of urinary catecholamines by HPLC with electrochemical detection (HPLC-ED) is routinely used in the detection and diagnosis of catecholamine-secreting tumours including pheochromocytoma and neuroblastoma (Bergquist *et al.*, 2002).

Fig. 1b. The Biosynthesis and Catabolism of Catecholamines



(Adapted from Bartlett WA, Birmingham Heartlands Hospital, personal communication)

Catabolytes: MOPET = 3-methoxy-4-hydroxyphenylethanol, HVA = Homovanillic acid, MOPEG = 3-methoxy-4-hydroxyphenylethylene glycol, DOPET = 3,4-dihydroxyphenylethanol, DOPAC = 3,4-dihydroxyphenylacetic acid, DOPEG = 3,4-dihydroxyphenylethylene glycol, DOMA = 3,4-dihydroxymandelic acid. Enzymes: 1 = Dopamine-β-mono-oxygenase, 2 = Phenylethanolamine-N-methyl transferase (PNMT), 3 = Catechol-O-methyl transferase (COMT), 4 = Monoamine oxidase (MAO), 5 = Aldehyde reductase, 6 = Aldehyde dehydrogenase, 7 = L-amino acid decarboxylase (L-AAAD), 8 = Tyrosine Hydroxylase.

1.2.1. General Principles of High Performance Liquid Chromatography (HPLC)

A wide variety of biological analytes can be measured quantitatively and qualitatively using HPLC, making it the method of choice within the pharmaceutical, biotechnology, environmental and food industries. The principles of HPLC are based upon those of standard chromatographic techniques, however in HPLC the sample, dissolved in a solvent (mobile phase), is applied to a tightly packed solid support (bound to the stationary phase) at high pressure. The separation of molecules using HPLC relies upon exploitation of the analytes properties, such as hydrophobicity, charge, and size (Bird, 1989). The composition of the mobile and stationary phases are designed to exploit one or more of these properties resulting in retention of the analytes by the column for a characteristic retention time. When the analyte is eluted from the column its properties are then analysed using electrochemical detection methods, or those which exploit the UV or fluorescent properties of the analyte.

1.2.1.1 Measurement of Urinary Catecholamines by High Performance Liquid Chromatography with Electrochemical Detection (HPLC-ED)

Application of HPLC-ED to the detection of catecholamines is favoured over methods such as GC-MS, in which the catecholamines must be derivatised prior to analysis. This process often requires the use of highly toxic organic solvents at relatively high temperatures (Bergquist *et al.*, 2002). When using HPLC to measure urinary catecholamines, a degree of sample treatment is required prior to injection onto the column. Alumina absorption pre-treatment of samples has been employed previously (Goldstein *et al.*, 1984; Ito *et al.*, 1984; Wu & Gornet, 1985; Eisenhofer *et al.*, 1986; Koller, 1988; Weiker, 1988), however methods with 'on-line' sample pre-treatment have now been developed (Bayly *et al.*, 1999).

Reverse-phase HPLC is employed for the analysis of catecholamines (Molnar and Horvath, 1976; Mayer & Shoup, 1983; Goldstein *et al.*, 1984; Ito *et al.*, 1984; Wu & Gornet, 1985; Eisenhofer *et al.*, 1986; Koller, 1988; Weiker, 1988; Green *et al.*, 1989; Cummings *et al.*, 1990; Pastoris *et al.*, 1995; Dutton *et al.*, 1999;Cosentino *et al.*, 2000). Here the stationary phase is made hydrophobic by the covalent binding of long carbon chains to the silica backbone of the solid support (Molnar & Horvath, 1976). The mobile phase, however, is less hydrophobic than the stationary phase, allowing partitioning of the catecholamines from other sample constituents. The catechol ring of dopamine, noradrenaline and adrenaline allow interaction of these compounds with the octadecyl-silica (C_{18}) stationary phase. Catecholamines exist as conjugate acids of strong bases, thus ion pairing is also employed to protect the silica backbone of the stationary phase. Here heptanesulphonic acid is used in the mobile phase allowing the sulphonate ion to bind to the catecholamine producing a neutral ion pair. Some methods employ a trace enrichment cartridge (TEC) (Bayly *et al.*, 1999; Dutton *et al.*, 1999) which is located upstream of the column, allowing the solid phase extraction (SPE) of the catecholamines onto a Hema SB (sulphobutylhydroxymethacrylate) cation exchange resin. This extracts the catecholamines from the biological material in which they are to be measured prior to entry into the mobile phase. Many HPLC systems include a 'pre-column' which is located just before the column and protects it from damage by any interfering compounds or particulate material which may block the column. The catechol ring structure of the catecholamines makes these compounds susceptible to oxidation at a given potential, to their respective quinones. This transfer of electrons is converted to a voltage signal which is sent to an integrator. The detector response is directly proportional to the concentration of the analyte (Bergquist *et al.*, 2002), with amperometric detection being most applicable to routine catecholamine analysis.

1.2.1.2 Measurement of the Sulphate Conjugates of Catecholamines

The direct measurement of catecholamine sulphates by HPLC-ED has been described (Elchisak, 1983[a]), however very high oxidation potentials are required. Most methods use pre- or post-column desulphation of catecholamine sulphates with the concentration of the sulphate conjugates taken as the difference between the total and free catecholamine concentrations (Weiker, 1988; Yoshimura *et al.*, 1993). A method has been described in which hot perchloric acid is used to deconjugate the catecholamines once they have eluted from the column but before they are detected (Elchisak, 1983[b]). However this method would be considered a safety hazard in most modern laboratories.

An automated method for the detection of urinary catecholamines and their sulphates has been described (Bayly *et al.*, 1999): this method employs reverse phase isocratic ion-paired HPLC with reductive mode electrochemical detection. Briefly, 'on line' sample pre-treatment is performed whereby an aliquot of the sample is spiked with the internal standard dihydroxybenzylamine hydrobromide (DHBA), and adjusted to pH 8.3. It is then injected onto a trace enrichment cartridge (TEC) allowing SPE of catecholamines. The TEC is then switched in line with the column, and the catecholamines eluted onto the column at pH 3.5. Detection occurs via an ESA Coulochem II (ESA Analytical Ltd, UK) electrochemical detector in reductive mode with a model 5021 conditioning cell set at +0.35V in series with a model 5011 analytical cell with settings E1 = -0.05V and E2 = -0.35 V. A second round of analysis is performed on the same sample after automated enzymatic desulphation of catecholamine sulphate by arylsulphatase. This method is employed in the measurement of urinary dopamine and dopamine sulphate (Chapter 2).

1.2.2 Dopamine

The chemical formula for Dopamine is $C_6H_5(OH)_2(CH_2)_2NH_2$ with its most well understood role being that of a neurotransmitter (Smit *et al.*, 1995). A deficiency of neural dopamine is seen in Parkinson's disease, and may be treated by the administration of the dopamine precursor, L-3,4-dihydroxyphenylalanine (L-dopa) (Routh *et al.*, 1971; Senard *et al.*, 1995). Metabolism of dopamine leads to the formation of noradrenaline, via dopamine-β-hydroxylase, which in turn forms adrenaline through the action of phenylethanolamine-N-methyl transferase (PNMT). Other metabolites of dopamine include dihydroxyphenylacetic acid (DOPAC) produced by monoamine oxidase (MAO) and methoxytyramine via the enzyme catechol-O-methyl transferase (COMT) (Fig.1b, page 23).

Developments in the study of plasma (Yoneda *et al.*, 1983; Yoshizumi *et al.*, 1995) and urine catecholamines (Sowers *et al.*, 1988; Gill *et al.*, 1991; Clark *et al.*, 1992), together with extensive animal studies (Baines & Chan 1980; Zimlichman *et al.*, 1988; Yoshizumi *et al.*, 1992[a,b]), have led to the finding that peripheral dopamine has an important role as a hormone. Although plasma free dopamine is often available in very low amounts, its measurement has illustrated the role of dopamine in the peripheral sympathetic nervous system (Miura *et al.*, 1995). However, the role of renal dopamine as a natriuretic hormone is more accurately determined through the measurement of urinary dopamine. The ingestion of protein causes an increase in urinary dopamine excretion which is accounted for by the measurement of urinary creatinine and subsequent correction for creatinine in the calculation of urinary catecholamine concentration (Williams *et al.*, 1986).

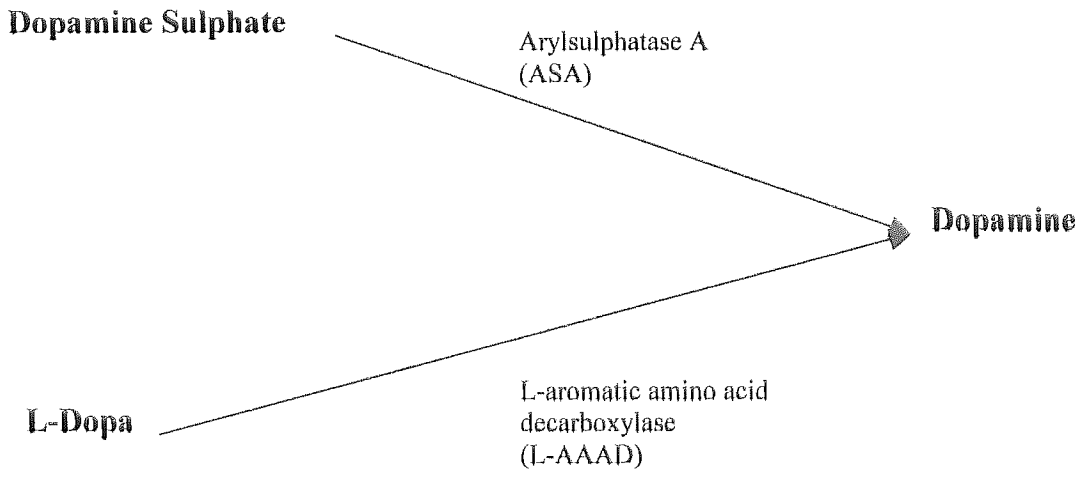
1.2.2.1 Sources of Renal Dopamine

Dopamine concentrations in urine are much greater than can be accounted for by glomerular filtration, suggesting that it is produced intrarenally (Oates *et al.*, 1979; Wolfovitz *et al.*, 1993; Goldstein *et al.*,1995). There is an increase in urinary dopamine levels in response to corticosteroid treatment, which has been shown to originate from an increase in the renal production of dopamine (Schoors *et al.*, 1990).

Catecholamine conjugates represent the predominant form of circulating catecholamines, with sulphation (as opposed to glucuronidation) being the major conjugate in humans (Imai *et al.*, 1970; Abenhaim *et al.*, 1980; Demassieux *et al.*, 1986). There are two proposed sources of renal dopamine. The first is the renal tubular uptake of L-dopa from the circulation, and its decarboxylation via the enzyme L-aromatic amino acid decarboxylase (L-AAAD) (Ball & Lee, 1977; Zimlichman *et al.*, 1988; Hayashi *et al.*, 1990; Meister & Apeira, 1993; Wolfovitz *et al.*, 1993; Goldstein *et al.*, 1995) which is present in the renal proximal tubules (Meister *et al.*, 1992). The second proposed source of renal dopamine is via the deconjugation of circulating dopamine sulphate by Arylsulphatase A (ASA) (Brown & Allison,1981; Demassiuex *et al.*, 1986; Goldstein *et al.*, 1999, figure 1c).

Dopamine sulphate exists in two isomeric forms, dopamine-3-O-sulphate and dopamine-4-O-sulphate (Elchisak, 1986) upon which arylsulphatase A acts via the mechanism proposed in section 1.4.2.2.

Fig. 1c. Sources of Renal Dopamine



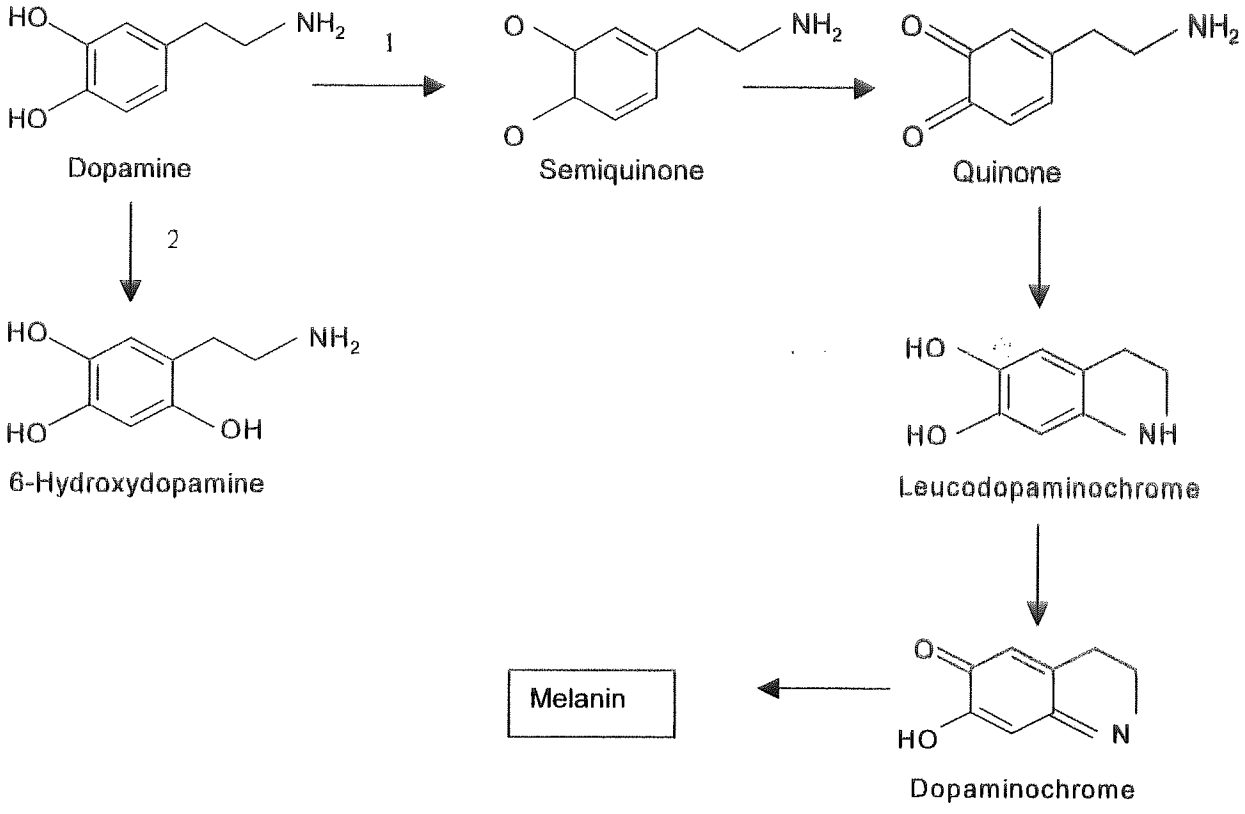
Renal dopamine is formed from the decarboxylation of circulating L-Dopa, and the desulphation of dopamine sulphate by ASA.

1.2.2.2 Oxidation of Dopamine

In the presence of molecular oxygen, dopamine, noradrenaline and adrenaline autoxidise (Graham *et al.*, 1978[a], Graham *et al.*, 1978[b], Chiueh *et al.*, 1993, Chetty *et al.*, 2002). Dopamine is more easily autoxidised than noradrenaline and adrenaline at alkaline pH. It is non-enzymatically converted to semi-quinone, quinone, and melanin. This autoxidation is also catalysed by transition metals (e.g. manganese) and the ferrous ion (Fe^{2+})(Chiueh *et al.*, 1993). *In vivo*, dopamine is thought to be autoxidised to 6-hydroxydopamine (6-OHDA) (Andrew *et al.*, 1993; Tong & Baines 1993) resulting in the generation of hydrogen peroxide (Bindoli *et al.*, 1992; Jellinger *et al.*, 1995), itself a catalyst for the conversion of dopamine to 6-OHDA via the release of Fe^{2+} (Linert *et al.*, 1996). The cytotoxic effects of 6-OHDA have been recognised for nearly 30 years (Lundstrom *et al.*, 1973; Kostrzwa & Jacobowitz, 1974). Previous literature suggests that dopamine oxidation products demonstrate cytotoxicity to neural cells and may contribute to the nigrostriatal neurodegeneration observed in parkinsons disease (Graham *et al.*, 1978[a]; Chiueh *et al.*, 1993; Jellinger *et al.*, 1995; Linert *et al.*, 1996).

Elevated urinary 6-OHDA has been observed in patients with diabetes mellitus complicated by diabetic nephropathy (Chetty *et al.*, 2002), and it has been postulated that dopamine autoxidation may contribute to the development of nephropathy, or could arise as a result of this complication of diabetes mellitus. The autoxidation of dopamine is illustrated in figure 1d.

Fig. 1d. Autoxidation of Dopamine



Dopamine is autoxidised to its quinone, which on cyclisation leads to the formation of dopaminochrome and melanin (pathway 1). An alternative pathway of dopamine oxidation involves the formation of the cytotxin 6-OHDA (pathway 2).

1.2.2.3 Proposed Natriuretic Mechanism of Dopamine

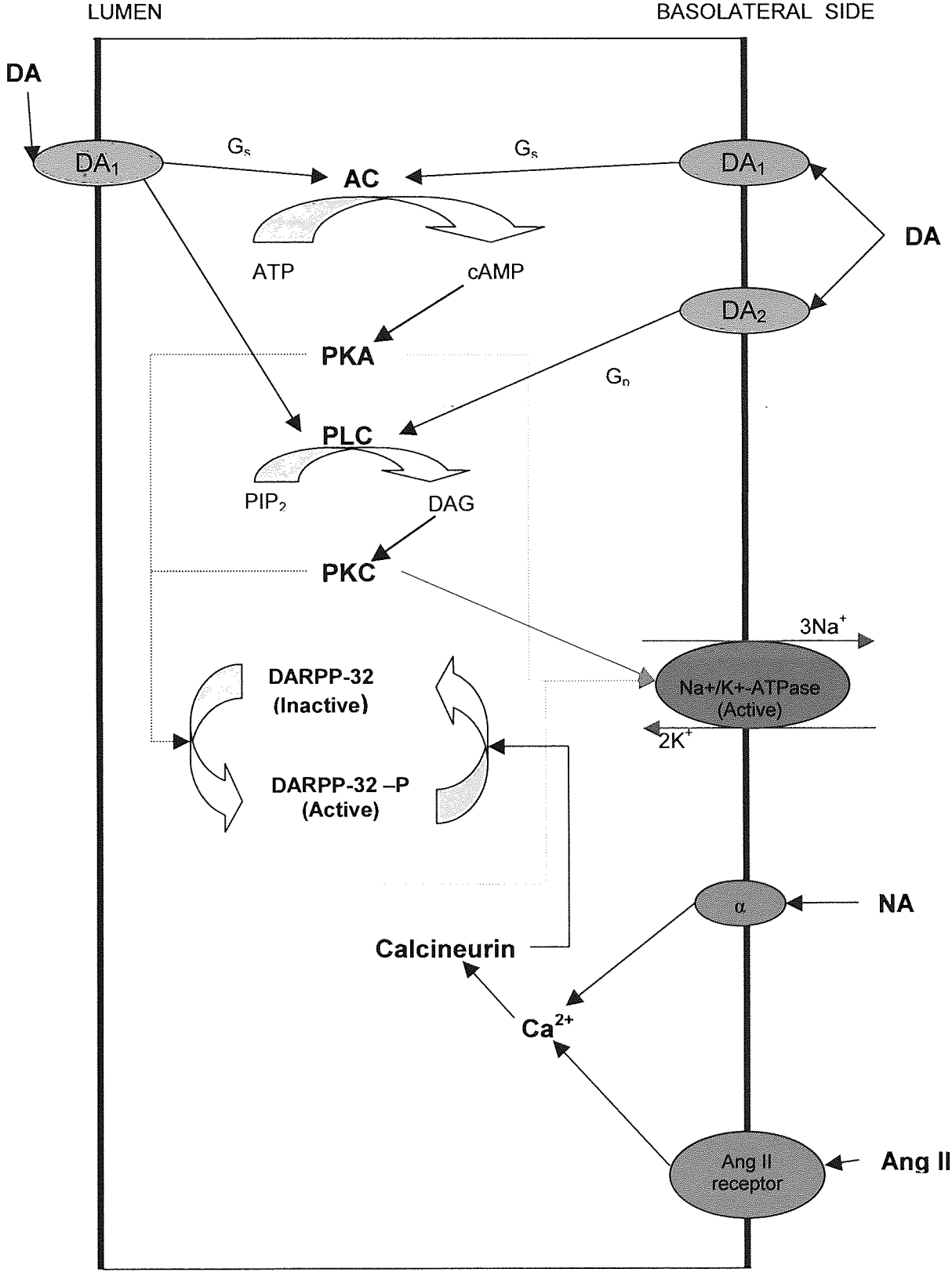
Dopamine is known to cause natriuresis by inhibition of tubular sodium reabsorption in the proximal convoluted tubule (PCT), the medullary thick ascending limb (MTAL) and the cortical collecting duct (CCD) of the kidney. Dopamine exerts its natriuretic effect via the inhibition of Na^+/K^+-ATPase, which is present on the basolateral membrane of all tubular cells in high concentrations (Aperia *et al.*, 1994). Dopamine binds to DA_1 receptors on both the luminal and basolateral sides of the proximal tubule cell (Hubbard & Henderson, 1995). In doing so, it stimulates adenylate cyclase via stimulatory G-proteins (G_s) and promotes the formation of cyclic AMP (cAMP). This then activates cAMP-dependent protein kinase (PKA) which phosphorylates the α subunit of Na^+/K^+-ATPase thereby rendering it inactive. Protein kinase A also phosphorylates the phosphoprotein dopamine-and-cAMP-regulated phosphoprotein (DARPP-32). In its phosphorylated form, DARPP-32 is a potent inhibitor of protein phosphatase 1 (PP_1), preventing the dephosphorylation of Na^+/K^+-ATPase (Aperia *et al.*, 1994). In binding to DA_1 receptors on the luminal side of the PCT cell dopamine also stimulates phospholipase C (PLC) via PLC-coupled G-proteins (G_p). This then promotes the formation of inositol trisphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol-4,5-bisphosphate (PIP_2). Protein kinase C is stimulated by DAG. This promotes the formation of the phosphorylated and therefore inactive form of Na^+/K^+-ATPase (Lokhandwala *et al.*, 1995) both directly and via DARPP-32. Binding of dopamine to DA_2 receptors on the basolateral membrane activates PLC via G_p subsequently stimulating the formation of IP_3 and DAG. The stimulation of PKC by DAG and the subsequent action of PKC upon DARPP-32 and Na^+/K^+-ATPase is as described previously. Conversely, noradrenaline (NA) and angiotensin II (via α adrenergic and angiotensin II receptors on the basolateral membrane respectively) raise intracellular calcium levels, and as a result activate the calcium / calmodulin-dependent phosphatase, calcineurin. When activated, calcineurin

dephosphorylates, and therefore activates Na^+/K^+-ATPase directly, as well as dephosphorylating DARPP-32. The deactivated DARPP-32 can no longer inhibit PP_1, thus promoting the formation of the dephosphorylated, and therefore active, form of Na^+/K^+-ATPase (Aperia *et al.*, 1994). The natriuretic effect of dopamine requires the presence of noradrenergic tone provided by the renal nerves, and the phosphorylation state of Na^+/K^+-ATPase may be affected by intracellular sodium concentration (Ibarra *et al.*, 1993). The mechanism of action of dopamine as a natriuretic is illustrated in figure 1e.

The exact mechanism of action of dopamine as a natriuretic in the MTAL and CCD remains unclear. Satoh *et al.* (1995) suggest that the binding of dopamine to DA_1 receptors alone causes the activation of PKA via cAMP in the MTAL and CCD, which leads to inhibition of Na^+/K^+-ATPase. The activation of PLC-PKC is not involved in dopamine-induced natriuresis in these tubular segments. These authors also propose that the inhibition of PCT Na^+/K^+-ATPase requires binding of dopamine to both DA_1 and DA_2 receptor subtypes leading to PKC activation. However, Lokhandwala *et al.* (1995) suggest that DA causes activation of PLC in renal cortical cells via DA_1 receptors. The subsequent formation of IP_3 and DAG then promote Na^+/K^+-ATPase inhibition as described previously. A review by Hubbard & Henderson proposes an AC-cAMP-DARPP-32 mediated pathway, but a consensus for dopaminergic natriuresis in the MTAL and CCD has not been reached.

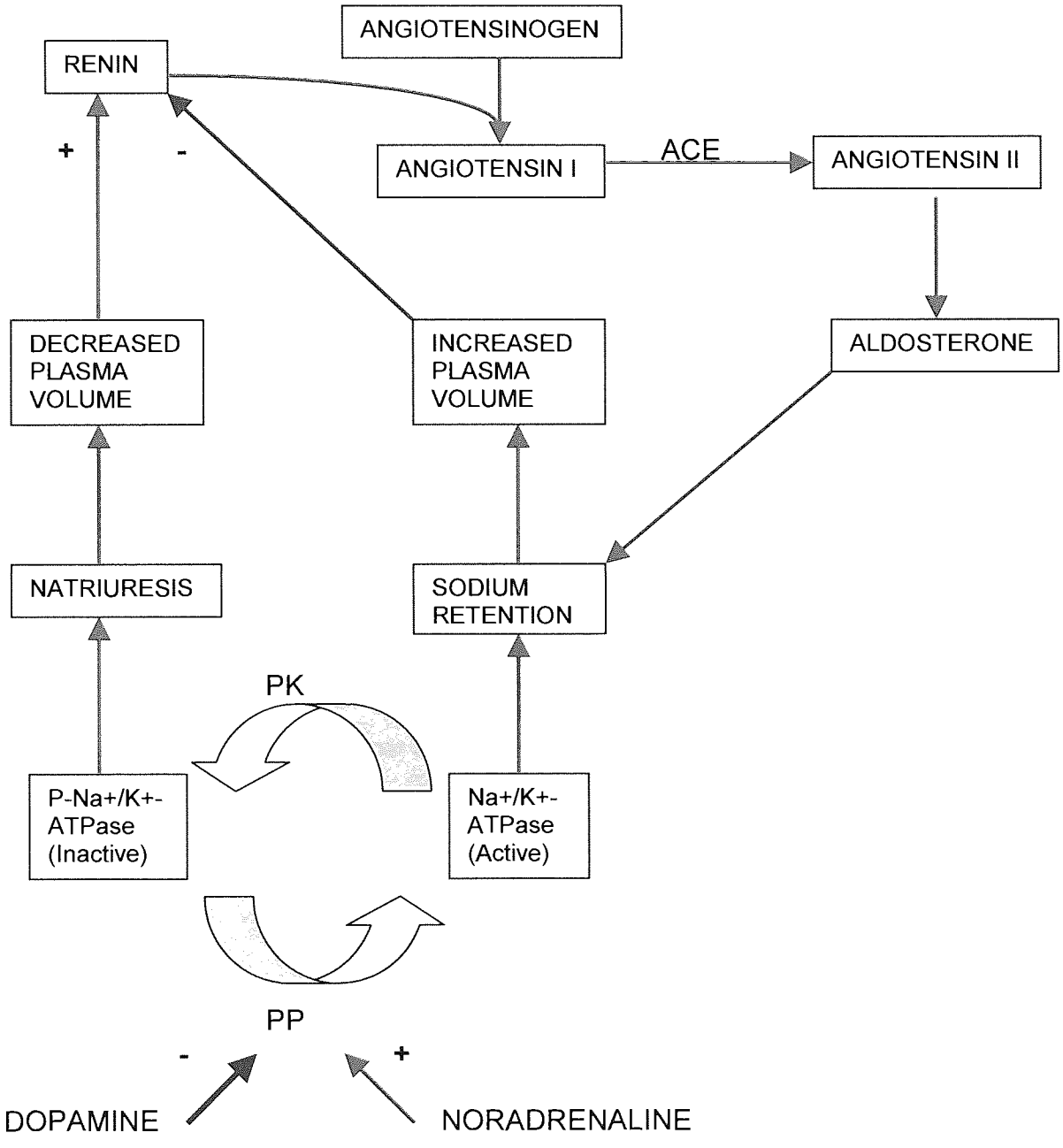
As a natriuretic, dopamine may play a role in the maintenance of sodium homeostasis as a blunted production of renal dopamine has been observed in a number of salt-retaining states. One proposed mechanism illustrating the interaction of the Renin-Angiotensin-Aldosterone system with that of dopamine and noradrenaline in the maintenance of sodium homeostasis, is illustrated in figure 1f.

Fig. 1e Proposed Mechanism of Action of Dopamine as a Natriuretic in the Proximal Convoluted Tubule



DA = dopamine, DA_1 = dopamine receptor type 1, DA_2 = dopamine receptor type 2, NA = noradrenaline, α = α-adrenergic receptor, Ang II = angiotensin II, G_s = stimulatory G-protein, G_p = phospholipase C-coupled G-protein, AC = adenylate cyclase, ATP = adenosine triphosphate, cAMP = adenosine-3,5-cyclic monophosphate, PKA = protein kinase A, PLC = phospholipase C, PIP2 = phosphoinositol trisphosphate, DAG = diacylglycerol, PKC = protein kinase C, DARPP-32 = dopamine and cAMP-dependent phosphoprotein. Arrows in green indicate inhibition; arrows in black indicate stimulation, activation or an increase in intracellular calcium.

Fig. 1f. Mechanism for the Role of Dopamine in the Maintenance of Sodium Homeostasis



PP = Protein Phosphatase; PK = Protein Kinase; ACE = Angiotensin Converting Enzyme

1.3 Salt-Retaining States

Congestive heart failure, nephrotic syndrome and cirrhosis are clinical conditions associated with the renal retention of sodium. Dopamine is known to cause natriuresis as described in section 1.2.1.3, and it is thought that a defective renal dopaminergic system may contribute to the retention of sodium in some salt-retaining states.

Arakawa *et al.* (1995) reported an increase in urinary free dopamine during the early stages of mild exercise, which correlated with urinary sodium excretion. Mild exercise appeared to activate the renal dopaminergic system thus promoting natriuresis and contributing to the regulation of plasma volume, and consequently blood pressure. This is further supported by the work of Sakai *et al.* (1995) who observed an increased plasma free dopamine in those completing a half-marathon. Strobel *et al.* (1990) found that decreasing the pH from 7.4 to 6.9 increased the affinity of both dopamine sulphate isomers for arylsulphatase A indicating a possible increase in deconjugation of dopamine sulphate to active free dopamine with exercise. The increase in plasma lactic acid which occurs during exercise may result in a decrease in the pH of blood from the physiological 7.4. This decreased pH may increase the affinity of dopamine sulphate for arylsulphatase A.

Nakaya *et al.* (1994) observed an increase in the production of dopamine from dopamine sulphate in heart failure patients. During the acute phase of the condition, plasma levels of free dopamine increased and levels of dopamine sulphate significantly decreased when compared to corresponding levels in a normal control group. However, as the condition improved, plasma free dopamine levels fell and those of plasma dopamine sulphate rose. One explanation for this could be that during the acute phase of the condition, dopamine sulphate

was deconjugated to form free dopamine in an attempt to compensate for the sodium-retaining effects of heart failure.

The pathology of hypertension and diabetes mellitus, two conditions which can be salt-retaining, are outlined below.

1.3.1 Hypertension

The sustained elevation of systemic arterial pressure is defined clinically as hypertension. The levels at which the blood pressure is considered abnormal are somewhat arbitrary, being based on an increased risk of cardiovascular complications notably coronary heart disease and stroke. A minority of cases can be attributed to a specific cause and such cases are known as secondary hypertension. Causes of secondary hypertension can be categorised as follows:

1.3.1.1 Cardiovascular Hypertension

Atherosclerosis can lead to chronically elevated total peripheral resistance and therefore an elevation of blood pressure. Coarctation of the aorta may have a similar effect and lead to an increased blood pressure. Both cases can be classified as cardiovascular hypertension.

1.3.1.2 Renal Hypertension

An occlusion of one or both renal arteries or hypertension as caused by renal disease e.g. glomerulonephritis, pyelonephritis, and polycystic disease are termed renal hypertension or renovascular hypertension. This is not to be confused with hypertension due to sodium retention of the kidney as described in section 1.3.1.6.

1.3.1.3 Endocrine Hypertension

Adrenocortical disease such as Conn's syndrome (hypersecretion of aldosterone), or Cushing's syndrome (hypersecretion of glucocorticoids) cause hypertension, as do pheochromocytoma and other catecholamine-secreting tumours. These cases are classified as endocrine hypertension.

1.3.1.4 Neurogenic Hypertension

Neural lesions e.g. a defect in the cardiovascular control centre or a defect of the baroreceptors can lead to an increase in blood pressure. Hypertension can also arise as a compensatory response to a reduction in blood flow caused by a neural tumour. Such cases are termed neurogenic hypertension.

1.3.1.5 Essential Hypertension

The majority of cases of hypertension are described as primary or essential hypertension (Ganong, 1991; Sherwood, 1993). Since dopamine plays a role in sodium balance and essential hypertension may be due in part to sodium retention and an expanded ECF volume, dopamine may have a pathogenetic role in hypertension. Two subtypes of essential hypertension have been described:

1.3.1.5 1 Low Renin Essential Hypertension (LRH)

Low renin essential hypertension (LRH) is defined as an increased blood pressure accompanying low plasma renin activity (PRA) without demonstrable hypersecretion of aldosterone, and may account for 20–30% of the hypertensive population (Re *et al.*, 1978). A decreased urinary excretion of dopamine has been observed in LRH as compared to that observed in normal renin essential hypertension and normotensive subjects. This indicates that there is a reduction in the renal production of dopamine from L-dopa in those with essential hypertension and low plasma renin levels (Aoki *et al.*, 1989; Iimura, 1996). Renal dopamine may therefore play a part in the maintenance of sodium homeostasis in such subjects.

1.3.1.5.2 Salt-Sensitive Hypertension

This form of hypertension is defined as a change in blood pressure in response to an alteration in body electrolyte and therefore water content. Furthermore, salt sensitive normotensive

subjects are more likely to develop hypertension than their salt-insensitive counterparts. The classification of salt sensitivity may be problematic as there is no single clinical index which has the power to predict salt-sensitivity (Mattes & Faulkner, 1999). An increased urinary excretion of L-dopa and a low dopamine : L-dopa ratio has been observed in a population of subjects with salt-sensitive normal renin essential hypertension, when compared with their non-salt-sensitive counterparts (Gill *et al.,* 1991). This indicates a decreased renal dopamine production from L-dopa and further supports the role of renal dopamine in sodium homeostasis. Salt-sensitivity has been observed in both hypertensive, and normotensive subjects of Afro-Caribbean origin (Sowers *et al.,* 1988; Flack *et al.,* 1991). Such subjects have a blunted increase in renal dopamine production in response to a salt load. Salt-sensitivity has been reported to be more common in subjects of this ethnicity than in Caucasian subjects, and it is believed that racial differences in the response to dietary sodium demonstrate a genetic component to salt-sensitivity (Svetkey *et al.,* 1996). However, it has recently been reported that salt-sensitivity in normotensive Afro-Caribbean subjects may be due to a deficiency of dietary potassium (Morris *et al.,* 1999).

Previous studies have demonstrated a defect in the renal dopaminergic system and the contribution of a defect in this system to salt-sensitivity (i.e. an increase in blood pressure as a result of an increased sodium load). There is a two-fold increase in activity of PCT Na^+/K^+-ATPase in Dahl salt-sensitive rats fed a high salt diet compared to salt resistant rats which showed no change in PCT Na^+/K^+-ATPase activity (Nishi *et al.*, 1993).

As described in section 1.2.1.3, dopamine inhibits Na^+/K^+-ATPase via PLC-PKC pathway. In salt-sensitive hypertensive rats, PKC activation following activation of DA_1 receptors is abolished and would account for the failure of dopamine to promote natriuresis in hypertensive animals. This may be due to the defective coupling of DA_1 to G_p on the basolateral membrane (Lokhandwala *et al.*, 1995).

1.3.2 Diabetes Mellitus

Diabetes mellitus affects 2% or more of the UK population with the majority of cases being due to insulin resistance (type 2 diabetes mellitus). The prevalence of type 2 diabetes is increasing in line with obesity in the population. In 1999, a WHO report described updated criteria for the diagnosis and classification of diabetes mellitus (Alberti & Zimmet, 1999). These are defined as a fasting plasma glucose concentration of $\geq$ 7.0 mmol/L, or a two-hour plasma glucose concentration of $\geq$ 11.1 mmol/L after 75g glucose load in an oral glucose tolerance test (OGTT), or a random plasma glucose concentration of $\geq$ 11.1mmol/L, accompanying symptoms of diabetes. Such symptoms include polyuria, polydipsia, blurring of vision and unexplained weight loss. The report highlighted the classification of conditions of abnormal glucose handling describing the terms 'Impaired Glucose Tolerance' (IGT) and 'Impaired Fasting Glycaemia' (IFG). Impaired glucose tolerance is a stage of impaired glucose regulation where the fasting plasma glucose concentration is < 7.0 mmol/L and OGTT two hour plasma glucose concentration $\geq$ 7.8mmol/L but < 11.1 mmol/L. Whereas IFG classifies individuals who have fasting glucose values above the normal range but below those diagnostic of diabetes, i.e. a fasting plasma glucose $\geq$ 6.1 mmol/L but < 7.0 mmol/L. (http://www.diabetes.org.uk/infocentre/carerec/newdiagnotic.htm). There are two distinct types of diabetes mellitus classified according to the ability of the pancreatic β cells to secrete insulin; type 1 or insulin dependent diabetes mellitus (IDDM) and type 2 or non-insulin dependent diabetes mellitus (NIDDM). Type 1 diabetes mellitus is caused by an inability to produce insulin due to an immune mediated destruction of pancreatic islet β cells. The genetic component of type 1 diabetes mellitus is not as strong as in type 2 diabetes mellitus, with the concordance rate of identical twins being approximately 50%. Type 2 diabetes mellitus is characterised by a normal, or on occasion elevated level of insulin secretion

coupled with a desensitisation of tissue to the metabolic effects of insulin. This can be due to a decrease in the number of insulin receptors in the adipose tissue although this is not usually the cause of insulin resistance. There is a strong genetic component to the development of type 2 diabetes mellitus with a concordance rate in identical twins of close to 100%. Management of both types of diabetes mellitus is based upon maintenance of a normal blood glucose concentration. This can be achieved by the administration of insulin in type 1 diabetes mellitus. Strict dietary management either with or without orally active drugs which either increase insulin secretion or enhance insulin action are administered in type 2 diabetes mellitus. However, if managed inadequately both type 1 and type 2 diabetes mellitus carry the risk of the development of chronic complications affecting both the large blood vessels (atherosclerosis) or small blood vessels (microangiopathy). In microangiopathy a thickening of the basement membrane leads to disease affecting principally the retinal, renal and neuronal microvasculature. The accumulation of sorbitol in the tissues and the formation of advanced glycated end-products (AGE) which cross-link matrix proteins are thought to contribute to the development of diabetic microangiopathy.

1.3.2.1 Retinopathy

Scarring of the retina can lead to visual impairment due to macular disease and blindness. Retinopathy can be classified according to the findings on retinal examination (fundoscopy) as non-proliferative diabetic retinopathy, proliferative diabetic retinopathy or maculopathy. Good glycemic control may reduce the incidence of retinopathy by 25% (http://medweb.bham.ac.uk/easdec/audit_commission.htm.).

1.3.2.2 Neuropathy

Here peripheral neuropathies predominantly of the sensory nerves, lead to impairments in sensation, and because of anaesthesia in the feet can be responsible for neuropathic foot ulcers.

1.3.2.3 Nephropathy

Increased extracellular matrix and mesangial matrix volume are characteristic of the development of diabetic nephropathy. However, glucose can lead to the production of advanced glycated end-products (AGE) and in doing so generates reactive oxygen species. The formation of AGE occurs non-enzymatically through the oxidation and glycation of proteins when incubated with ketosamines (amadori products of the Maillard reaction). The generation of oxygen free radicals can be induced by AGEs at the site of receptor-AGE interaction and can be inhibited by the presence of antioxidants (Yan *et al.*, 1994). The formation of reactive oxygen species through AGEs may promote the autoxidation of dopamine to 6-OHDA, itself a cytotoxin (Lundstrom *et al.*, 1973; Kostrzwa & Jacobowitz, 1974). Increased intracellular glucose can stimulate PKC in mesangial cells resulting in the activation of cytokines and growth factors which ultimately leads to the 'laying down' of both physiological and non-physiological collagen species, causing microangiopathy. Protein kinase C is also indirectly activated by dopamine in the PCT and probably the MTAL and CCD. This brings into play one of the possible roles of dopamine in the development of diabetic nephropathy. A second proposed role of renal dopamine in diabetic nephropathy is via its autoxidation to 6-OHDA. An elevated 6-OHDA is seen in diabetic subjects with nephropathy when compared to non-nephropathic patients with diabetes mellitus and it has

been proposed that the formation of 6-OHDA from dopamine may contribute to the development of nephropathy (Chetty *et al.*, 2002). A decrease in urinary excretion of dopamine has been observed in patients with type 1 diabetes mellitus (Segers *et al.*, 1995). Stenvinkel *et al.* (1991) demonstrated that urinary dopamine excretion was not influenced by sodium chloride infusion in patients with type 1 diabetes, whereas in a normal control group an increase in urinary dopamine of approximately 15% was observed. The type 1 group also displayed an impaired urinary sodium excretion when compared with controls. In response to a dietary salt load, type 1 patients showed no change in urinary dopamine excretion as opposed to a control group whose urinary dopamine excretion increased significantly (Rudberg *et al.*, 1997). A blunted renal dopamine production was also observed in patients with type 2 diabetes mellitus (Shigetomi *et al.*, 1995). Subjects with chronic renal failure have been shown to have a blunted response to dopamine infusion (Casson *et al.*, 1983). Furthermore, patients with type 2 diabetes mellitus complicated by nephropathy demonstrate a decreased excretion of urinary dopamine as compared with normal control subjects and non-nephropathic patients with type 2 diabetes (Murabayashi *et al.*, 1989).

In both type 1 and type 2 diabetes mellitus there is a lack of renal dopamine. This may be due to either decreased renal dopamine production, or the rapid autoxidation of dopamine. Hence dopamine may be less able to promote natriuresis leading to sodium retention and this ineffective natriuresis may contribute to the development of hypertension in diabetes mellitus. The increased autoxidation of dopamine to 6-OHDA in patients with diabetes mellitus may contribute to the development of nephropathy via the effect of 6-OHDA itself together with the resulting formation of reactive oxygen species.

1.4 Arylsulphatase A

One proposed source of renal dopamine is via the desulphation of circulating dopamine sulphate to dopamine by Arylsulphatase A (ASA, EC 3.1.6.1; Stinshoff, 1972). This lysosomal enzyme is a member of the sulphatase enzyme family, which comprises arylsulphatases A, B, C (steroid sulphatase), D and E, N-acetylgalactosamine 6-sulphatase, N-Acetylglucosamine 6-sulphatase, Irudonate Sulphatase, and Sulphamidase (Waldow *et al.*, 1999). Arylsulphatase A (ASA) was first purified from *Aerobacter aerogenes* strain 9621 in 1963 (Fowler & Rammler, 1963). Arylsulphatase A activity has been observed in most human tissues (Kreysing, *et al.*, 1990). Of note is the finding that the renal cortex of the rat demonstrates a high ASA activity (Leznicki & Rozanska, 1991). As a consequence of its ubiquitous nature ASA is thought to be of physiological significance in the deconjugation of catecholamine sulphate isomers, and thus it may be of importance in the regulation of catecholamine levels (Strobel, *et al.*, 1990), most notably the levels of dopamine.

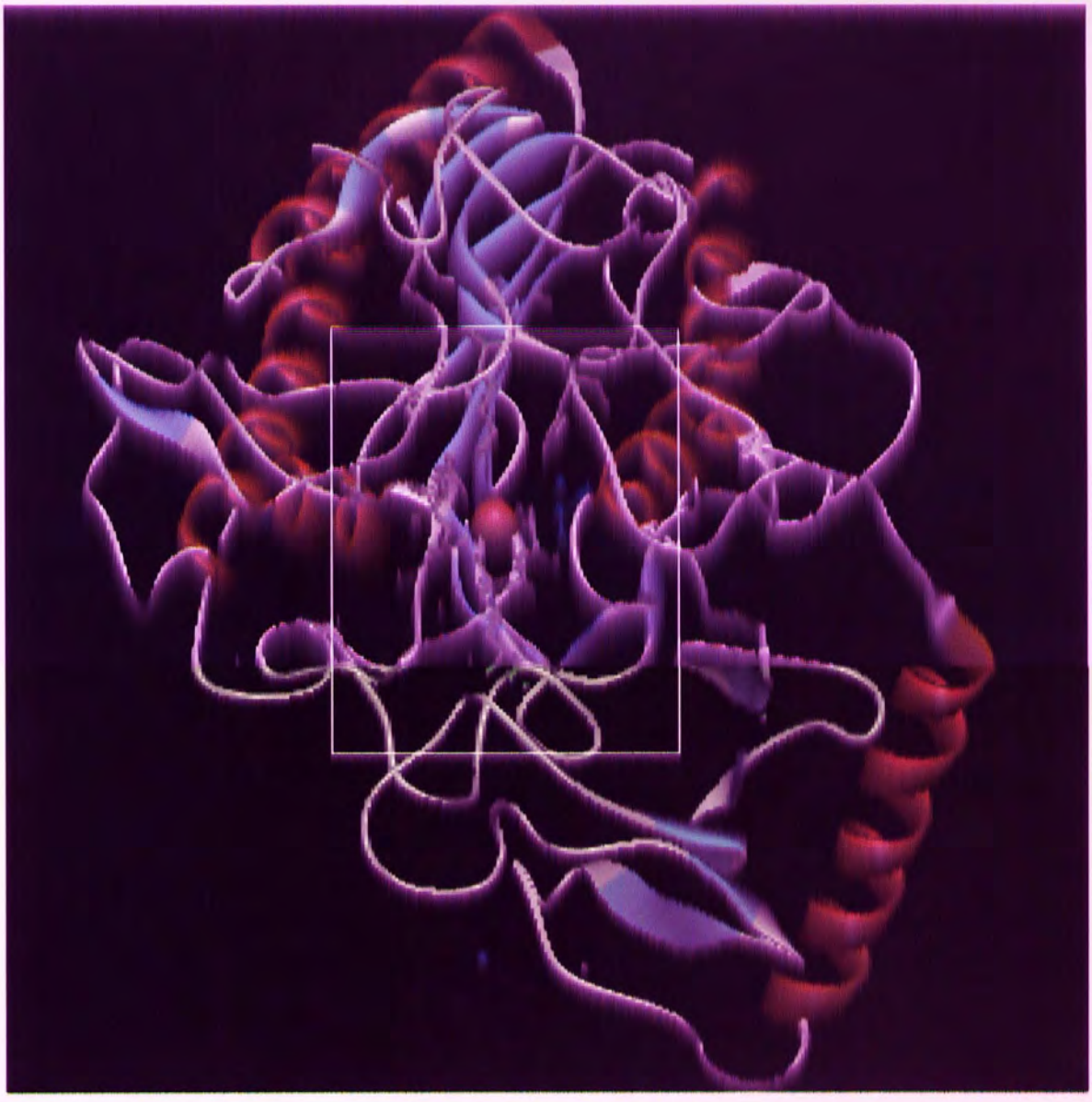
1.4.1 Structure of Arylsulphatase A

By X-ray diffraction crystallography Lukatela *et al.* (1998) determined the crystal structure of ASA. It is composed of a homo-octamer comprising a tetramer of dimers $(\alpha_2)_4$ at acidic pH (characteristic of lysosomes), which at neutral pH dissociates into its four dimer components. Several amino acid residues make up the active site of ASA with one residue being particularly important in catalysis. The thiol group ($-CH_2SH$) of the conserved cysteine residue at position 69 is post-translationally oxidised at the endoplasmic reticulum to L-Cα-formylglycine (FGly 69) (which has an aldehyde as the functional side group) to render it catalytically active. A lack of FGly 69 formation renders ASA inactive and results in the rare

but fatal disorder known as multiple sulphatase deficiency (MSD) (Waldow *et al.*, 1999). This functionally essential FGly 69 is located at the active site of ASA in a positively charged cavity at the C-terminal end of the major β-sheet and is lined with charged amino acids that act as ligands to an octahedrally co-ordinated magnesium ion (Mg^{2+}) (Knaust *et al.*, 1998; Luketala *et al.*, 1998). The amino acid residues forming the active site include Asp 29, Asp 30, Asp 281, Asn 282, His 125, His 229, Lys 123, Lys 302 and Ser 150. These residues complex the Mg^{2+} and are thought to be involved in the cleavage of sulphate esters (Waldow *et al.*, 1999). Formylglycine participates in sulphate ester hydrolysis as an aldehyde hydrate, as described in section 1.4.2.2 (Luketala *et al.*, 1998; Waldow *et al.*, 1999). Substitution of the ten amino acid residues listed above results in partial or total loss of ASA activity, although there is uncertainty regarding the role of Ser 150 (Knaust *et al.*,1998; Lukatela *et al.*,1998; Waldow *et al.*, 1999). There are numerous residues that are conserved in all human sulphatases and a number of residues that are conserved across species. Few of these residues appear to be critical for the catalytic activity of ASA or for the formation of FGly 69 but may be essential to the stability of ASA in the lysosomal environment (Knaust *et al.*, 1998). There are three N-glycosylation sites in ASA, Asn 158, Asn 184 and Asn 350, which are located at the periphery of the molecule (Lukatela *et al.*, 1998). Patients with the pseudodeficiency of arylsulphatase A (PD-ASA section 1.4.1.3.1) gene lack the Asn_{350}*N*-glycosylation site of their ASA, a potential polyadenylation signal which may result in the blunted ASA activity observed in PD-ASA (Park *et al.*, 1996). However, substitution of Asn 350 with Ser 350 does not affect the rate of synthesis, stability or catalytic properties of ASA in transfected baby hamster kidney cells, indicating that this mutation may not contribute to the lack of ASA activity observed in PD-ASA (Gieselmann *et al.*, 1989). The structure of ASA, including the location of the active site residues, is illustrated in figures 1g(i) and 1g(ii). Using polyacrylamide gel electrophoresis (PAGE) ASA has been shown to display a number of different electrophoretic banding patterns in subjects with alcoholism and schizophrenia

(Manowitz *et al.*, 1978; Manowitz *et al.*, 1981; Georgopolous & Manowitz, 1982; Hullyukar *et al.*, 1984; Park *et al.*, 1996).

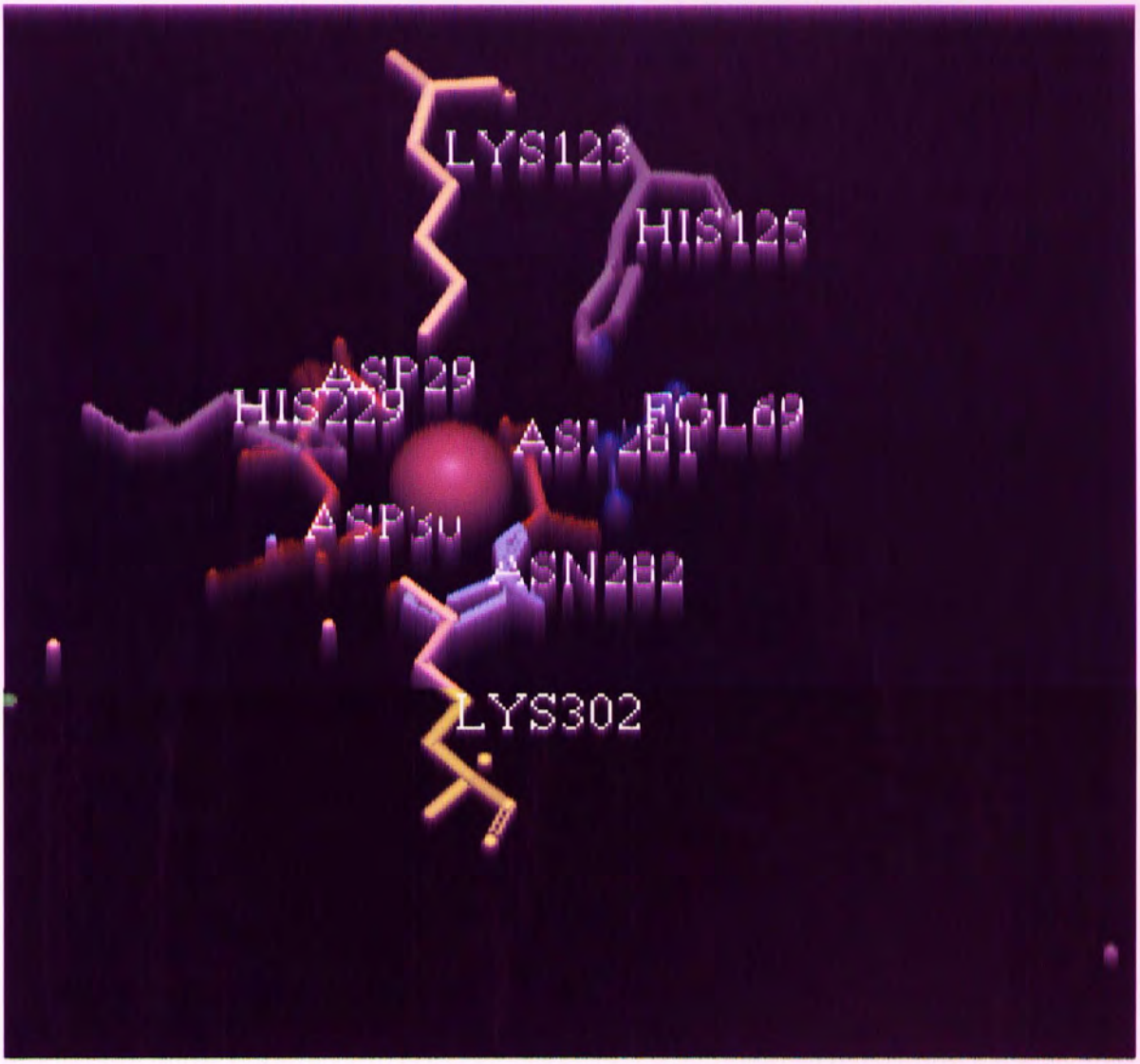
Fig. 1g(i). Structure of Arylsulphatase A (ASA)



(PDB file downloaded into ViewerLite, 4.2 (Accelerys Inc.) For manipulation).

Alpha helices are coloured red, beta sheets are turquoise, amino acid residues of the active site are illustrated in stick formation and are coloured green, Fgly 69 is illustrated in ball formation and is coloured blue and the magnesium ion at the active site is shown as a pink ball. The active site of ASA is illustrated in more detail in Fig. 1g(ii) as the area enclosed within the white box (above).

Fig .1g(ii). Structure of Active Site of Arylsulphatase A (ASA)



Illustrated are the eight amino acid residues which form the active site of arylsulphatase A together with the octahedrally-co-ordinated magnesium ion. The secondary structure of the protein has been removed for clarity.

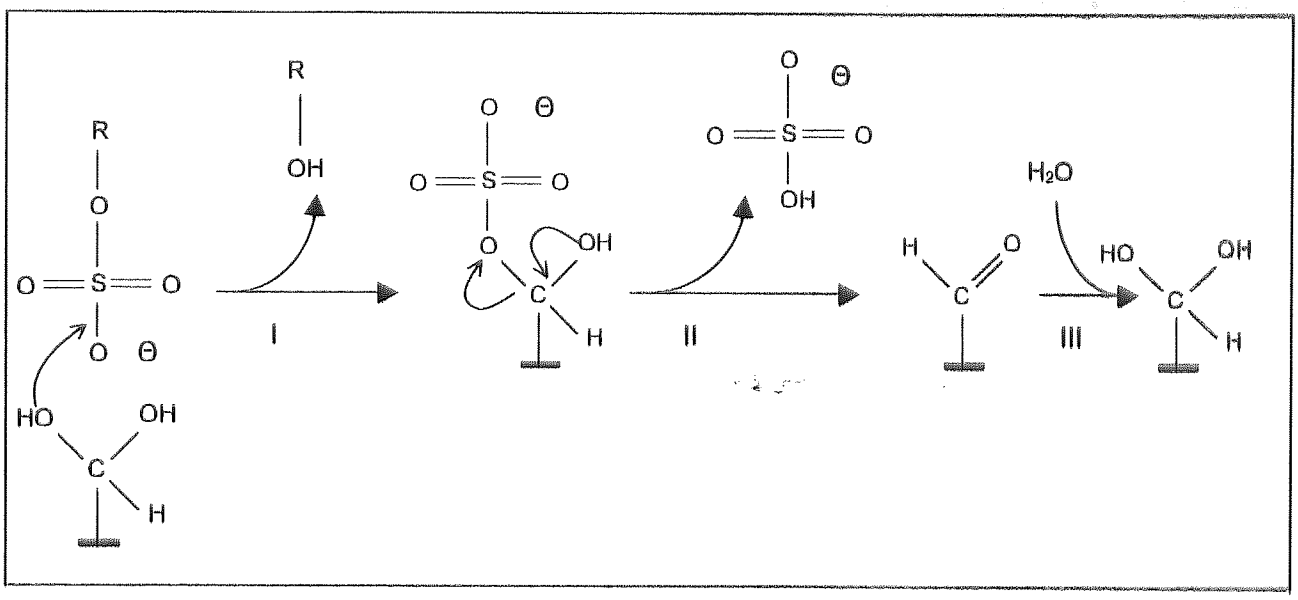
1.4.2. Mechanism of Action of Arylsulphatase A

The reaction between ASA and sulphate ester is initiated with the addition of water to the aldehyde side group of FGly 69 to form aldehyde hydrate and by binding of the substrate RO-SO_3^- to the active site. One of the aldehyde hydrate hydroxyl groups attacks the sulphur of the substrate leading to trans-esterification of the sulphate group onto the aldehyde hydrate forming the intermediate E-$CH(OH)OSO_3H$. The substrate alcohol is simultaneously released. In a subsequent step the second hydroxyl of the aldehyde hydrate group is activated to induce the elimination of the SO_4^{2-} anion and the aldehyde is regenerated. The catalytic cycle is closed by the release of sulphate and the deprotonation of His 125 (Lukatela *et al.*, 1998; Waldow *et al.*, 1999).

The exact role of $Mg^{2+}$is uncertain, however, it may co-ordinate the oxygen of the hydroxyl group in the first step of sulphate ester cleavage. The histidine residue at position 125 of ASA is a candidate for the deprotonation of the second hydroxyl group of the aldehyde hydrate in the second step of this reaction.

A mechanism for the action of ASA has been described (Stinshoff, 1972; Lukatela *et al.*, 1998; Waldrow *et al.*, 1999) and is illustrated in Fig 1h.

Fig. 1h. Mechanism of Action of Arylsulphatase A

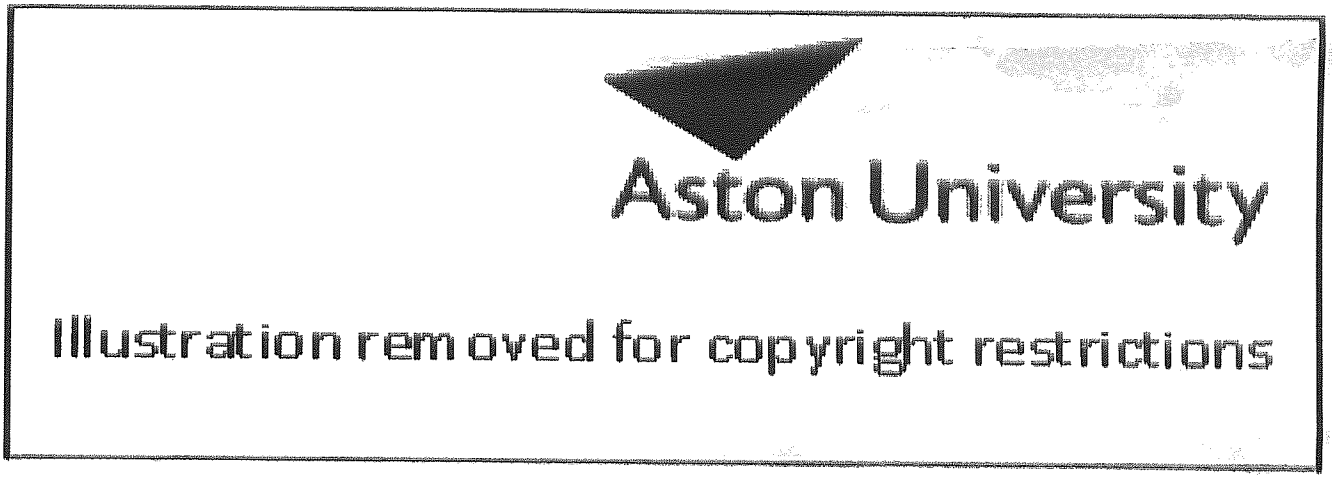


In the first half-cycle (I) one of the hydroxyls of the aldehyde hydrate attacks the sulphur of the sulphate ester forming an enzyme-sulphate ester, whereby the residual alcohol is released. Sulphate is eliminated in the second half-cycle (II) by an intramolecular rearrangement cleaving the ester bond and forming an aldehyde group at the enzyme. Finally the aldehyde hydrate is regenerated by the entry of water (III). This scheme is based on the reaction mechanism described by Luketala *et al.*, (1998) (Waldrow *et al*, 1999).

1.4.3 Structure of The Gene for Human Arylsulphatase A (*arsa*)

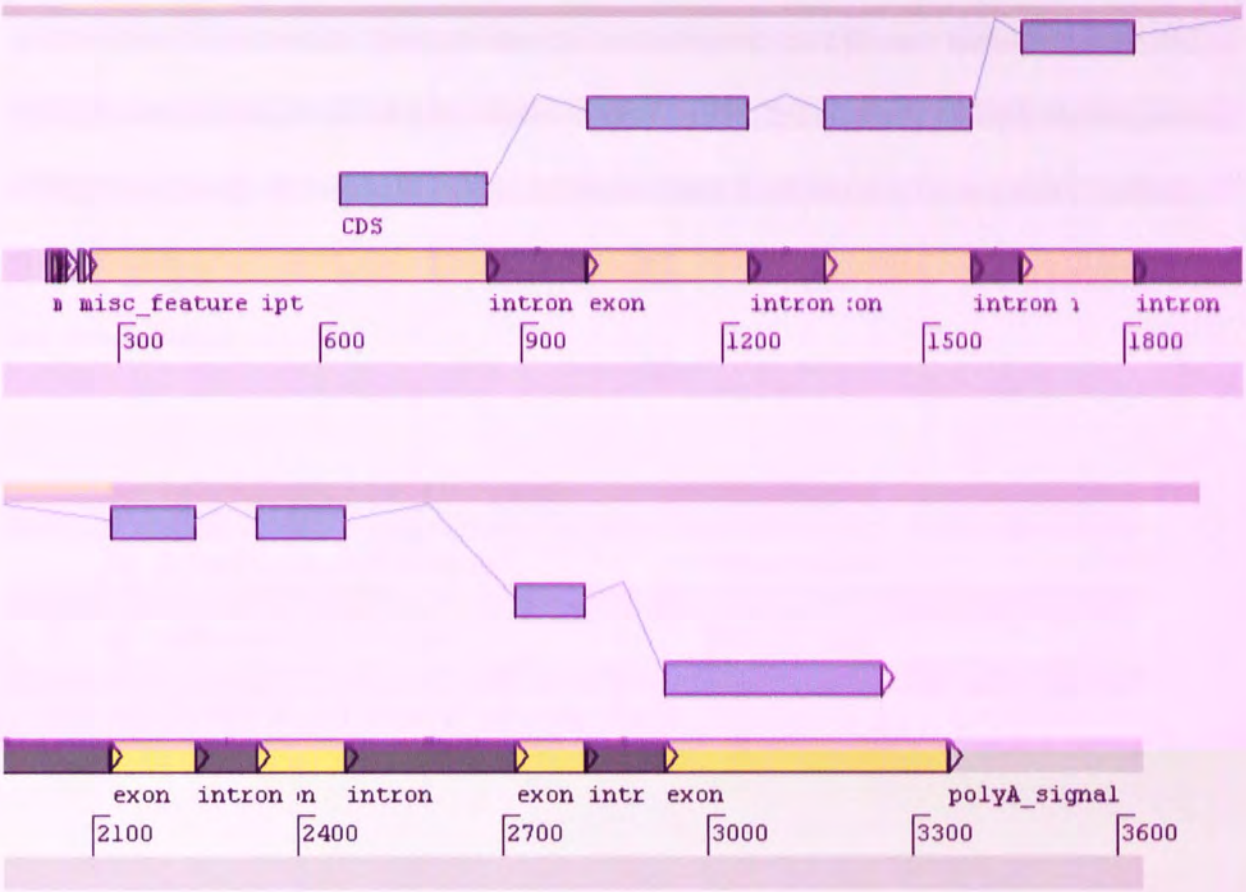
The gene for human ASA (*arsa*) has been mapped to chromosome 22q13.33 – qter (http://www.ensembl.org), and its cDNA (Stein, *et al.*, 1989) and genomic DNA (Kreysing, *et al.*, 1990) sequences have been described. The *arsa* gene is 3637bp in length and contains eight exons as illustrated in figure 1j. The sequence of *arsa* has been deposited in the Genbank database accession number X52150. The location of *arsa* within chromosome 22 of the human genome is illustrated in figure 1i.

Fig. 1i. Position of *arsa* on Chromosome 22



Reproduced from (http://www.ensembl.org). The area enclosed in a red box illustrates the position of *arsa* with respect to chromosome 22. The box entitled 'overview' displays the q.13.33 region of the chromosome, illustrating the position of all genes at this region, including the genes flanking *arsa*.

Fig. 1j. Structure of the Gene for Arylsulphatase A (*arsa*)



(Composed using Artemis release 4, Genome Research Ltd, The Sanger Centre, UK). The first ~1950 bases are displayed in the top half of the figure, from bases 1950 onwards are displayed below.
Light grey bar = Entire sequence of *arsa*; Green shaded areas = Promoter motifs; Yellow shaded areas = Exonic regions; Blue shaded areas = Coding sequence; Dark grey shaded areas = Intronic sequence.

1.4.3.1 Arylsulphatase A Polymorphisms and Disease

Arylsulphatase A is responsible for the desulphation of cerebroside sulphate which, when it accumulates in lysosomes, characterises the neurodegenerative disease metachromatic leukodystrophy (MLD) (Rodriguez-Soriano, *et al.*, 1978; Stein, *et al.*, 1989; Kreysing, *et al.*, 1990; Ben-Yoseph & Mitchell, 1995). Metachromatic Leukodystrophy is a rare (1:40000 (Gieselmann, *et al.*, 1994; Ben-Yoseph & Mitchell, 1995)), autosomal recessive disorder that has three clinical variants:

a) Late-infantile onset (1-2 years) 60% of cases

b) Juvenile onset (3-16 years) 30% of cases

c) Adult onset (>16 years) 10% of cases

(Stein, *et al.*, 1989; Ben-Yoseph & Mitchell 1995).

Metachromatic leukodystrophy is thought to be the result of deficient activity of ASA which leads to an increase in the sphingolipid cerebroside sulphate (CS) affecting primarily the oligodendrocytes, which are progressively de-myelinated (Gieselmann, *et al.*, 1994). Rodriguez-Soriano *et al* (1978) described a case of late infantile metachromatic leukodystrophy in which the patient demonstrated a decrease (increase?) in renal tubular sodium reabsorption. This may be the result of a blunted renal dopamine formation from dopamine sulphate due to the decreased ASA activity observed in MLD. Pseudodeficiency of ASA (PD-ASA) results in a marked decrease in intracellular ASA activity without diminished ability to catabolise sulphatides. Despite their decreased ASA activity patients with PD-ASA display normal pathology (Park *et al.*, 1996).

There are numerous mutations of the ASA gene, which affect the apparent rate of synthesis, stability, and/or catalytic properties of the enzyme in MLD (Ben-Yoseph & Mitchell, 1995; Berger, *et al.,* 1996; Coulter-Mackie & Gagnier, 1997). These include amino acid substitutions, splice donor site mutations, and small deletions (Gieselmann *et al.*, 1994). One of the most common mutations associated with MLD results in the substitution of proline 426 by leucine. This is thought to affect the stability of ASA within the lysosomal environment, but has no affect upon catalytic activity (Knaust *et al.*, 1998). Arylsulphatase A pseudodeficiency is linked to mutations in the ASA gene, although so far these mutations have not been linked to those of MLD. In pseudo-deficiency, subjects display a marked decrease in ASA activity without clinical symptoms (Ben-Yoseph & Mitchell, 1995; Berger, *et al.*, 1996; Coulter-Mackie & Gagnier, 1997).

A deficiency in the activity of ASA may play a role in the impaired production of dopamine observed in response to a salt load in salt-retaining states. As a reduced dopaminergic response is found in normal subjects with a family history of hypertension, it is possible that this be under genetic control. There may be a mutation in *arsa* that affects the activity of the enzyme, and thus the renal production of dopamine in salt-retaining states

1.5 Molecular Biological Techniques for the Analysis of the Human Genome

The elucidation of the working draft of the human genome in 2001(www.nature.com; www.wellcome.ac.uk), has given the scientific community a vast array of information allowing the analysis of human pathology and the diagnosis of disease at the gene level.

1.5.1 Structural Analyses

The analysis of the sequence of human genes allows the detailed investigation of genetic mutations that may be associated with disease states. One of the tools which has facilitated gene sequence analysis is the polymerase chain reaction (PCR).

1.5.1.1 Polymerase Chain Reaction (PCR)

Since its conception in 1986 (Mullis, 1986), the polymerase chain reaction (PCR) has become one of the most widely used molecular biological techniques. It is a versatile technique which can be adapted for numerous analyses, from the investigation of gene expression to the screening of gene clones. Its specificity allows the administration of numerous downstream applications such as DNA sequencing. Here the basic principles of PCR and the application of this technique to the analysis of gene sequencing and expression analyses will be discussed.

1.5.1.1.1 The Theory of PCR

The polymerase chain reaction (PCR) is the *in vitro* amplification of DNA using thermostable DNA polymerase and gene-specific oligonucleotide primers. The basic principles of PCR are described in figure 1k.

The polymerase chain reaction theoretically doubles the amount of target DNA with each cycle, however the efficiency of PCR is never 100%. A PCR with 20 cycles does not give the expected million-fold amplification, rather a 10^5 fold amplification of the target DNA is achieved. This degree of amplification is usually sufficient for most PCR-based investigations, notably DNA sequencing.

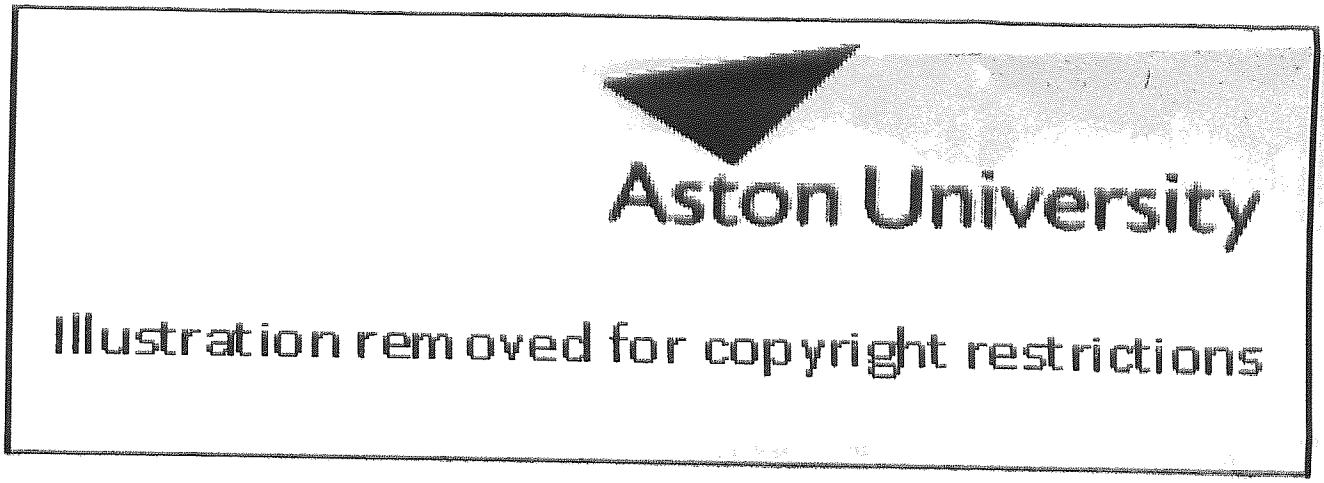
1.5.1.1.2 Practical Considerations of PCR

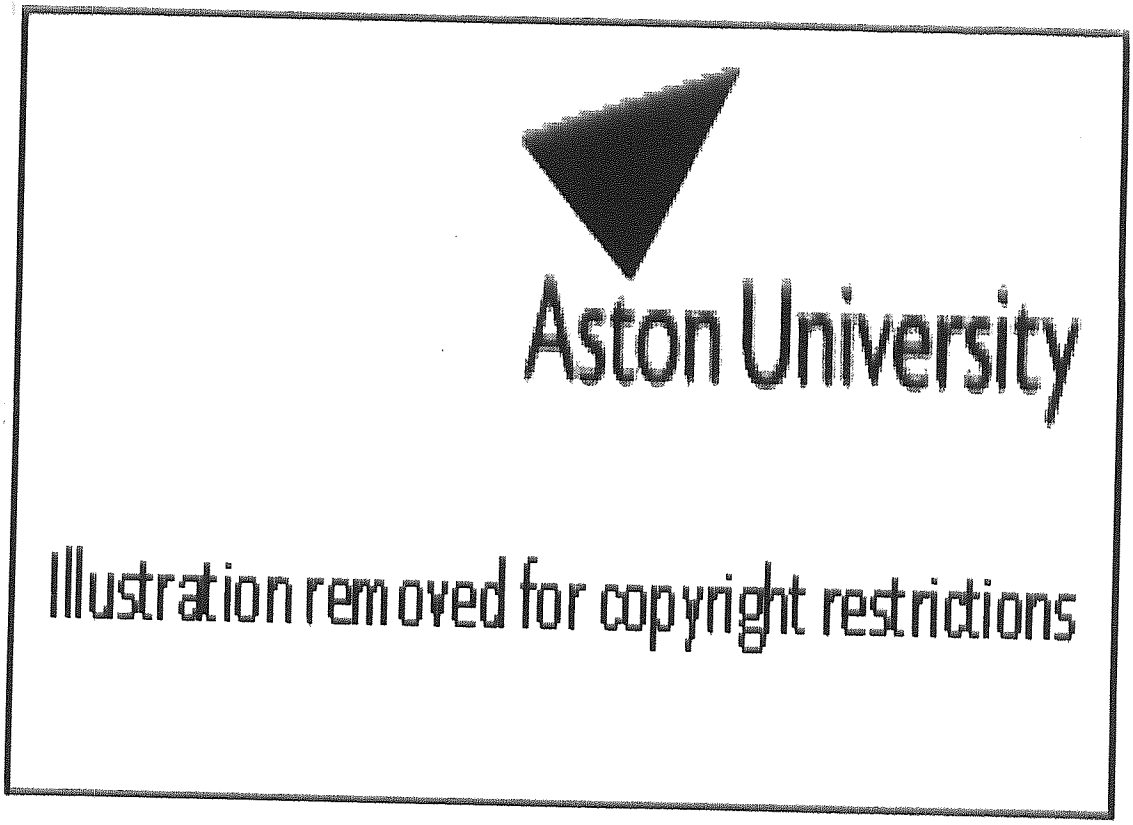
Numerous factors should be considered when designing and optimising a PCR reaction. These include the composition of the PCR reagents including the concentrations of *Taq* DNA polymerase, dNTPs, $MgCl_2$, and gene-specific primers.

1.5.1.1.2.1 DNA Polymerase

There are various commercially available DNA polymerases with different properties tailored towards the type and size of PCR product under investigation. They are all derived from *Taq* DNA polymerase which has 5'-3' exonuclease activity, 5'-3'polymerase activity but no 3'-5' exonuclease activity. This enzyme is suited to PCR due to its origin i.e. the thermostable

Fig 1k. Polymerase Chain Reaction





(Reproduced from www2.Kenyon.edu/Depts/Biology/courses/biol14/images/per.jp)
The double-stranded DNA of interest (a) is heat-denatured at 92 – 98°C to separate the two complementary strands. Once separated each strand is subject to the annealing of sequence-specific and therefore gene-specific primers, by lowering the temperature of the reaction mixture to between 40 - 72°C (b). Once these primers have hybridised to the template strands of DNA, the thermostable DNA polymerase then extends the primer sequence by the introduction of individual deoxynucleotides (dNTPs) which hybridise to the template strand in an order which is complementary to the template strand (c). This extension or elongation step and takes place at 72°C when *Taq* DNA polymerase is employed The cycle of denaturation, primer annealing, and extension is repeated (e, f & g) from 25 - 40 times such that after 20cycles over 1million copies of the double-stranded target DNA, together with 42 copies of single DNA strands of variable length have been generated.

bacterium *Thermus aquaticus* allowing its activity to remain at the high temperatures required during PCR.

1.5.1.1.2.2 Deoxynucleotides

The four deoxynucleotides (dNTPs) are incorporated by *Taq* DNA polymerase to form the bases which comprise the new complementary DNA strand with each cycle of PCR. They should be present within a PCR at equimolar concentrations, usually between 50 and 200μM.

1.5.1.1.2.3 PCR Buffer

The buffer for PCR contains Tris-HCl, and KCl which may assist primer-template annealing. The buffer is at pH 6.8 – 8.3 and this varies with temperature (*Taq* DNA polymerase has a higher fidelity at the lower pH achieved at the higher temperatures employed during PCR). *Taq* DNA polymerase is reliant upon the presence of the divalent cation Mg^{2+} which is often included in the buffer at a working concentration of 1.5mM. Magnesium (Mg^{2+}) is thought to form a complex with the triphosphate group of each dNTP facilitating the formation of phosphodietser bonds between each nucleotide and therefore the formation of complementary DNA sequence with each cycle of PCR. The concentration of Magnesium is therefore critical in the optimisation of PCR.

1.5.1.1.2.4 PCR Primers

The design of primers for PCR is crucial to successful PCR optimisation. For sequence-specific amplification there are a number of properties of the primers which should be carefully considered:

a) That each primer be 15 – 25 nucleotides in length.

b) That repetitive sequences or regions containing the same nucleotide be avoided.

c) That runs of 3 or more G or C nucleotides at the 3' end be avoided to prevent mispriming at GC-rich regions.

d) That internal complementarity be avoided to prevent the formation of secondary structure within a primer sequence.

e) That the sequences at the 3' end do not allow base pairing within the primer or with the second primer of a pair leading to secondary structure or the formation of primer-dimers respectively.

f) That the theoretical melting temperature of each primer matches that of its counterpart as closely as possible, to allow the use of a compatible annealing temperature. The theoretical melting temperature of PCR primers can be calculated using the following equation:

$$Tm = 69.3 + 0.41 \times \%GC - \left[\frac{650}{\text{sequence length}} \right]$$

(McPherson & Moller, 2000)

DNA amplified using PCR can be subsequently analysed using DNA sequencing and *in silico* manipulation of the resulting sequence data. This will be discussed in detail in chapter 4.

1.5.2 Expression Analyses

The amplification of specific gene sequences can be employed in the analysis of gene expression. A number of techniques for gene expression analysis do not utilise PCR, e.g Northern blotting and *in situ* hybrdisation (ISH). However, PCR is increasingly used in the expression analysis of low copy number genes and as such can be incorporated into reverse transcription and microarray analyses.

1.5.2.1 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The amplification of DNA is employed as a tool for the analysis of gene expression using reverse transcription polymerase chain reaction (RT-PCR). Here RNA (either total RNA or mRNA) is extracted from the sample under investigation and is reverse transcribed using reverse transcriptase derived from either avian myeloblastosis virus (AMV) or moloney murine leukaemia virus (M-MLV). A single strand is produced initially from a primer which is either gene-specific, contains an oligo dT motif (for the binding of the primer to the poly-A tail of mRNA), or is randomly designed to amplify the entire RNA population of the cell, such as a random hexamer or decamer. After heat-denaturation of any secondary structure of RNA, the primer binds to the RNA and reverse transcriptase and extends this bound sequence using free dNTPs. The complementary DNA (cDNA) produced is then used as a template for PCR. This technique can be employed to assess gene expression both quantitatively, semi-quantitatively, or qualitatively, and the methods employed are discussed in detail in chapter 6.

1.6 Hypotheses

The catecholamine dopamine is a natriuretic hormone which is formed from dopamine sulphate via ASA in the kidney. A blunted excretion of dopamine has been observed in disease states which may demonstrate sodium retention, notably diabetes mellitus. This blunted excretion of dopamine indicates a decrease in the renal production of this catecholamine. It is postulated that the activity of arylsulphatase A be lower in patients with salt-retaining states, resulting in diminished dopamine formation from dopamine sulphate and an impaired ability to excrete sodium.

1.7 Aims and Objectives of The Study

This study aims to investigate the formation of renal dopamine from dopamine sulphate via ASA in diabetes mellitus, a condition which may be salt-retaining. Despite theoretical formation of renal dopamine from dopamine sulphate, there is a lack of direct evidence regarding the measurement of urinary catecholamines in subjects whose sodium intake has been altered. Measurements of the urinary free and sulphoconjugated dopamine of individuals whose sodium intake has been altered, will be determined to establish the source, and confirm the role of renal dopamine as a natriuretic in such individuals.

A decreased dopamine excretion has been observed in individuals with both type 1 and type 2 diabetes mellitus and may contribute to the development of hypertension in such patients. Whether this blunted dopamine output is due to a decreased renal production of dopamine, or to an increase in the autoxidation of dopamine to its oxidation products, notably 6-OHDA, is as yet unknown. To assess the renal production of dopamine from dopamine sulphate in subjects with diabetes mellitus the activity of arylsulphatase A (ASA) in normal individuals as compared with a diabetic population will be investigated. Furthermore, a mutation in the gene for arylsulphatase A (*arsa*) may result in a decreased activity and therefore an impaired renal production of dopamine from dopamine sulphate. To assess the formation of renal dopamine from dopamine sulphate at the molecular level the sequence and expression of the gene for *arsa* in diabetic subjects will be compared with those of a normal population. This will involve the assessment of the expression of ASA RNA from paraffin-embedded renal tissue and the development of a novel RT-PCR to compare the expression of renal ASA in normal individuals and patients with diabetes mellitus. This will allow the elucidation of whether ASA is down-regulated in diabetes mellitus resulting in an impaired renal production of dopamine.

CHAPTER 2

2. THE EFFECT OF DIETARY SODIUM RESTRICTION ON URINARY FREE DOPAMINE AND DOPAMINE SULPHATE CONCENTRATION

2.1 Introduction

2.1.1 Renal Dopamine

Renal dopamine promotes natriuresis as described in section 1.2.2.3. There are two proposed sources of renal dopamine:

a) Decarboxylation of L-dihydroxyphenylalanine (L-dopa) by the enzyme L-aromatic-amino-acid decarboxylase (L-AAAD).
b) Desulphation of dopamine sulphate by arylsulphatase A (ASA).

Once formed from L-Dopa, dopamine has a very short half life and is quickly metabolised to its acid, alcohol and ethylene glycol metabolites depending upon the metabolic pathway involved. There is evidence to suggest that dopamine is also conjugated to the physiologically inert dopamine sulphate which may be desulphated when renal dopamine is required for natriuresis.

2.1.2 Renal Dopamine in Salt-Retaining States

Impaired renal production of dopamine has been reported in salt-retaining states. Nakaya *et al.* (1994) observed an increase in the production of dopamine from dopamine sulphate in congestive heart failure patients. Furthermore, in both type 1 and type 2 diabetes mellitus there is a blunted renal production of dopamine (Stenvinkel *et al.*, 1991; Segers *et al.*, 1995; Shigetomi *et al.*, 1995; Rudberg *et al.*, 1997). This decreased dopamine concentration may be due in part to the rapid autoxidation of dopamine to, among other oxidation products, 6-hydroxydopamine (6-OHDA). Thus in patients with diabetes mellitus, dopamine may be less available to promote natriuresis leading to the development of hypertension.

2.1.2.1 Renal Dopamine in Essential Hypertension

Numerous studies demonstrate a decreased renal production of dopamine from both L-dopa and endogenous dopamine sulphate in essential hypertension. A blunted renal production of dopamine from L-dopa (Clark *et al.*, 1992; Kuchel & Shigetomi, 1992), and a decrease in the ratio of urinary dopamine:L-dopa (Gill *et al.*, 1991) have been observed in hypertensive patients. Miura *et al.* (1989) found that plasma dopamine levels were decreased in essential hypertension when compared with those seen in a normal control group. However, such a phenomenon was not observed in hypertension due to renal disease, indicating that a suppressed dopaminergic system may be an underlying cause of essential hypertension. Numerous studies have illustrated a decreased deconjugation of plasma dopamine sulphate to plasma free dopamine in essential hypertension. Subjects with essential hypertenaion have increased levels of plasma dopamine sulphate (Kuchel *et al.*, 1979, 1984[b]), which have been

found to return to values similar to those of control subjects when the hypertension is treated (Yoshizumi *et al.*, 1996). Thus dopamine sulphate may play a role in the formation of plasma free dopamine in patients with essential hypertension.

2.1.2.2 Renal Dopamine and Low Renin Essential Hypertension (LRH)

Renal dopamine excretion is blunted in patients with low renin essential hypertension when compared to that of normal renin essential hypertensives and normotensive subjects (Aoki *et al.*, 1989; Iimura, 1996). The maintenance of sodium homeostasis may therefore be influenced by renal dopamine in patients with low renin essential hypertension.

2.1.2.3 Renal Dopamine and Salt-sensitivity

Ethnicity may affect dopaminergic responses to dietary sodium. Sowers *et al.*, (1988) found that both hypertensive and normotensive subjects of Afro-Caribbean ethnicity demonstrate an increase in mean arterial pressure (MAP) and systolic blood pressure (SBP) with a sodium load, and they can therefore be described as 'salt-sensitive'. Previous studies demonstrate an impaired renal dopamine production in response to an increase in sodium intake in 'salt-sensitive' subjects (Gill *et al.*, 1991). Moreover, it has been suggested that a deficiency of dietary potassium may be the cause of salt-sensitivity in normotensive Afro-Caribbean subjects (Morris *et al.*, 1999).

2.1.3 Dopamine Sulphate is a Physiological Reservoir of Active Free Dopamine

Plasma dopamine concentration appears to increase, and dopamine sulphate concentration decreases where there is a physiological requirement for natriuresis. This suggests that plasma dopamine sulphate is not merely a metabolite of free dopamine, but that it may act as a physiological reservoir of the catecholamine that can be utilised as required. However, the measurement of urinary dopamine and dopamine sulphate provides a more accurate representation of the renal dopaminergic response to altered dietary sodium. The measurement of urinary catecholamines is now routinely performed in pathology laboratories for the diagnosis of catecholamine secreting tumors, in particular pheochromocytoma. Urinary dopamine sulphate can be measured using a modification of this technique whereby urinary free dopamine is measured both before and after desulphation by arylsulphatase A (ASA). The difference between these two measurements, and therefore the dopamine sulphate concentration may be is subsequently calculated. This method was employed to test the hypothesis that dopamine sulphate is a pool of active free dopamine which can be called upon as required. The urinary free dopamine (UFDA) and urinary dopamine sulphate ($UDASO_4$) concentration in 52 subjects of Afro-Caribbean origin was measured by HPLC-ED, both before and after dietary salt restriction.

This study was originally designed to assess the blood pressure and renal dopaminergic responses to both a decreased and increased dietary sodium intake. However the director of the study returned to his home country after only decreased dietary sodium was assessed, and in doing so took the blood pressure response data with him. He did not allow myself or my collegues access to this, or any other clinical data at any time.

2.2 Materials and Methods

2.2.1 Subjects

One hundred and sixty one subjects of Afro-Caribbean origin, aged between 18 and 79 years were recruited into the study from two churches in the West Midlands (table 2a).

The following inclusion criteria were applied:

a) Hypertensive and normotensive individuals between the ages of 18 and 80 yrs on no medication, or prescribed no more than one drug for the treatment of hypertension.

b) African Caribbean individuals who were both first and second-generation migrants or citizens of the UK.

Subjects with ischaemic heart disease, recent disabling stroke, any other chronic condition (e.g. renal failure, malignancy, any condition which was expected to end life within the next year or was disabling such that the subject was not able to participate in regular examination and testing), those using oral contraceptives, or any woman known to be pregnant at the time of recruitment, and subjects with systolic blood pressure (SBP) greater than 180 mmHg and / or diastolic blood pressure (DBP) greater than 105 mmHg, were excluded. For the purpose of this study, hypertension was defined as SBP greater than or equal to 140 mmHg and / or DBP greater than or equal to 90 mmHg. All the investigational procedures conform to the tenets of the Declaration of Helsinki and were approved by the research and ethics committee at Birmingham Heartlands and Solihull NHS Trust.

2.2.2 Sample Collection and Storage

Each subject was asked to collect six 24 hr urine samples. Three were collected while each subject consumed his or her regular diet (baseline), and three were collected after dietary salt depletion (low salt). For the purpose of this study, salt depletion was defined as the consumption of a diet containing approximately one half to two thirds of the subjects habitual amount of sodium. This was estimated to be approximately 391-434mmol (9-10g) per day, which was reduced to approximately 261mmol (6g) per day during the two week reduced sodium period. The low salt period was preceded by instructions from a dietician which were reinforced periodically by nurses and physicians. The reduced sodium intake included avoidance of adding salt to the diet, use of sodium free bread, and the elimination of foods known to have a high salt content. After at least two weeks of sodium depletion, the low salt 24 hr urine samples were collected. A decrease in urinary sodium concentration of at least 10 mmol/L with salt restriction was used to ascertain compliance with the low sodium regimen. Eighteen non-compliant subjects were excluded from data analysis. Eighty six of the 161 subjects who originally took part in the study, failed to supply complete collections and were therefore excluded from data analysis. Five of the remaining compliant subjects who had supplied complete collections were excluded due to one of their laboratory results being of outlier status (i.e. ≥ 3 SD from the mean). This left 52 subjects remaining in the study.

All 24 hr samples were collected into 20 mL of 50% ($^{v}/_{v}$) HCl. All samples were pre-diluted to 3L with normal saline and stored at -20°C prior to analysis for dopamine and dopamine sulphate. Immediately prior to HPLC-ED analysis, samples were thawed and mixed thoroughly, and an aliquot centrifuged for five minutes to separate any sediment. Urine

samples in excess of 3L volume did not undergo pre-dilution and the results were corrected accordingly.

Two undiluted 20 mL aliquots of each urine sample were taken into universal containers, and stored at -20°C. These were used to measure urine sodium, potassium and creatinine.
One blood sample was taken from each subject (at baseline). Serum samples were stored at -20°C prior to analysis for plasma renin activity (PRA) and aldosterone concentration.

2.2.3 Methods

High Performance Liquid Chromatography with Electrochemical Detection (HPLC-ED) was employed to measure UFDA and $UDASO_4$ using a method described in section 1.2.1.2 (Bayly *et al.*, 1999). The Gilson ASTED-XL sample processing unit (Anachem, Luton, UK) and Unipoint software were used to measure the concentration of urinary dopamine both before and after desulphation with ASA. Urinary sodium (UNa) and potassium (UK) were analysed using ion-selective electrodes, and urinary creatinine measured using the Jaffe reaction (Monarch analyser, IL, Lexington, MA, USA).

Plasma renin activity (PRA) and aldosterone concentration were measured using commercially available kits. The Biodata Renin MAIA kit (Biodata diagnostics, Rome, Italy) was used to measure PRA at pH 6.0 and Aldosterone concentration was measured using coated tube radioimmunoassay (Coat-A-Count Aldosterone, Diagnostic Products Corporation, LA, California, USA).

2.2.4 Data Analysis and Statistics

A subgroup of the compliant cohort were designated as a Low Renin subgroup. This was defined as a PRA <0.39 nmol/L/hr and was observed in 19 subjects. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) for Windows v6.1. Data found not to be normally distributed was logarithmically transformed to normality. All comparative statistical analyses used the Students paired t-test for significance on either raw or logarithmically transformed data.

2.3 Results

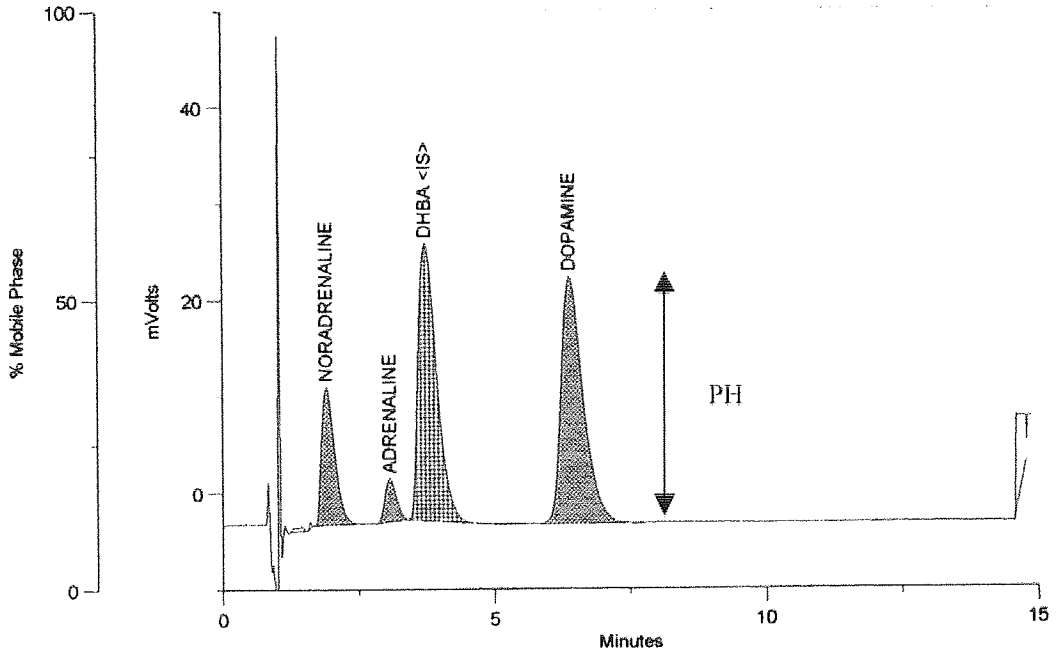
The clinical characteristics of the study population are illustrated table 2a. Figure 2a illustrates the chromatographic data used to measure the concentration of urinary dopamine.

Table 2a. Clinical Characteristics of the Study Population.

	MALE	FEMALE	COMBINED
n	9	43	52
AGE (years)	53 (14)	45 (16)	47 (16)
BMI	28.2 (4.2)	28.7 (5.9)	28.6 (5.4)
SBP (Baseline)	124 (25)	136 (20)	133 (21)
DBP (Baseline)	76 (15)	79 (8)	77 (9)

A description of the clinical characteristics of the patients included in the final analysis. n = number of subjects; BMI = Body mass index; SBP = Systolic blood pressure; DBP = Diastolic blood pressure. Data is shown as Mean (SD)

Fig. 2a. Example Chromatogram Illustrating the Separation of Urinary Catecholamines using HPLC-ED



Amperometric detection was used to determine the concentration of urinary catecholamines using peak height (PH). The equation below was applied using Gilson Unipoint software. For each sample the concentration of free catecholamines were measured initially and the same sample analysed for a second time after enzymatic deconjugation by ASA. The concentration of catecholamine sulphate was subsequently taken as the difference between these two values. DHBA (IS) = Dihydroxybenzylamine hydrobromide (internal standard).

Calculation of urinary free dopamine concentration (UFDA):

$$UFDA = \left[\frac{\text{PH of DA in unknown / PH of DHBA in unknown}}{\text{PH of DA in Std / PH of DHBA in Std}} \right] \times \text{Conc. DA in Std}$$

Where PH = Peak height
DA = Dopamine
DHBA = Dihydroxybenzylamine hydrobromide (internal standard)
Std = Standard

The same calculation was carried out on the chromatogram obtained from a 1:4 dilution of the same sample which had been enzymatically desulphated by ASA, and the result multiplied by four to give the total urinary dopamine concentration (UTDA). The urinary dopamine sulphate concentration ($UDASO_4$) was calculated as follows:

$$UDASO_4 = UTDA - UFDA$$

Each subject supplied three baseline and three low salt samples. The mean of all three analyte results in each category (baseline or low salt) was calculated, and this figure taken as the baseline or low salt result for the individual.

In the compliant cohort, five subjects results were excluded due to at least one parameter being of outlier status (i.e. greater than or equal to three standard deviations (SD) from the mean). After salt restriction, mean UNa concentration decreased significantly from 112 mmol/L to 76 mmol/L, ($p<0.0001$). With the low salt regimen UFDA concentration decreased, but this change failed to reach statistical significance. However mean $UDASO_4$ concentration increased significantly from 4758 nmol/L to 6223 nmol/L ($p=0.0090$) with the low sodium diet (table 2b).

Table 2b. Mean UNa, UK, UFDA, and $UDASO_4$ Concentrations in the Compliant Cohort under Baseline Conditions and After Salt Restriction.

	Compliant Cohort n = 52		
	Baseline	**Low Salt**	**p***
UNa (mmol/L)	112 (45)	76 (37)	<0.0001
UK (mmol/L)	50 (15)	52 (15)	0.2820
UFDA (nmol/L)	649 (198)	631 (194)	0.3490
$UDASO_4$ (nmol/L)	4758 (5409) [2689(1017-33023)]#	6223 (5684) [4140(1082-22745)]#	0.0090

Data were log transformed if not normally distributed, and parametric statistics used. Data is shown as Mean (SD). # = [Median (Range)] shown to illustrate the distribution of overtly non-normally distributed data. * p values <0.05 are considered significant

In the low renin subgroup, UNa concentration fell significantly, although less so than in the entire cohort, from 104 mmol/L to 82 mmol/L ($p = 0.0002$). In this subgroup UFDA concentration decreased significantly from 703 nmol/L to 624 nmol/L ($p = 0.0100$), and although the $UDASO_4$ concentration increased to the same extent as it did in the compliant cohort, this change just failed to reach statistical significance (table 2c).

Table 2c. Mean UNa, UK, UFDA, and $UDASO_4$ Concentrations, in the Low Renin Subgroup under Baseline Conditions, and with Salt Restriction.

	Low Renin Subgroup n = 19		
	Baseline	**Low Salt**	**p***
UNa (mmol/L)	104 (38)	82 (40)	0.0002
UK (mmol/L)	49 (13)	54 (13)	0.1850
UFDA (nmol/L)	703 (239)	624 (184)	0.0100
$UDASO_4$ (nmol/L)	4284 (2850) [3657(1253-11156)]#	6146 (4552) [4744(1104-19472)]#	0.0700

Data were log transformed if not normally distributed, and parametric statistics used. Data is shown as Mean (SD). # = [Median (Range)] shown to illustrate the distribution of overtly non-normally distributed data. * p values <0.05 are considered significant.

Urinary potassium concentration did not change with salt restriction in either the compliant cohort or the low renin subgroup (tables 2b and 2c). None of the subjects had urinary potassium concentrations outside the reference range, indicating that none were deficient in dietary potassium.

2.4 Discussion

Dopamine is typically considered as a neurotransmitter, and a deficiency of neural dopamine is seen in Parkinson's disease. However, peripheral (renal) dopamine is also known to have an alternative role as a natriuretic hormone. Previous studies have demonstrated an increase in plasma dopamine concentration and a decrease in plasma dopamine sulphate concentration in parallel with the physiological requirement for natriuresis (Aoki *et al.*, 1989; Yoshizumi *et al.*, 1992[b]; Nakaya *et al.*, 1994; Yoshizumi *et al.*, 1996). Furthermore, a decrease in urinary excretion of dopamine has been observed in patients with type 1 and type 2 diabetes mellitus (Stenvinkel *et al.*, 1991; Segers *et al.*, 1995; Shigetomi *et al.*, 1995; Rudberg *et al.*, 1997), in which sodium retention may underlie the development of hypertension.

Decreased dietary sodium intake reduces the requirement for natriuresis, and thus should lessen the physiological demand for active free dopamine. If dopamine sulphate is the precursor of free dopamine, then dietary salt restriction should also reduce the conversion of dopamine sulphate to dopamine and thus may increase the size of the precursor pool. The compliant cohort of this study showed a significant increase in $UDASO_4$ concentration with sodium restriction consistent with this hypothesis.

The sodium depleting effect of dopamine is thought to oppose the sodium retaining effects of the renin-angiotensin-aldosterone (RAA) system on the kidney. Previous studies have shown that renal dopamine production is decreased in hypertensive subjects with low plasma renin activity (PRA) (Aoki *et al.*, 1989). Such individuals are thought to have a suppressed RAA system, and in these subjects dietary salt restriction has been associated with an impaired renal dopaminergic response (Drayer *et al.*, 1981; Aoki *et al.*, 1989). However, in this study the

UFDA concentration of the Low Renin subgroup decreased significantly with salt restriction, indicating a normal dopaminergic response. The fact that $UDASO_4$ concentration did not change significantly with dietary salt restriction may be due to the production of renal dopamine from an alternative source, possibly from the decarboxylation of L-dopa. The observed increase in $UDASO_4$ with salt restriction was greater in this subgroup than in the compliant cohort but did not reach statistical significance possibly as consequence of the small number of samples analysed. However, the trends in dopamine and its sulphate concentration in relation to alterations in dietary sodium in these subjects is consistent with our original hypothesis that $UDASO_4$ is a reservoir of active free dopamine.

Both hypertensive and normotensive Afro-Caribbeans demonstrate an increase in mean arterial pressure (MAP) and systolic blood pressure (SBP) with a sodium load (Sowers *et al.*, 1988). Any subject who demonstrates such a phenomenon can be described as 'salt-sensitive'. The reproducibility of the classification of salt-sensitivity, in terms of the blood pressure responses to dietary sodium, has been investigated (Mattes & Faulkner, 1999; Gerdts *et al.*, 1999), however, this work has been unable to determine distinct criteria for the definition of salt-sensitivity (Mattes & Faulkner, 1999). Previous studies have used arbitrary changes in blood pressure with alterations in dietary salt intake to discriminate between salt-sensitive and non salt-sensitive (salt resistant) subjects. Morris *et al.* (1999) used an increase in mean arterial pressure (MAP) of greater than or equal to 3 mmHg with an increase in dietary sodium from 15 to 250 mmol/day to define salt-sensitivity. However, Gerdts *et al.* (1999) classify salt-sensitivity as an increase in mean blood pressure of greater than 10% with a dietary salt load from 50 to 250 mmol/day.

Previous studies have observed a blunted dopaminergic response to dietary sodium alteration in both hypertensive, and normotensive Afro-Caribbeans (Sowers *et al.*, 1988), but we were unable to confirm this in our cohort. No change in urinary potassium concentration was observed in response to restricted dietary sodium which may indicate that the subjects recruited into this study are not salt-sensitive (Morris *et al.*, 1999) and should therefore demonstrate a normal dopaminergic response to alterations in dietary sodium intake. However, salt-sensitivity status cannot be assessed accurately in the absence of the blood pressure response to changes in dietary sodium. The clinical data, in particular the blood pressure responses of the subjects recruited into this study were unavailable due to the mitigating circumstances described in section 2.1.3.

Plasma catecholamine sulphate levels are influenced by diurnal variation. Kuchel & Buu (1985) observed a prevalent plasma dopamine sulphate peak at 11.00pm. If this diurnal variation is mirrored in the pattern of urinary dopamine and dopamine sulphate excretion, then this has been accounted for by measuring urinary dopamine and dopamine sulphate in 24hr urine samples.

2.5 Conclusion

In conclusion, the results of this study support the hypothesis that dopamine sulphate is not only a metabolite of dopamine, but also that it acts as a physiological reservoir of active free dopamine (Kuchel *et al.*, 1986). These results demonstrate that measurement of urine dopamine sulphate levels could be used as an informative tool in assessing the role of sulphoconjugation in renal handling of sodium, and the control of blood pressure.

CHAPTER 3

3. URINARY ARYLSULPHATASE A ACTIVITY IN PATIENTS WITH DIABETES MELLITUS

3.1 Introduction

Dopamine, in addition to being a neurotransmitter (Smit *et al.*, 1995), has a role as a natriuretic factor and hormone. Dopamine exerts its natriuretic effect via the inhibition of proximal tubular Na^+/K^+-ATPase (Bertorello *et al.*, 1988; Ibarra *et al.*, 1993; Aperia *et al.*, 1994; Goldstein *et al.*, 1995) and this is demonstrated by evidence that urinary dopamine excretion correlates with urinary sodium excretion both under normal conditions and after alterations in dietary salt intake (Alexander *et al.*, 1974; Carey *et al.*, 1981).

The increase in urinary dopamine levels in response to corticosteroid treatment, has been shown to originate from an increase in the renal production of dopamine (Schoors *et al.*, 1990). Peripheral dopamine is derived from both the renal decarboxylation of plasma dihydroxyphenylalanine (L-dopa) (Ball & Lee, 1977; Zimlichman *et al.*, 1988; Hayashi *et al.*, 1990; Meister & Apeira, 1993; Wolfovitz *et al.*, 1993; Goldstein *et al.*, 1995), and the desulphation of dopamine sulphate by arylsulphatase A (ASA) (Brown & Allison,1981; Demassiuex *et al.*, 1986; Goldstein *et al.*, 1999). Appreciable arylsulphatase activity has been demonstrated in the kidney of dogs (Yoshizumi *et al.*, 1992[a]), and Demassieux *et al.*(1986) have shown that catecholamine sulphates are hydrolysed by the aryl sulphatases *in vitro*. Furthermore, previous studies have confirmed the hypothesis that dopamine sulphate is not merely a metabolite of active free dopamine, but that it acts as a reservoir of dopamine which can be utilised with the physiological requirement for natriuresis (Chapter 2).

Sodium retention has been demonstrated in normotensive subjects with diabetes mellitus (Feldt-Rasmussen *et al.*, 1987; Stenvinkel *et al.*, 1991; Rudberg *et al.*,1997), and this may contribute to the development of hypertension and nephropathy in diabetes. Urinary dopamine excretion has been studied in relation to sodium retention in normal subjects and patients with diabetes mellitus, however measurements of basal urinary dopamine excretion have yielded conflicting results. Patrick *et al.* (1990) found no difference in urinary dopamine concentrations between control subjects and those with diabetes mellitus. Other studies have shown reduced urinary dopamine concentration in patients with diabetes mellitus compared to control subjects (Murabayashi *et al.*, 1989; Segers *et al.*, 1995). The correlation between urinary dopamine and sodium concentrations which is seen in normal subjects is not observed in patients with diabetes mellitus (Segers *et al.*, 1995). Furthermore, with a high salt load, an increase in urinary dopamine excretion occurred in controls but not in those with diabetes mellitus (Stenvinkel *et al.*, 1991; Rudberg *et al.*,1997) and these studies demonstrated no correlation between urinary dopamine and sodium in diabetic patients.

Diabetic nephropathy and hypertension may develop as complications of diabetes mellitus (Chapter 1). Established diabetic nephropathy is associated with persistent proteinuria of a degree (> 300mg/L) that can be readily detected by reagent strips. Incipient nephropathy is associated with albuminuria which is above the normal range but below that detected by reagent strips (> 10mg/L but < 300mg/L). This degree of proteinuria is known as 'microalbuminuria'. Chan *et al.* (1992) found that in patients with type 2 diabetes mellitus urinary dopamine concentrations decreased as proteinuria increased.

Previous investigations have demonstrated that dopamine is autoxidised to 6-hydroxydopamine (6-OHDA). It has been observed that urinary excretion of 6-OHDA is elevated in diabetic patients with nephropathy as compared to those without this complication

(Chetty *et al.*, 2002). The aforementioned evidence suggests impaired renal dopamine production or an increase in the autoxidation of dopamine to 6-OHDA in patients with diabetes mellitus. A reduction in ASA activity may contribute to the reduction in urinary dopamine excretion seen in subjects with diabetes mellitus.

The measurement of urinary ASA activity has been demonstrated previously (Baum *et al.*, 1959) using a simple colorimetric technique in which the desulphation of nitochatechol sulphate to nitrochatechol is assayed. Here, this method is employed to investigate the activity of ASA in the urine of patients with diabetes mellitus in comparison to control subjects.

3.2 Materials and Methods

3.2.1 Preliminary Studies

Preliminary studies undertaken in the clinical chemistry laboratory at Birmingham Heartlands Hospital demonstrated a significant increase in urinary ASA activity in patients with diabetes mellitus as compared to a control group. This result contradicts the hypothesis that the blunted renal dopamine concentration observed in patients with diabetes mellitus, as compared with normal controls, is due to a decrease in the activity of ASA in patients with diabetes mellitus. This phenomenon will be discussed further in section 3.4.

3.2.2 Power

The preliminary study discussed in section 3.2.1 allowed the application of the power calculation to assess the number of samples required to show a significant difference, if present, in urinary ASA activity between the diabetic and control populations. At the 0.05 level of significance (α) with a 90% chance of detecting a true difference between the population means as small as δ^2, the following equation was applied:

$$n \geq \frac{2s^2p}{\delta^2}(t_{\alpha,v} + t_{\beta(t),v})$$

Where: n = minimum number of samples required to show a significant difference

$$\delta = \frac{\text{mean of population 1} - \text{mean of population2}}{2}$$

$$s^2p = \frac{SS_1 + SS_2}{v_1 + v_2}$$

SS = population variance x v

v = assumed or predicted n – 1

$\beta = 1 - \alpha$

t = critical values of t distribution

(reproduced from Zar, 1996)

Using this equation, and assuming a minimum number of samples required to show a significant increase in ASA activity of 20, gave a calculated $n \geq 2.77$. The increase in ASA activity observed in the preliminary study was highly significant and was taken from a sample size of 51 patients with diabetes mellitus, and 37 normal controls. However, a calculated sample size of three was thought to be too small despite the distinct difference in ASA activity between the normal and diabetic populations observed in the preliminary study. The value of δ (the difference in ASA activity observed in the preliminary study) was halved and an assumed population size of 20 was used giving $n \geq 11.09$. Assuming sample sizes of ten and twelve gave $n \geq 11.79$ and $n \geq 11.54$ respectively. Thus a sample size of twelve was required to show a significant difference in the urinary ASA activity between normal and diabetic subjects at half the difference observed in the preliminary study. To ensure the study was sufficiently powered, fifteen samples were collected from each of two groups:

a) Patients with Type 2 diabetes mellitus

b) Normal control subjects

3.2.3 Patients

All the investigational procedures conformed to the tenets of the Declaration of Helsinki and were approved by the research and ethics committee at Birmingham Heartlands and Solihull NHS Trust.

Fifteen patients with type 2 diabetes mellitus were recruited into this study from the weekly diabetic clinic at Birmingham Heartlands Hospital. Twelve sex-matched control subjects were recruited from either the Lipid and General Medicine Clinics, or the Department of Clinical Chemistry, Birmingham Heartlands Hospital. A further three sex-matched control subjects were recruited from the Department of Microbiology, Aston University. Each was asked to fill in a questionnaire (figure 3a) and supply two urine samples as follows:

a) Random urine

This was a sample collected into a universal container for ASA, albumin and creatinine analyses.

b) 24hr urine

This sample was collected into hydrochloric acid for catecholamine, sodium and creatinine analyses.

Fig. 3a. Arylsulphatase Activity Study Questionnaire

ASA ACTIVITY STUDY

Name:.....

Sex: ☐ M ☐ F

PID:.....

D.O.B:.....

Race: Caucasian / Asian / Afro-Caribbean / Other

Smoker? Y / N

CLINICAL INFORMATION

Type of Diabetes Mellitus: ☐ 1 ☐ 2

Duration of Diabetes Mellitus: ☐ yrs ☐ months

Treatment of DM: (please circle)
Insulin Y / N
OHA Y / N
Diet Y / N
Other (please specify)

.....

BP...../.....mmHg

Previous MI? Y / N

Previous CVA? Y / N

Previous PVD? Y / N

Prior Diagnosis of Hypertension? Y / N

If yes, drug treatment of Hypertension (please specify).....

Retinopathy? Y / N

Nephropathy? Y / N

Neuropathy? Y / N

LABORATORY INFORMATION

Urine ASA.....mIU/L

Urine Sodium.....mmol/L

Urine Dopamine:

Volume of Collection.....L

Urine Dopamine.....nmol/24hr

Urine Dopamine.....nmol/L

Urine Retinol Binding Protein.....ug/L

Urine Microalbumin.....mg/L

Recent HBA1c.....%

Urine Creatinine.....mmol/ L

All patients were requested to fill in this questionnaire at the time of sample collection. The laboratory information was supplied at the time of sample analysis.

3.2.4 Arylsulphatase A assay

Arylsulphatase A activity was measured using the method of Baum *et al.* (1959). Briefly a 4mL aliquot of each urine sample was dialysed against deionised water overnight at 4°C to remove phosphate ions which inhibit the enzyme. The dialysates were then made up to 6mL and incubated at 37°C for one hour with a reagent containing nitrocatechol sulphate (NCS) (0.01M NCS, 0.05M Sodium acetate 0. 5mM Sodium pyrophosphate 10% Sodium chloride, pH 5.0). The ASA within each sample catalysed the desulphation of NCS to nitrocatechol, the absorbance of which was measured spectrophotometrically at 515nm (PU8700 series UV/Visible spectrophotometer, Phillips, UK). Considerable inhibition of Arylsulphatase B occurs as a result of the low substrate concentration used and the presence of pyrophosphate and chloride ions in reagent A, which ensures desulphation occurs by ASA only (Baum *et al.*, 1959). Urine ASA activity was corrected for creatinine concentration.

Urinary albumin concentrations were measured in all random urine samples and creatinine concentration was measured in all random and 24hr urine samples. Urine sodium was measured in all 24hr samples. All routine analyses were performed using the Roche P800 photometric measuring unit (Roche, UK). Urinary dopamine (In-house HPLC-ED, Chapter 2) was measured in all 24hr urine samples. The most recent glycated haemoglobin (HbA1c, in-house cation exchange HPLC) result was obtained from patients with diabetes mellitus.

Blood pressure measurements of patients with diabetes mellitus were taken at the time of sample collection.

3.2.5 Statistics

All statistical tests were performed on normally distributed data using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Urinary microalbumin concentration was the only data set which was not normally distributed, this was logarithmically transformed to normality. One microalbumin result was removed from correlation analysis because it was considered an outlier being > 3SD from the mean. Two patients failed to provide recent HbA1c results, and one patient failed to provide their blood pressure data. The corresponding ASA activity results were removed from correlation analyses.

An unpaired unequal variance Students t-test was used to compare urinary ASA activity in the controls and the group with diabetes mellitus. Pearsons correlation analysis was used to investigate the correlation between ASA activity and age, urinary albumin, HbA1c, urinary dopamine, urinary sodium, systolic blood pressure, and diastolic blood pressure in patients with diabetes mellitus. This analysis was also performed to investigate the correlation between urinary dopamine and sodium in each group.

An unpaired Students t-test was used to compare the urinary dopamine concentrations in patients with diabetes with those in normal subjects.

3.2.6 Kinetics analysis

Samples from six normal subjects, and six patients with diabetes mellitus with a range of ASA activities were analysed for their kinetic properties. The ASA activity of each sample was measured at six different NCS concentrations: 0.5mM, 1mM, 2mM, 3mM, 4mM, 5mM, and the K_m calculated for each data set using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Michaelis constants (K_m) obtained from the patients with diabetes mellitus were compared with those of the control group using an unpaired Students t-test.

3.3 Results

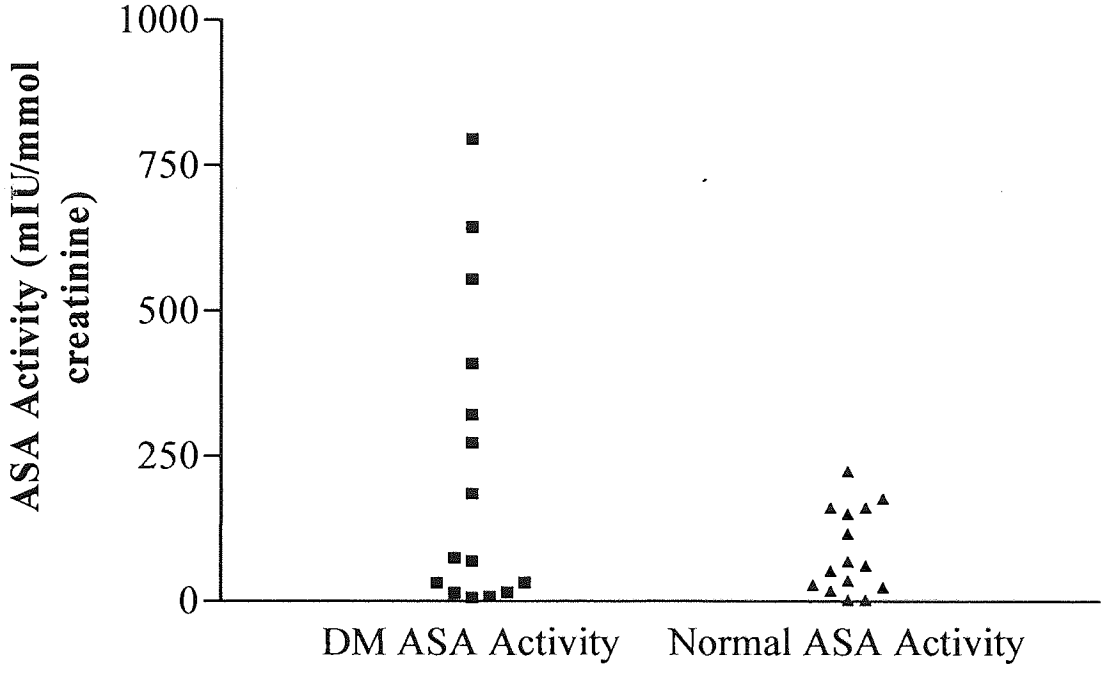
Within batch precision was 2.16% at an ASA activity of 4495 mIU/L (n = 10). Between batch precision, was 8.44% at ASA activity of 8.39 mIU/mmol creatinine, and 3.97% at an ASA activity of 625.1mIU/mmol creatinine. Figure 3b shows the urinary ASA activity in the two groups. The activity of ASA in the group with diabetes mellitus were found to be higher than in the control group but this difference failed to reach significance (p = 0.0503). The mean urinary ASA activity in the group with diabetes mellitus was 229.9 mIU/mmol creatinine (SD = 262.1), whilst in the control group it was 86.2 mIU/mmol creatinine (SD = 72.85). This is highly suggestive of a trend towards significance as illustrated in figure 3b.

The two groups were sex matched, however, the mean age in the group with diabetes mellitus was 63.4 years (range 49 – 73) whilst in the control group it was 41.5 (range 19-70). There was no correlation between urinary ASA activity and age in either the group with diabetes mellitus ($r^2 = 0.0079$, p = 0.7527) or in the control group ($r^2 = 0.04141$, p = 0.4669).

Non linear regression analysis showed that there was no correlation between urinary ASA levels and urinary albumin ($r^2 = 0.09825$, p = 0.2752), HbA1c ($r^2 = 0.1205$, p = 0.2451), urinary dopamine ($r^2 = 0.09388$, p = 0.2667), urinary sodium ($r^2 = 0.002571$, p = 0.8576), systolic blood pressure ($r^2 = 0.003332$, p = 0.8446) or diastolic blood pressure ($r^2 = 0.2163$,p = 0.0938) in those with diabetes mellitus.

Correlation analysis using non-linear regression showed a strong correlation between urinary dopamine and urinary sodium in the control group ($r^2 = 0.6748$, p = 0.0002) and a correlation in the group with diabetes mellitus ($r^2 = 0.2845$, p = 0.0406).

Fig. 3b. Urinary ASA Levels in the Control Group and the Group with Diabetes Mellitus

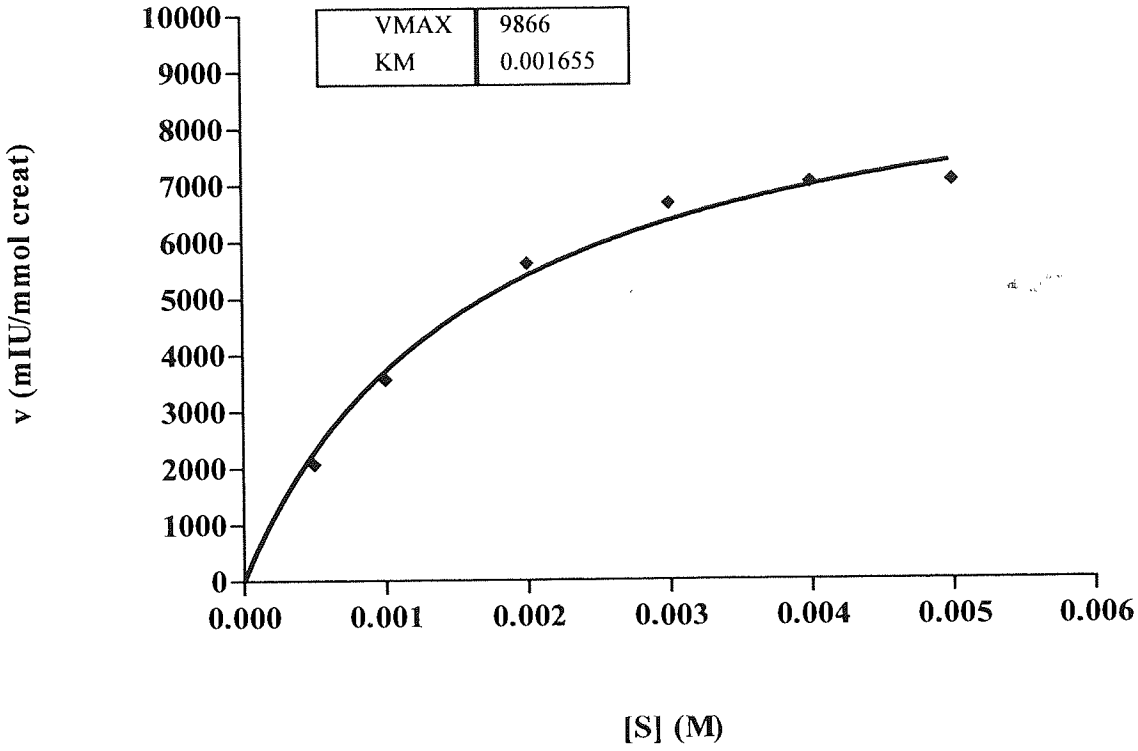


The activity of ASA in patients with diabetes mellitus and a normal control group. The data demonstrate a trend towards increased ASA activity in patients with diabetes mellitus.

The urinary dopamine excretion in patients with diabetes mellitus was significantly lower (mean = 792.1 nmol/L, SD = 317.9) than that observed in the normal control group (mean = 1199 nmol/L, SD = 431.7) ($p = 0.0065$), with no difference in the urinary sodium excretion between the two groups (mean urinary sodium in group with diabetes mellitus = 96.6 mmol/L (SD = 30.45), mean in normal controls = 96.93 mmol/L (SD = 33.17) $p = 0.9773$.)

There was no difference in the K_m values obtained in normal subjects as compared to patients with diabetes mellitus. The mean K_m in the control group was 1.02×10^{3}M (SD = 5.0×10^{4}), and the mean K_m observed in the group with diabetes mellitus was 1.62×10^{3}M (SD = 6.8×10^{4}) ($p = 0.1110$).

Fig. 3c. Example Michelis-Menten Curve for the Determination of K_m



Example Michaelis-Menten plot illustrating the initial velocity (v) of the desulphation of NCS by ASA at five substrate concentrations (S) and subsequent calculation of K_m and V_{max}.

3.4 Discussion

Impaired renal dopamine production in response to salt loading may be one of the mechanisms underlying the development of hypertension in patients with diabetes mellitus. There is often a relationship between nephropathy and hypertension in diabetes mellitus. This reduction in urinary dopamine may be due, at least in part, to a reduction in ASA activity, however the results of preliminary studies together with this investigation have demonstrated that the converse appears to apply. Urinary ASA activity is increased in patients with type 2 diabetes mellitus as compared with normal control subjects. This increase failed to reach significance, however it is clear from the raw data that a trend towards increased ASA in diabetes mellitus is demonstrated. Application of the power calculation described in section 3.2.2 to the preliminary data resulted in a theoretical n = 12. Since there were 15 samples in each group, this study was theoretically sufficiently powered. However in the preliminary investigation a relatively large number of samples were analysed (n = 51 diabetic, n = 37 normal controls) and these preliminary studies demonstrated a significant increase in urinary ASA activity in the diabetic group. The preliminary data are obtained from numerous groups of patients and controls whose results were supplied by different technicians at different times and as a consequence they are without a complete set of laboratory results. It is clear that the results obtained in this investigation show a trend towards increased ASA activity in diabetes mellitus, however a low ASA activity was observed in some patients with diabetes mellitus, and this may have skewed the data such that significance was not reached. Further analysis of samples from both groups may be required in order to confirm a significant increase in ASA activity in diabetes mellitus.

Since the ages of the two groups were not closely matched, it may be that the cause of the increased urinary ASA levels in patients with diabetes mellitus is the increased age of this

group. However non linear regression analysis showed no correlation between urinary ASA and age, hence age cannot explain this observation. Any difference in ASA activity between males and females is accounted for by the analysis of samples from sex-matched groups. Furthermore, the analysis of data in the preliminary study confirms that gender is not a confounder.

No significant correlation between urinary ASA and glycaemic control as measured by HbA1c was found. We could find no correlation between urinary ASA and urinary albumin as a marker of glomerular damage. Furthermore, preliminary studies have shown no correlation of urinary ASA activity with urinary retinol binding protein as a marker of tubular damage. The increased urinary ASA activity appears unrelated to glycaemic control or conventional markers of glomerular or tubular dysfunction.

Urinary ASA levels were not related to urinary sodium or dopamine output in patients with type 2 diabetes mellitus or normal subjects. This may be due to the formation and subsequent excretion of renal dopamine from L-dopa.

Previous studies have demonstrated that urinary dopamine and sodium concentrations are correlated in normal subjects but that this correlation is absent in diabetes mellitus (Rudberg *et al.,* 1997). In this investigation, the correlation between urinary dopamine and sodium in normal control subjects was considerably stronger than that observed in patients with diabetes mellitus. However, the correlation between these two parameters maintained statistical significance in the diabetic group. This may indicate that previous observations are mirrored in this study but that further investigations are required to confirm the breakdown in the dopaminergic response to sodium in patients with diabetes mellitus.

There is an increase in the excretion of 6-OHDA in patients with diabetic nephropathy as compared with non-nephropathic diabetic patients (Chetty *et al.*, 2002). In this study three of the patients with diabetes mellitus had nephropathy as a complication. Of these, only two had ASA levels which may be considered high. This study has not assessed the levels of 6-OHDA in normal subjects as compared to patients with type 2 diabetes mellitus. Since there are only three patients who suffer from nephropathy in the study group then a correlation between urinary ASA and 6-OHDA would not be statistically viable. However, a further four of the patients with diabetes mellitus demonstrated urinary albumin levels which are associated with incipient nephropathy as compared with the control group, of which only one subject demonstrated such albumin excretion.

It could be suggested that the increase in ASA activity may result in an increased renal dopamine production, however there is no correlation between urinary ASA and dopamine in patients with diabetes. Furthermore, urinary dopamine levels are significantly lower in the diabetic group as compared with the normal control group. The increased presence of oxidative stress in the kidney of patients with nephropathy may accelerate the autoxidation of dopamine to 6-OHDA which itself may increase the oxidative stress of renal cells and contribute to the acceleration of diabetic nephropathy in such patients. The autoxidation of dopamine may sequester free dopamine available for natriuresis and result in the decreased dopamine excretion observed in diabetes mellitus.

The analysis of the kinetics of ASA illustrates that the increased ASA activity observed in patients with diabetes mellitus is not due to an alteration in the affinity of ASA for dopamine sulphate as has been observed *in vitro* (Strobel *et al.*,1990). This increased activity may therefore be due to an increased expression of the gene for arylsulphatase a (*arsa*).

3.5 Conclusion

The increased urinary ASA levels observed in diabetes mellitus are likely to reflect an increased production of ASA, the main sites of which are the kidney and white blood cells. ASA catalyses the conversion of renal dopamine sulphate to dopamine, and an increased expression of the arylsulphatase A gene (*arsa*) would result in increased levels of ASA and an increase in the desulphation of dopamine sulphate to active free dopamine. An alternative source of renal dopamine is L-dopa. A decreased renal dopamine production from L-dopa has previously been demonstrated in salt-retaining states (Aoki *et al.*, 1989; Gill *et al.*, 1991; Kuchel & Shigetomi, 1992). Furthermore, in this investigation the concentration of urinary dopamine is significantly lower in patients with diabetes mellitus. The renal expression of the *arsa* may be increased in patients with diabetes mellitus, leading to increased ASA activity, promoting the formation of dopamine from dopamine sulphate as a compensatory mechanism for the decreased production of renal dopamine from L-dopa, and increased sequestering of dopamine by autoxidation to 6-OHDA.

CHAPTER 4

4. THE STRUCTURE OF THE ARYLSULPHATASE A GENE (*arsa*)

4.1 Introduction

Arylsulphatase A (ASA) catalyses the conversion of the physiologically inert dopamine sulphate, to the natriuretic hormone dopamine. The structure of ASA (Lukatela *et al.*, 1998 EC 3.1.6.1) and its mechanism of action have been described (Waldow *et al.*, 1999). Furthermore, the gene for Arylsulphatase A (*arsa*) has been mapped to chromosome 22q13.33 – qter (www.ensembl.org), and the genomic (Kreysing, *et al.*, 1990) and cDNA (Stein, *et al.*, 1989) sequences of *arsa* have been characterised.

Dopamine excretion is blunted in type 1 and type 2 diabetes mellitus (Segers *et al.*, 1995; Shigetomi *et al.*, 1995), and other conditions which may be salt-retaining, including essential hypertension (Aoki *et al.*, 1989; Gill *et al.*, 1991; Iimura, 1996). This may be due in part to the decreased production of dopamine from L-dopa or dopamine sulphate. Decreased excretion of dopamine may also be due to an increase in the sequestering of free dopamine by autoxidation to, among other oxidation products, 6-hydroxydopamine (6-OHDA) (Tong & Baines, 1993).

Diminished ASA activity is observed in patients with metachromatic leukodystrophy (MLD) where polymorphisms of *arsa* result in a decreased ASA activity and the subsequent accumulation of sphingolipid cerebroside sulphate. This leads to the progressive de-myelination of oligodendrocytes (Gieselmann, *et al.*, 1994). Blunted ASA activity as a result of polymorphic mutations of *arsa* (Ben-Yoseph & Mitchell, 1995; Berger, *et al.*, 1996; Coulter-Mackie & Gagnier, 1997) may also result in the blunted dopamine excretion observed

in salt-retaining states. To investigate this hypothesis, the following study was undertaken in parallel with the investigation described in Chapter 3, the ASA activity study. However the results observed demonstrate an increase in urinary ASA activity in patients with diabetes mellitus. This contradicts the hypothesis that there is a decreased ASA activity which results in a blunted dopamine excretion in diabetes mellitus and salt retaining states. The increased ASA activity observed in patients with diabetes mellitus is not due to an increased affinity of ASA for dopamine sulphate in such patients as demonstrated by no change in the K_m of ASA between patients with diabetes mellitus and normal control subjects (Chapter 3). To investigate the possible existence of polymorphisms within *arsa* and to confirm that the increased ASA activity observed in patients with diabetes mellitus is not due to the existence of an isoform of ASA with a higher affinity for dopamine sulphate, the sequence of *arsa* in patients with diabetes mellitus was analysed and compared with that of normal subjects.

The development of automated sequencing instrumentation such as the Applied Biosystems 3700 high thoughput capillary sequencer coupled with fluorescent dye terminator reactions, allows the large scale sequencing analysis of large numbers of samples with a relatively low turnover time. Furthermore DNA analysis *in silico* allows sequence evaluation to be performed with relative ease and efficiency. This investigation employed specifically designed primers to amplify a large proportion of *arsa* using the polymerase chain reaction (PCR), and subsequent sequence analysis of the amplicons generated.

4.2 Materials and Methods

4.2.1 Samples

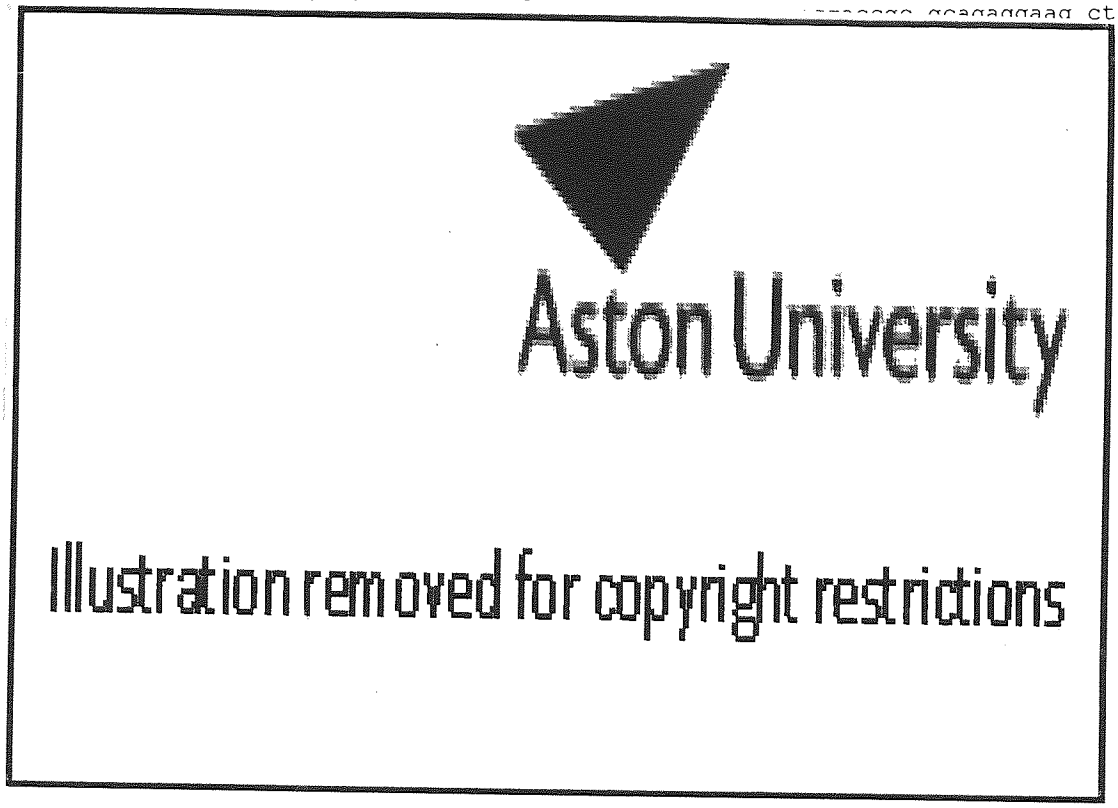
Deoxyribonucleic acid was extracted from the whole blood of three normal subjects and three patients with diabetes mellitus. Samples were quantitated using UV spectrophotometry at 260nm and 280nm. An absorbance of 1.0 at 260nm corresponded to approximately 50μg /mL for double stranded DNA, and the ratio of absorbance at 260nm:280nm gave an indication of the purity of the DNA extracted. Pure preparations gave ratios close to 1.8 and protein contaminated samples gave a significantly lower ratio. Once quantitated, DNA was reconstituted in H_2O to a concentration of 20ng/μL and stored at -20°C until use.

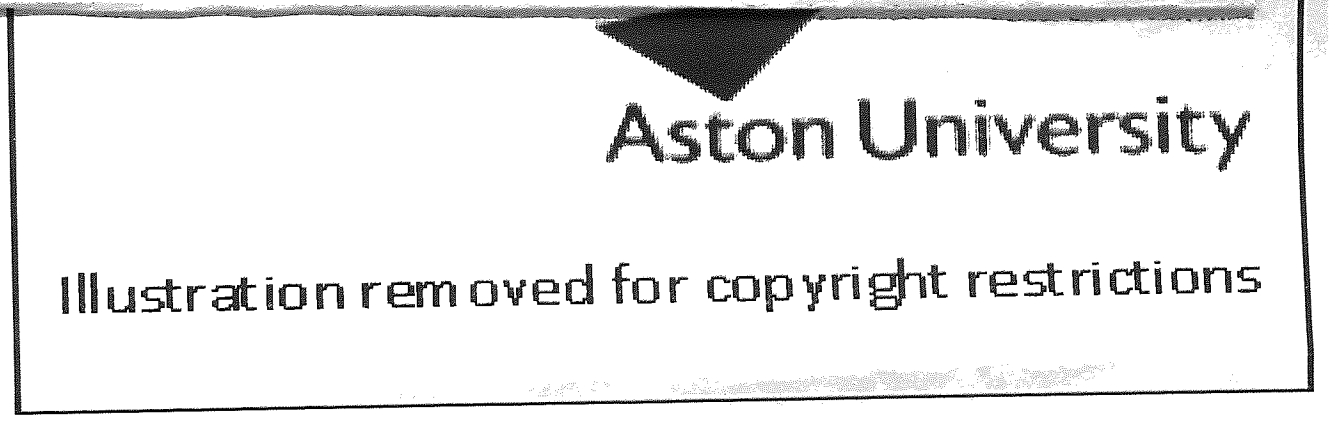
4.2.2 Primer Design

Primers were designed to span 77.4% (1179bp / 1524bp) of the coding sequence of *arsa* (Accession No. X52151, Stein *et al.*, 1989). The factors which were taken into consideration when designing PCR primers are discussed in detail in section 1.5.1.1.2.4. Briefly the %G+C content, theoretical melting temperature and therefore annealing temperature of each primer set, together with the length of the 3' G+C clamp of each primer were assessed. The genomic sequence of *arsa* (Accession No. X52150, Kreysing *et al.*, 1990) was obtained from the databases searched within the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). The position of primer binding was evaluated such that as much of the exonic region of the *arsa* sequence was incorporated into the analysis as possible. This is illustrated in figure 4a.

Fig. 4a. Position of Primer Binding To The Genomic DNA Sequence of *arsa*

```
1 agccgctcct cctctgagaa gctccggacc cgagaggaca ccgacactgc gcagcgccga
                                                  gcagaggaag ctcccagagc
```





nnnn = coding region of *arsa*; nnnn = position of forward primer binding; nnnn = position of reverse primer binding; nnnn = multiple transcription initiation sites; nnnn = four potential Sp1 binding sites (Kreysing *et al.*, 1990).

Primers were manufactured by Alta Biosciences (University of Birmingham, UK) and were reconstituted in double-distilled H_2O to stock concentrations of 70 - 120μM. All primer pairs were diluted to a working concentration of 10μM using double distilled H_2O prior to use in PCR. The properties of each primer are described in table 4a.

4.2.3 PCR Optimisation

The annealing temperature of each primer set was used as guide for the empirical elucidation of the optimal annealing temperature for each primer set as described in section 1.5.1.1.2.4 and table 4a.

For each PCR a master mix containing: 1x PCR buffer, 1.0mM $MgCl_2$, dNTPs each at a concentration of 0.2mM, 1.25U *Taq* DNA polymerase (Promega, UK), and 1.0μM of each primer (Alta Biosciences, University of Birmingham, UK) was prepared. Template DNA was added (2μL,40ng) and each reaction mix made up to 25μL with double distilled H_2O. A negative control using double distilled H_2O in place of DNA template was set up for each PCR reaction to test for reagent contamination. Samples were cycled as follows: 94°C, 1min 30s; the annealing temperature for each primer set as illustrated in table 4a, 60s; 72°C, 60s. This was repeated 30 times using a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA). The reaction mixes were analysed using electrophoresis through a 2% agarose gel (Seakem LE agarose) using 0.5x Tris-acetate running buffer (TAE) with ethidium bromide staining. Amplicons were visualised under UV illumination and the gel image captured using a Grab-IT annotating grabber version 2.59 for windows 3.1 (Ultra-Violet Products Ltd, UK).

Table 4a. Properties of Primers Designed for the Amplification the *arsa* Gene

			Start Position				Size of Product (bp)	
Primer	**Sequence**	**Size**	**cDNA**	**gDNA**	**T_m**	**AT**	**cDNA**	**gDNA**
arsa F1 Forward	5' GCGCCCGCAGCCCGGTAC 3'	18	N/A	261	67.4	64	540	540
arsa F1 Reverse	5' CAGCCCTCCCGCCGCCAG 3'	18	171	800	67.4			
arsa F2 Forward	5' CGGCAAGTGGCACCTTGGGGTG 3'	22	372	1141	67.7	65	382	570
arsa F2 Reverse	5' CCCAAATGGCCCGCGGCCTG 3'	20	738	1710	67.6			
arsa F3 Forward	5' GGCGGCTGCTCCGGTCTCTTG 3'	21	873	2152	67.6	66	218	308
arsa F3 Reverse	5' CCCAGCAGCAGGGGGCTGAG 3'	20	1091	2459	67.6			
arsa F4 Forward	5' GCCCTCGGCAGTCTCTCTTC 3'	20	1102	2721	63.5	61	408	523
arsa F4 Reverse	5' CTGGGCAATGGCAGCAAGCTG 3'	21	1510	3243	63.7			

F1 – F4 = Assignment of each fragment of *arsa*; gDNA = genomic DNA; T_m = theoretical melting temperature; AT = annealing temperature.

4.2.4 Isolation and Purification of DNA from Agarose Gels

A novel method was employed to recover PCR products from agarose gels. DNA amplicons were visualised by ultraviolet illumination and a sterile scalpel blade used to remove each PCR product from the gel. This gel 'slice' was then placed into the tip of a 100µL filter tip (Axygen Scientific Inc., USA) and this placed into a 1500µL microfuge tube (Eppendorf, UK). Each sample was centrifuged at 3000 rpm for 10 minutes and the DNA collected in the microfuge tube. The DNA was precipitated overnight using ethanol or isopropanol and sodium acetate (Sambrook *et al.*, 1989). The precipitated DNA was separated from the remaining solution by centrifugation at 13,000 rpm for 20mins and the pellet washed with 70% ethanol, dried and resuspended in 10-50µL double distilled H_2O. All samples were stored at -20°C until use.

4.2.5 Quantification of Isolated DNA

All samples were quantified by comparison with a range of concentrations of standard λ phage DNA with ethidium bromide (2µg/mL) staining under UV illumination.

4.2.6 Bulk preparation of PCR products

To ensure sufficient yield of product to allow successful sequencing analysis, the PCR products from five separate PCR reactions using the same template were pooled and analysed as a single sample at agarose gel electrophoresis.

4.2.7 DNA sequencing

Sequencing of 0.4 μg of DNA was performed using an automated fluorescent dye terminator sequencing reaction (Alta Biosciences, University of Birmingham, UK). The appropriate forward primer of each set was used as the sequencing primer.

4.2.8 Sequence analysis

Sequence data obtained for each sample was analysed using the GCG Bestfit and Pileup sequence alignment tools within the Wisconsin package (Devereux *et al.*, 1984) on the University of Birmingham Academic Computing Service VAX facilities. The quality of sequence data was measured as follows:

$$\% \text{ Successful sequence data} = \left[\frac{N_2}{N_1} \right] \times 100$$

Where: N_1 = Total number of bases of *arsa* fragment

N_2 = Number of bases of readable sequence

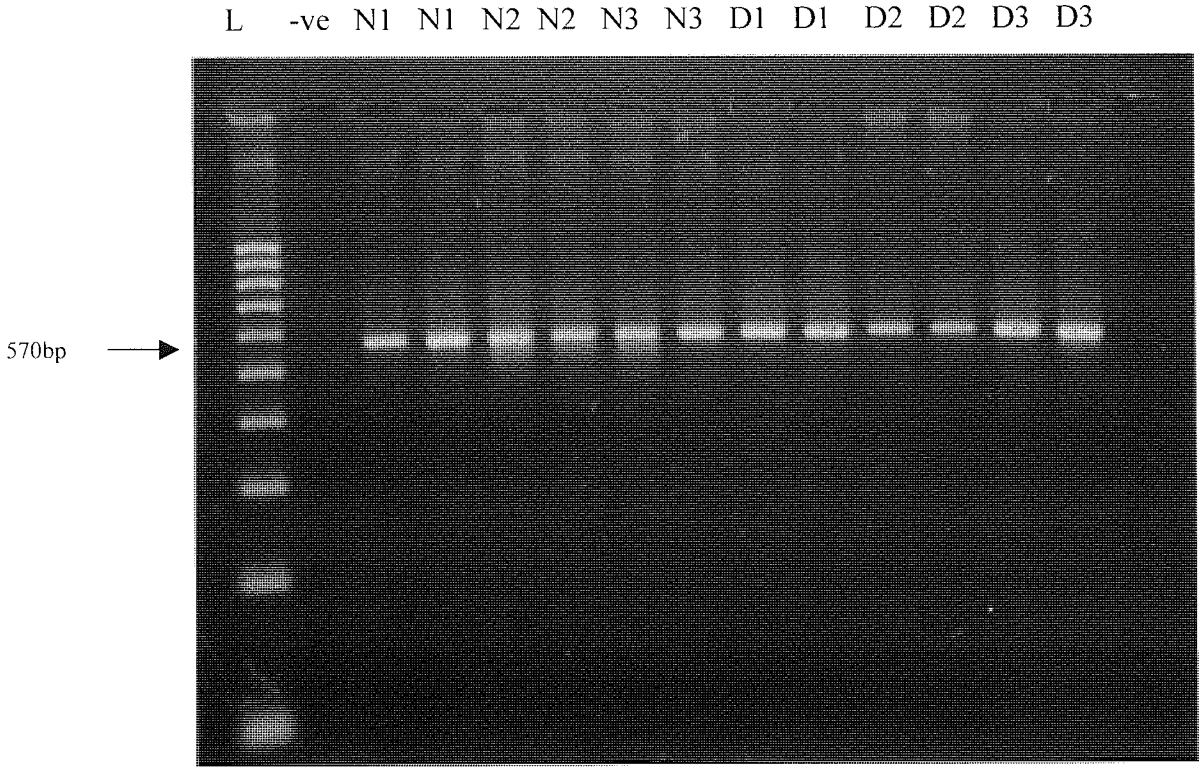
Each fragment of the published sequence of *arsa* (X52150) was aligned with the sequence data obtained from all normal and diabetic samples and any discrepancies between the two sequences was noted. The sequences obtained from all three patients with diabetes, together with those obtained for normal subjects, and X52150, were aligned and all seven sequences per fragment compared.

4.3 Results

The results of PCR amplification of each fragment of the *arsa* gene indicated that there were no insertions or deletions in the *arsa* gene in patients with diabetes mellitus as compared with normal controls. However, small differences in size cannot be easily detected by PCR. Sequencing analysis of each fragment of the gene confirmed these initial findings. Figure 4b illustrates the formation of a 570bp PCR product of fragment 2 of the *arsa* sequence from normal subjects and patients with diabetes mellitus. Each fragment gave similar results with both the normal and diabetic samples showing the amplification of PCR products of the same size.

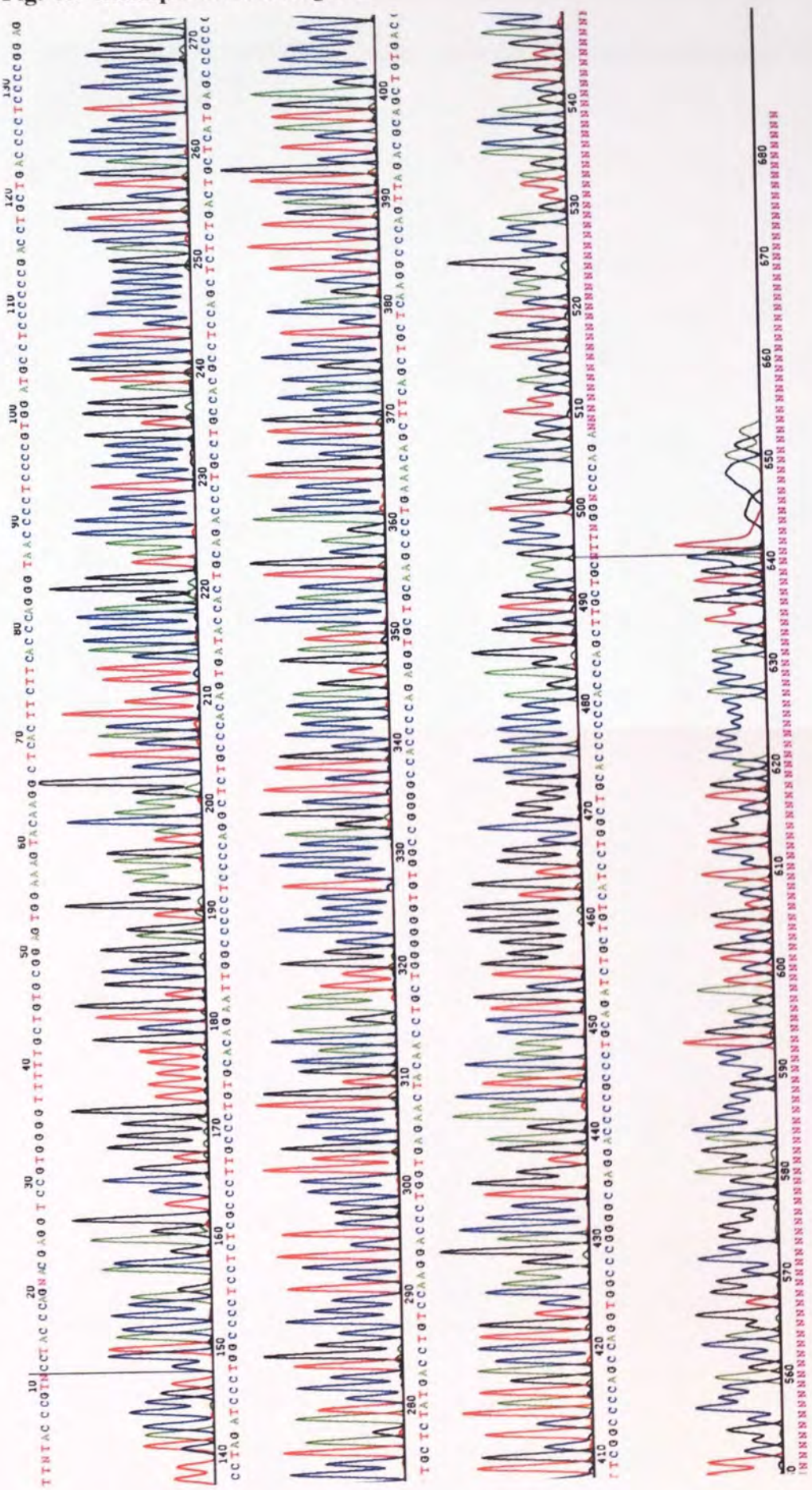
The quality of sequence data obtained in this investigation is illustrated in figure 4c; an example chromatogram of the sequencing data of fragment 4 from a normal subject.

Fig. 4b. PCR Products Obtained from the Amplification of Fragment 2 of *arsa*



L = 100bp ladder (Sigma, UK); N1 – N3 = genomic DNA template from normal subjects; D1 – D3 = genomic DNA template from patients with diabetes mellitus.

Fig. 4c. Example Chromatogram



Figures 4d – 4g illustrate the sequence alignments between each fragment of the *arsa* gene sequence, and the published genomic sequence of *arsa* (Accession No. X52150, Kreysing *et al.*, 1990).

Fig. 4d. Pileup Analysis of Fragment 1 (F1) of *arsa*

Applicable to figures 4d – 4g: F1 – F4 = fragments 1 - 4; N1 –N3 = Normal samples; D1 – D3 = Diabetic samples; ASA = published sequence of *arsa*; A G C T = purine and pyrimidine bases; N = unreadable sequence; . = gap in sequence data; ~ = positions for which there is no sequence data available, e.g. at the beginning and end of a set of sequence data.

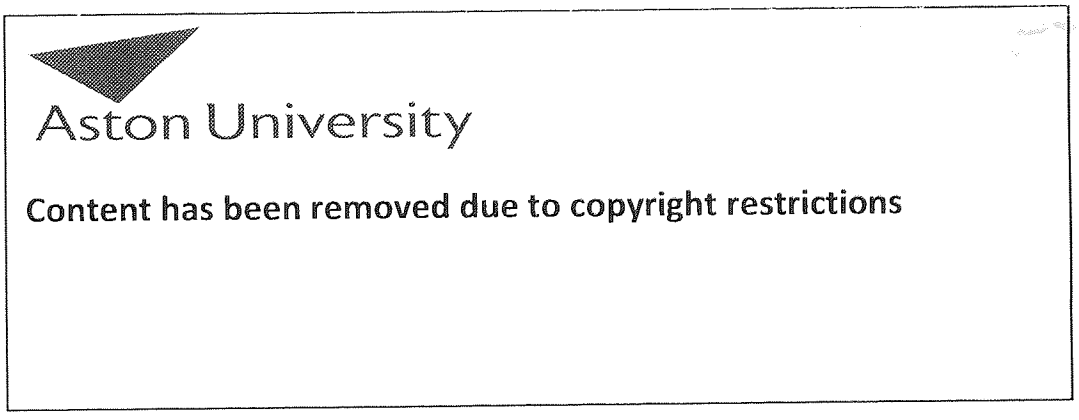
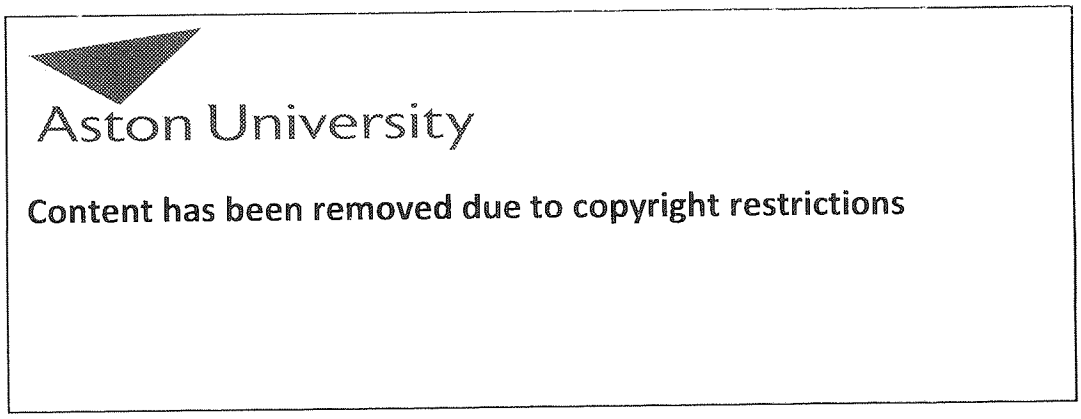


Fig. 4e. Pileup Analysis of Fragment 2 (F2) of *arsa*

F2D1

Fig. 4f. Pileup Analysis of Fragment 3 (F3) of *arsa*

Fig. 4g. Pileup Analysis of Fragment 4 (F4) of *arsa*



The sequence data obtained for each fragment is summarised in table 4b.

Table 4b. Sequence Data of Four Fragments of *arsa* – Normal and Diabetic Subjects

Fragment No.	Normal or Diabetic	% Successful Sequence Data Obtained	Discrepancy between Sequence Data and X52150
F1	N1	74	Substitutions between 324 – 467; Insertion of G at 325; Insertion of T at 464.
F1	N2	56	Substitutions between 466 – 628; Insertion of T at 464.
F1	N3	63	Substitutions between 324 – 610; Insertion of G at 325; Deletion of T at 447; Insertion of T at 464.
F1	D1	46	Substitutions between 466 – 467; Deletion of T at 447; Insertion of T at 464.
F1	D2	49	Substitutions between 324 – 467; Insertion of G at 624; Deletion of T at 447; Insertion of T at 464.
F1	D3	63	Substitutions between 324 – 467. Insertion of G at 324; Deletion of T at 447; Insertion of T at 464.
F2	N1	95	All discrepancies intronic / silent mutations
F2	N2	93	All discrepancies intronic / silent mutations
F2	N3	94	All discrepancies intronic / silent mutations
F2	D1	95	All discrepancies intronic / silent mutations
F2	D2	94	All discrepancies intronic / silent mutations
F2	D3	95	All discrepancies intronic / silent mutations
F3	N1	93	No discrepancies
F3	N2	93	No discrepancies
F3	N3	92	No discrepancies
F3	D1	91	No discrepancies
F3	D2	91	All discrepancies intronic / silent mutations
F3	D3	93	No discrepancies
F4	N1	93	C → G at 2790; Thr391Ser; Exon 7
F4	N2	69	C → G at 2790; Thr391Ser; Exon 7; C → G at 2983; Ser417Cys; Exon 8
F4	N3	92	C → G at 2790; Thr391Ser; Exon 7; G → A at 3220; Arg496His; Exon 8
F4	D1	72	C → G at 2790; Thr391Ser; Exon 7
F4	D2	94	No discrepancies
F4	D3	60	No discrepancies

4.4 Discussion

4.4.1 Mutations of *arsa*

Numerous mutations of *arsa* have been characterised, the most common of these are described in table 4c. Fifty seven mutations associated with metachromatic leukodystrophy (MLD) have been located: five small deletions, three splice mutations, 47 amino acid substitutions, one nonsense mutation, and a combined missense/splice donor site mutation (Berger *et al.*, 1996).

The 'I' and 'A' alleles are those most commonly associated with MLD. The 'I' allele occurs in 45% of infantile and 27% of all cases of MLD, and the 'A' allele is found in 45% of juvenile or adult onset cases and 25% of all cases of MLD (Coulter-Mackie & Gagnier, 1997).

Table 4c Common Mutations of *arsa*

Mutation	Position			Allele	Disease
	gDNA	cDNA	AA Residue		
G → A[1,2,3]	1238 Intron 2	459+1	N/A	I	MLD
G → C or T[1]	1471 Exon 3	579	Trp193Cys	I	MLD
A → T[1]	2791Exon 7	1172	Thr391Ser	I	MLD
C → T[1,2,3]	3010 Exon 8	1277	Pro426Leu	A	MLD
A → G[1,3]	2417 Exon 6	1049	Asp350Ser	Apd	ASA pseudodeficiency Loss of N-Glycosylation site
AATAAC →AGTAAC[1,3]	N/A	1619 Poly-A signal	N/A	Apd	ASA pseudodeficiency
C → T[1]	1787 Exon 4	820	Thr274Met	N/A	Common *arsa* mutation
C → T[1]	1140 Exon 2	362	Ala121Val	N/A	Common *arsa* mutation
T → G[1,2]	1428 Exon 3	536	Ile179Ser	N/A	MLD
G → A[1]	1074 Exon 2	296	Gly99Asp	N/A	Common *arsa* mutation
T → C[2]	1005 Exon 2	227	Leu76Pro	N/A	Not related to MLD / Apd
G → A[2]	1029 Exon 2	251	Arg84Gln	N/A	
C → T[2]	1065 Exon 2	287	Ser96Phe	N/A	
C → T[2]	1527 Exon 3	635	Ala212Val	N/A	
G → A[2]	2823 Intron 7	1204 +1	N/A	N/A	

[1] = Coulter-Mackie & Gagnier, 1997; [2] = Berger *et al.*, 1996; [3] = Ben-Yoseph & Mitchell, 1995; gDNA = Genomic DNA; AA = amino acid; Apd = arylsulphatase A pseudodeficiency allele;

4.4.2 Sequence Interpretation

There were no discrepancies which would affect the activity of ASA, between the established sequence of *arsa*, and that obtained by sequencing.

4.4.2.1 Fragment 1 (Exon 1)

Sequence data of fragment 1 of *arsa* from normal and diabetic subjects was poor in all cases with the percent successful data obtained ranging from 46 –74%. This may be due to the suitability of the forward primer for sequencing analysis. It is recommended that the primer which is used for DNA sequencing is designed such that the desired sequence is located between 80 and 150 nucleotide bases away from the position of primer binding, and that this sequence be less than 300 nucleotide bases long. However, the sequence data obtained provided >400 nucleotide bases of accurate sequence (http://seqcore.brcf.med.umich.edu/doc/dnaseq/primers.html). All other factors considered when designing primers specifically for sequencing analysis correlate with the considerations of primer design for PCR amplifcation as described in Chapter 1.

All discrepancies between the sequencing data obtained and the sequence of X52150, occurred within the sequence between the promoter motifs and the coding sequence of the gene. This region is not transcribed and so will not affect the sequence, and therefore the activity of ASA. Insertions or deletions in the sequence between promoter motifs and the start codon of eukaryotic genes have been reported to have little or no effect upon transcription (Lodish *et al.*, 2000). Furthermore these discrepancies were observed in all samples analysed indicating that they are not sequence polymorphisms associated with

diabetes mellitus. The significance of the discrepancies in the promoter region of *arsa* may be investigated further.

4.4.2.2 Fragment 2 (Exons 2,3 and 4)

The sequence data of fragment 2 from normal and diabetic subjects was very good, with the percent successful data obtained ranging from 93 - 95%. Several discrepancies were found, however these were located within the intronic regions of *arsa* , or were silent mutations which caused no change in the translated sequence of ASA. Thus no difference in the structure and therefore activity of ASA was observed.

4.4.2.3 Fragment 3 (Exons 5 and 6)

Fragment three of *arsa* provided good sequence data, with the percent successful sequence data obtained from all samples ranging from 91 – 93%. Any discrepancies that were observed occurred within the intronic region of the gene resulting in no difference in the coding sequence of *arsa.*

4.4.2.4 Fragment 4 (Exons 7 and 8)

Accurate sequence data obtained from fragment 4 varied between 60 – 94%. Several discrepancies were observed between sequence data and the published sequence of arsa (X52150). Most were either intronic and therefore would not affect the coding sequence of

arsa. However, there was one discrepancy which occurred in four of the six samples. This resulted in the amino acid substitution illustrated in table 4b. Here a Threonine residue was substituted by a Serine residue at amino acid position 391. This substitution has been described previously and contributes to the 'I' allele associated with metachromatic leukodystrophy (table 4c). This may be a common mutation of arsa which alone has no effect upon the activity of the enzyme. Furthermore both Threonine and Serine are aliphatic with hydrophilic side chains, and are therefore similarly reactive. This substitution is unlikely to affect the conformation of ASA. This amino acid residue is not located at the active site of ASA and so is unlikely to affect the activity of the enzyme. This substitution occurred in samples from both normal subjects and patients with diabetes mellitus indicating that this amino acid substitution is not a polymorphism associated with diabetes mellitus. However the effect of this amino acid substitution has not been extensively investigated.

Two of the sequences obtained from normal subjects had additional amino acid substitutions within Exon eight of *arsa*, neither of which have been described previously. The first was the replacement of a Serine residue for a Cysteine residue at position 417. The effect of this amino acid substitution is not known. Cysteine is a hydrophobic amino acid with a highly reactive sulphydryl side chain, and so it may affect the conformation of ASA in replacing the hydrophilic amino acid Serine. However, this substitution is located at the periphery of ASA and not near the active site and is therefore unlikely to affect the activity of the enzyme. The second substitution was the replacement of an Arginine by Histidine at amino acid position 496. These amino acids are both basic and have hydrophilic side chains. Although Histidine is more reactive than Arginine it is unlikely that this amino acid substitution will affect the conformation of ASA, and again this substitution is not located at the active site of ASA and so is unlikely to affect the activity of the enzyme. Furthermore, these two amino acid substitutions occurred in two different samples, both of which were from normal subjects.

Therefore the discrepancies in the *arsa* sequence observed here are unlikely to represent polymorphisms related to diabetes mellitus. The effect of the nucleotide discrepancies and the resulting amino acid substitutions observed here may be candidates for further investigation.

This study employed the sequencing of a representative number of samples in order to look for similarites in the sequence of *arsa* in normal subjects and patients with diabetes mellitus. Wider screening methods such as single strand conformational polymorphism (SSCP) analysis screen large numbers of samples for sequence differences and were unsuitable for application to this study.

4.5 Conclusion

The *arsa* sequences obtained from all samples in this investigation differed from that of the published sequence. However, none of these discrepancies are likely to affect the activity of ASA. Previous studies (Chapter 3) have illustrated an increased ASA activity in patients with diabetes mellitus as compared with normal controls which was not due to an increased affinity of ASA for dopamine sulphate in such patients. The conserved sequence of *arsa* seen here supports the hypothesis that the increase in activity of ASA observed in diabetes mellitus is not a consequence of polymorphisms in *arsa*. However, the increased activity of ASA in diabetes mellitus may be due to an increase in the expression of *arsa* in patients with this condition as a compensatory mechanism for diminished renal dopamine production, and sequestering of free dopamine by autoxidation. To assess this hypothesis, the expression of *arsa* in patients with diabetes mellitus will be investigated and discussed in chapter 6.

CHAPTER 5

5. USE OF *gapd* AS A TRANSCRIPTIONAL BENCHMARK IN REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION: DESIGN OF PRIMERS WHICH DO NOT BIND TO *gapd* PSEUDOGENE SEQUENCES

5.1 Introduction

Gene expression analysis has been investigated using a variety of molecular biological techniques including Northern Blotting (Engler-Blum *et al.*, 1993; Goldsworthy *et al.*, 1993; Barroso *et al.*, 1999), *in situ* hybridisation (ISH) (Heppner *et al.*, 1996; Guiot & Rahier, 1997), differential display analysis (Barisani *et al.*, 2000), ribonuclease protection (Lemay *et al.*, 1996), and methods based on reverse transcription polymerase chain reaction (RT-PCR, Finke *et al.*, 1993; Luetjens *et al.*, 2001) including microarray technology (Chin *et al.*, 2002). Quantitative and semi-quantitative RT-PCR (Chapter 6) have found increasing application in the study of gene expression (Witsell & Schook, 1990; Specht *et al.*, 2001), particularly when the yield of mRNA is limited, as the sensitivity of this technique allows the detection of low levels of message (Raff *et al.*, 1997). A low yield of nucleic acid is often obtained from archive samples (Rupp & Locker, 1988; Stanta & Schneider, 1991; Weizsacker *et al.*, 1991; Stanta *et al.*, 1998; Coombs *et al.*, 1999; Specht *et al.*, 2000) or tissue biopsies (Miyazake *et al.*, 1994; Del Prete *et al.*, 1998) and is complicated further by the potential degradation of mRNA by ubiquitous RNAses.

A common approach in such studies is to 'normalise' expression of the gene under investigation against that of a non-competitive standard which is under weak transcriptional regulation. These so-called 'housekeeping genes' include the genes for β actin (*actb*, Raff *et*

al., 1997), 18S rRNA (Spencer & Christensen, 1999) and glyceraldehyde-3-phosphate dehydrogenase (*gapd*, Del Prete *et al.*, 1998). The advantages and disadvantages of the use of a wide variety of housekeeping genes have been reviewed (Suzuki *et al.*, 2000; Sturzenbaum & Kille, 2001). Although such genes were initially thought to be constitutively expressed, evidence suggests that housekeeping genes may be up-or down-regulated under particular experimental conditions and in certain disease states (table 5a). Meticulous experimental design is therefore required when planning a gene expression protocol to ensure the appropriate housekeeping gene is used as an internal control. The analysis of *arsa* expression will require the use of an internal control gene and this investigation will ensure the employment of an appropriate 'housekeeping' gene.

As illustrated in table 5a, there are many advantages and flaws associated with the use of numerous genes used as internal controls in expression analyses. In situations where tissue is undergoing extensive morphological changes, the use of actin is not recommended. Also, expression studies using genes whose transcripts have more than one isoform should be designed to prevent cross-reactivity. This applies to the use of both actin and cyclophilin. The ribosomal RNA subunits have compared favourably against *gapd*, actin and cyclophilin in expression studies (Sturzenbaum & Kille, 2001), however there are disadvantages to the use of rRNA as an internal control.

The lack of polyadenylation of rRNA means it cannot be exploited when dealing with poly(A)$^+$ RNA or cDNA derived from total RNA using oligo-dT primers in the reverse transcription. Ribosomal subunits do not show the same degree of sequence conservation between species as does *gapd*. Also, one must ensure that mitochondrial RNA is not isolated if using rRNA as an internal control, as the expression of mitochondrial genes fluctuate according to the needs of cellular energy and metabolism (Sturzenbaum & Kille, 2001).

Table 5a. Comparison of Housekeeping Gene Regulation Under Experimental Conditions and in Disease States

Housekeeping Gene	Factor	Effect	Target
GAPD[1]	Hypoxia[3,17]	Upregulation	Cultured bovine vascular endothelial cells
	Transition metals[17]	Upregulation	Cultured bovine vascular endothelial cells
	Deferoxamine[17]	Upregulation	Cultured bovine vascular endothelial cells
	Manganese[12]	Upregulation	Astrocytes
	Glucose	Upregulation	INS-1 glucose responsive β-cell line
	$1,25(OH)_2D_3$	Upregulation	BT-20 human breast carcinoma cell line[7]
	Insulin	Upregulation	Differentiating brown adipocytes[18], hepatoma cell lines, 3T3-F442A adipocytes[8], 3T3-L1 adipocytes[9]
	Programmed cell death	Upregulation	Neurones
	Heat Shock[27]	Upregulation	BALB/c 3T3 and Hsp70 cells
	Oxidative Stress by diethyldithiocarbamate[21]	Increased Steady-state level	Rabbit Aortic Vessel rings
	Iron-chelating agent	Upregulation	
	UV irradiation[16]	Modulation	Human keratinocytes
	2,3,7,8-tetrachlorodibenzo-p-dioxin[4]	Upregulation	Human keratinocytes
	Platelet-derived Growth Factor	Upregulation	Rat smooth muscle cells
	Interleukin-2	Upregulation	Murine T-lymphocytes
	NAD^+ precursors[24]	Upregulation	Jurkat cells
	Noradrenaline[18]	Upregulation	Differentiating brown adipocytes
	Tri-iodothyronine[18,]	Upregulation	Differentiating brown adipocytes, Mouse Skeletal muscle[23]
	Retinoic acid[18]	Down regulation	Differentiating brown adipocytes
	Caerulein (causing acute pancreatitis)[6]	Upregulation	Rat Pancreas
	Pregnancy	Upregulation	Uterine artery endothelial cells
	Food deprivation[25]	Down-regulation	Oxyntic, antral and duodenal mucosa, pancreas (rat)
	Rapamycin[14] (immunosuppressant)	Upregulation	Rat hepatocytes
	Imipramine	Upregulation	Rat Brain
	Tumor implantation[13]	Upregulation	Mouse spleen
		Upregulation	Mouse tumorigenic and malignant cell lines[5]
	Glucose[10]	Upregulation	INS-1 cells
		Upregulation	Human hepatocellular carcinoma tissue[19]
	Breast cancer cell proliferation[7]	Positive correlation	Human breast cancer cell
		Inter-individual variation[20]	Rat liver
	Cell proliferation by carbon tetrachloride treatment[26]	Upregulation	Rat liver
β-ACTIN[1]	Hypoxia[3]	Upregulation	
	Bacterial exposure	Down-regulation	Human uroepithelial cell lines
	Ionising radiation	Down-regulation	Syrian hamster embryo cell lines

Housekeeping Gene	Factor	Effect	Target
	Growth factors	Upregulation	Low serum-cultured mammalian cells
	High Serum	Upregulation	Low serum-cultured mammalian cells
	Caerulein	Upregulation	Pancreas
	Vitamin B6 deficiency	Upregulation	Rat liver
	Adrenocorticotrophin	Upregulation	Adrenal glands of hypophysectomised rats
	Abdominal aortic banding	Upregulation	Rat myocardium
	Gonadotrophin	Upregulation	Rat granulosa cells
	Epidermal growth factor and cyclohexamide[15]	Upregulation	AKR-2B mouse embryo
	Matrigel treatment[22]	Dose-dependent inhibition	Membrane Type-1 Matrix Metalloproteinase
	Cell proliferation by carbon tetrachloride treatment[26]	Upregulation	Rat liver
Cyclophilin (CYP)[2]	Mercuric Chloride	Upregulation	Plants
	Heat shock	Upregulation	Plants
	Viral infection	Upregulation	Plants
	Ethephon	Upregulation	Plants
	Salicylic acid	Upregulation	Plants
	Heat stress & hypoxia[3]	Protein induction	H9c2 Myogenic cells[11]
Elongation factor-α[2]		Modulation	Rapidly growing tissue
		Modulation	Plant meristems
		Modulation	Gametophytes
	Growth arrest	Modulation	
	Transformation	Modulation	
	Ageing	Modulation	
	Cell death	Modulation	
Albumin[2]	Phenobarbitone	Modulation	
	Methylclofenapate	Modulation	
	Limitation of amino acid availability	Modulation	
	Cell proliferation by carbon tetrachloride treatment[26]	Upregulation	Rat liver
Tubulin[2]	Cell density	Modulation	
	Amino acid limitation	Modulation	
HPRT[2]		Invariable expression	Alveolar macrophages, spleen cells, cytokines

[1]Reviewed in Suzuki *et al.*, 2000; [2]Reviewed in Sturzenbaum & Kille, 2001; [3]Zhong & Simons, 1999; [4]McNulty & Toscano, 1995; [5]Bhatia *et al.*, 1994; [6]Calvo *et al.*, 1997; [7]Revillion *et al.*, 2000; [8]Ercolani *et al.*, 1988; [9]Alexander *et al.*, 1985; [10]Roche *et al.*, 1997; [11]Andreeva *et al.*, 1997; [12]Hazell *et al.*, 1999; [13]Aledo *et al.*, 1999; [14]Francavilla *et al.*, 1992; [15]Elder *et al.*, 1984; [16]Garmyn *et al.*, 1991; [17]Graven & Farber, 1998; [18]Barroso *et al.*, 1999; [19]Gong *et al.*, 1996; [20]de Leeuw *et al.*, 1989; [21]Ito *et al.*, 1996; [22]Selvey *et al.*, 2001; [23]Shimokawa *et al.*, 1997; [24]Yan *et al.*, 1999; [25]Yamada *et al.*,1997; [26]Goldsworthy *et al.*,1993; [27]Nakai *et al.*, 1991.

Thus far the scientific community has not reached an agreement upon the use of one particular gene as the ultimate internal control for expression analyses. The investigator must choose the most appropriate gene to use in the light of the intended experimental conditions and/or disease state in question.

As illustrated in table 5a, the use of *gapd* as a standard for comparison in quantitative RT-PCR should be interpreted with caution. The expression of *gapd* however, is not effected by stimulation with anti-IgE or incubation with cyclosporin A or dexamethasone (Williams & Coleman, 1995), indicating that expression studies involving immunosuppression can employ *gapd* as an internal control without a problem. Furthermore, as is the case for several of the commonly used housekeeping genes, recent literature has described the characterisation of numerous pseudogenes for *gapd* in the human genome sequence (Benham *et al.*,1984; Arcari *et al.*, 1989; Benham *et al.*,1989; Garbay *et al.*,1996). Of particular note are the processed pseudogene sequences formed by the incorporation of processed gene transcripts into genomic DNA. Due to the high level of sequence homology of *gapd* pseudogenes with *gapd* transcript, the majority of primer pairs designed to bind to *gapd* cDNA in semi-quantitative and quantitative RT-PCR assays (Chapter6) will bind to both genuine and pseudogene sequences. As such, *gapd* pseudogene targets generate amplicons of approximately the same size as that obtained from *gapd* cDNA. This leads to the interference of pseudogene sequence in subsequent densitometry of the RT-PCR product, making normalisation against the message of *gapd* potentially erroneous. Treatment of RNA samples with DNase may exclude the possibility of amplification of genomic DNA pseudogene sequence, however, the absence of genomic DNA from RNA preparations cannot be guaranteed. Also, the extraction of RNA from small amounts or difficult starting material, for example paraffin-embedded tissue will not permit the use of DNase treatment without significant loss of yield. The phenomenon of

pseudogene interference has been reported previously for *actb* when used as comparator gene (Raff *et al.*, 1997), however there are limited data on the significance of *gapd* pseudogenes.

To asses the expression of *arsa* in normal subjects as compared with those with diabetes mellitus (Chapter 6), the use of *gapd* as an internal control gene was proposed. However, during expression analysis it became apparent that the primers designed for the amplification of *gapd* were in fact amplifying *gapd* pseudogene sequence (section 6.3.3.). To avoid the potential amplification from genomic DNA in the form of *gapd* pseudogene sequence, a primer pair capable of supporting amplification from *gapd* cDNA and not from any of the currently described pseudogenes is required. Here a RT-PCR assay and primer set for the pseudogene free amplification of *gapd* as a semi-quantitative reference is described.

5.2 Materials and Methods

5.2.1 Design of *gapd* Primers

The genomic DNA sequence (including both intronic and exonic regions) of human *gapd* (Accession No. J04038) was obtained from the 'National Centre for Biotechnology Information' (NCBI) (http://www.ncbi.nlm.nih.gov). From this, a number of primer pairs were designed to amplify regions of the gene which spanned at least one intron. The BLASTN tool provided by 'The Sanger Centre' (http://www.sanger.ac.uk) was used to search for binding of each primer to any sequence within the human genome. Sequences to which both primers of a pair bound were identified using NCBI and 'European Bioinformatics Institute' (EBI, http://www.ebi.ac.uk).

The homology of *gapd* to the sequences of each of the *gapd* pseudogenes was assessed using the CLUSTALW sequence alignment tool provided by 'Biology Workbench' (http://workbench.sdsc.edu). Primers were designed such that they did not bind to the *gapd* pseudogene sequences available on either the NCBI or EBI databases ('*gapd* Primers'). An additional primer pair was designed such that they bound to both *gapd* and *gapd* pseudogene sequences ('pseudogene binding primers').

Each set of primers was manufactured by Alta Biosciences (University of Birmingham, UK), reconstituted in DEPC treated water to a stock concentration of 100mM and stored at -20°C until required. Working solutions were made using a 1:10 dilution of the stock.

5.2.2 Samples

5.2.2.1 Genomic DNA

Genomic DNA was extracted from human whole blood using the Wizard genomic DNA purification kit (Promega UK Ltd) and stored in 1 x Tris-EDTA Buffer (TE) at -20°C until PCR.

5.2.2.2 RNA

Total RNA was extracted from fresh blood samples taken into 4mL EDTA vacuette tubes (Greiner Labortechnik, UK). Briefly, 13mL of Red Cell Lysis Buffer (RCLB) (144mM NH_4Cl, 1mM $NaHCO_3$ in DEPC treated water) was added to 1mL blood, mixed by inversion and incubated at 4°C for 15mins. Samples were then centrifuged at 2,300rpm for 10mins in a benchtop microfuge, the supernatant removed and a further 13mL RCLB added. Samples were mixed thoroughly and centrifuged again at 2,300rpm for 10mins. The supernatant was discarded and any remaining red cells removed. The pellet was resuspended in Buffer RLT (containing β-mercaptoethanol) as supplied with the RNeasy RNA extraction kit, (QIAGEN Ltd, UK) and RNA extracted as per the manufacturers instructions for animal cells. Ribonucleic acid was eluted into 30µL RNase-free water and stored at -20°C until use. One sample was treated with DNase as described in the Ambion protocol, and a second sample was stored immediately at -20°C until Reverse Transcription and Polymerase Chain Reaction (RT-PCR).

5.2.3 Reverse Transcription

Reverse transcription was performed using ABgene 'Reverse-IT' 1st strand synthesis kit protocol (ABgene, Surrey, UK). A 4μL aliquot of each total RNA sample was added to 5.6μL nuclease-free H_2O and random decamer primers (6μM final concentration, Ambion, UK) and any secondary RNA structure denatured by heating to 70°C for 5 mins using a PTC-100 thermal cycler (MJ Research, Watertown, USA). Samples were immediately placed on ice. First strand synthesis buffer (5x, Promega, UK) was added along with 2μL dNTP mix (0.5mM final concentration, ABgene, UK), and M-MLV reverse transcriptase (200units/reaction, Promega UK). Reverse transcription occurred at 42°C for one hour and the reaction stopped by heating to 75°C for five minutes. The use of random primers in this reaction resulted in the formation of total cDNA which was stored at -20°C until use as template for PCR.

5.2.4 *gapd* Polymerase Chain Reaction

A master mix containing the following components was prepared: 1 x PCR buffer containing 1.5mM $MgCl_2$ (final concentration), *Taq* DNA polymerase (0.625units, Roche Molecular Biochemicals, UK), dNTP mix (0.2mM each final concentration, Promega UK Ltd), forward and reverse primers (0.4μM final concentration) as described, 0.5μL of either human genomic DNA or total cDNA reverse transcription mix as template, made up to a final reaction volume of 25μL per reaction with double distilled filter-sterilised water. Temperature cycling was performed in a PTC-100 Thermal cycler (MJ Research, Watertown, MA, USA) as follows: For *gapd* primers: 95°C for 15mins, then 35 cycles of 94°C for 60s, 70°C for 30s, 72°C for 60s, followed by a final elongation step at 72°C for 10mins. For pseudogene binding primers:

95°C for 15mins, then 35 cycles of 94°C for 60s, 61°C for 30s, 72°C for 60s, followed by a final elongation step at 72°C for 10mins. All amplification products were size separated by electrophoresis through 2% agarose (Seakem LE agarose) in 0.5X Tris-acetate running buffer (TAE) and visualised by staining with ethidium bromide (1μg/mL final concentration, Sigma,UK) and viewed under UV illumination. Amplicon size was determined by comparison to a 100bp low ladder (Sigma , UK), and the gel image captured using a Grab-IT annotating grabber version 2.59 for windows 3.1 (Ultra-Violet Products Ltd, UK).

5.2.5 Amplicon Sequence Analysis

Bands were extracted from a 2% Seaplaque, Low melting temperature agarose gel (Biowhittaker molecular applications, Maine, USA) using the Agarose Gel DNA Extraction kit (Roche, UK). Samples were quantified by comparison with a range of concentrations of standard λ phage DNA with ethidium bromide (2μg/mL) staining under UV illumination. Recovered DNA samples were sequenced by a fluorescent dye terminator reaction with the *gapd* forward primer as sequencing primer (Lark Technologies, UK).

5.3 Results

Following extensive sequence alignment of *gapd* and the available pseudogene sequences a primer pair was designed which only supported amplification from functional *gapd* transcripts. In designing primers the criteria discussed in section 1.5.1.1.2.4 were considered. The sequences of each *gapd* primer are:

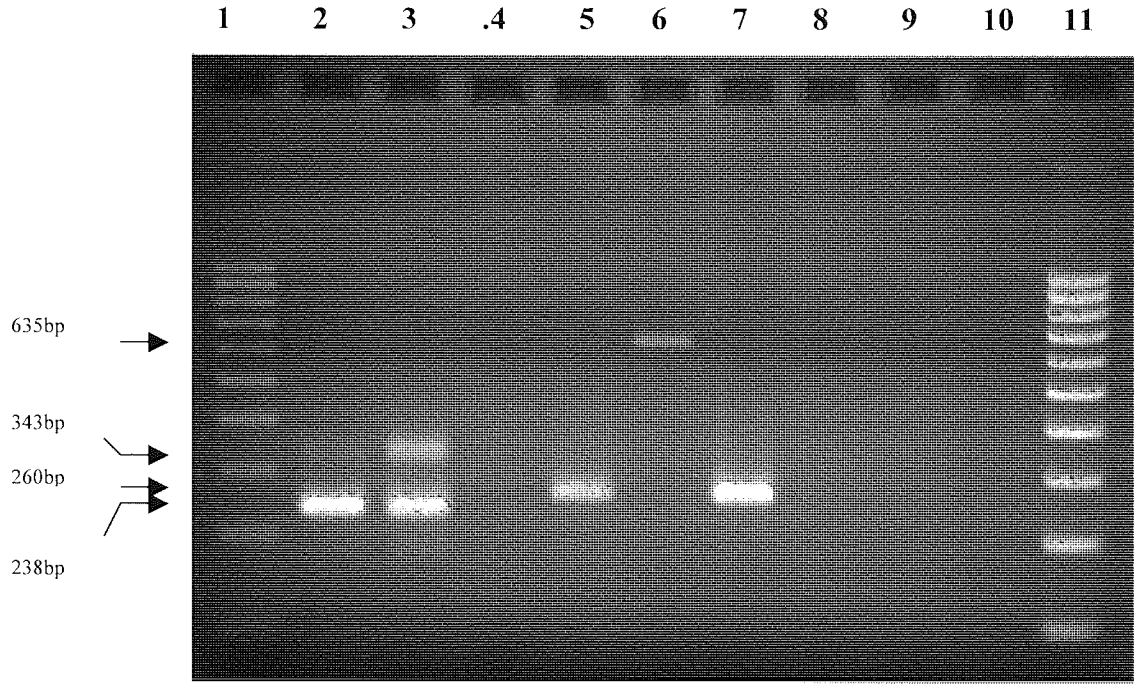
Forward Primer	5' GTCGTGGAGTCCACTG 3'
Reverse Primer	5' GTGGCAGTGATGGCATGG 3'

An alternative set of primers ('Pseudogene binding primers') which will bind to both *gapd* pseudogene sequences and that of *gapd* were used to illustrate the formation of PCR product from contaminating genomic DNA. The sequences of the pseudogene binding primers are:

Forward Primer	5' ACATCAAGAAGGTGGTGAAGC 3'
Reverse Primer (R2)	5' TTACTCCTTGGAGGCCATGTG 3'

Figure 5a illustrates the formation of PCR products using both sets of primers. Figure 5b. illustrates the positions of binding of each of these *gapd* primer pairs to *gapd*.

Fig. 5a. PCR Products Obtained Using Pseudogene-Binding Primers and Non Pseudogene-binding Primers



Lanes 2 – 4 used pseudogene-binding primers for PCR as described. Lanes 5 – 10 used non-pseudogene-binding primers for PCR. Templates: 1 & 11 = 100 base pair DNA ladder (Sigma, UK); 2 = Total cDNA with DNase digestion prior to RT-PCR; 3 = genomic DNA; 4 = none (negative control); 5 = Total cDNA with DNase digestion prior to RT-PCR; 6 = genomic DNA; 7 = Total cDNA without DNase digestion prior to RT-PCR, 8 = No RNA negative for RT; 9 = No reverse-trancriptase negative for RT; 10 = none (negative control). This figure illustrates PCR product formation from both primer pairs. Using pseudogene-binding primers with genomic DNA as template (Lane 3), two amplicons were formed. The larger of the two products (343bp) was due to the amplification of *gapd*, whereas the smaller product (which is approximately the same size as that produced with cDNA as template (Lane 2 – 238bp)) was formed from the amplification of *gapd* pseudogene sequence. This was confirmed by sequencing. Using the *gapd* primers with genomic DNA as template, a single product was formed from the amplification of *gapd* (Lane 6, 635 bp) without amplification of pseudogene sequences. Again these results were confirmed by sequencing. Contamination of RNA samples with genomic DNA is a common occurrence. This is illustrated by the results of RT-PCR using cDNA which is derived from RNA that had not been DNase digested (Lane 7 – 260 and 635bp products formed from cDNA and contaminating genomic DNA respectively). Digestion of RNA with DNase prior to reverse transcription can prevent such contamination (Lanes 2 and 5) but may not always be appropriate and as illustrated is not always 100% effective.

Fig. 5b. Sequence Alignment of *gapd* Gene, Coding Sequence and Pseudogene Sequences. Illustration of Position of Binding of Primer Pairs

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dJ585I14b.1     ------------------------------------------------------------
bA378G13.2      ----------------------------------------GCTCTCTGCTCCTCCC 16
BWB29074        ------------------------------------------------------------
HUMGAPDHG       CTTTACTCCTGCCCTTTGAGTTTGATGATGCTGAGTGTACAAGCGTTTTCTCCCTAAAGG 2760
dJ871G17.5      ------------------------------------------------------------
dJ357D13.1      ------------------------------------------------------------
dJ871G17.3      ------------------------------------------------------------

dJ585I14b.1     ------------------------------------------------------------
bA378G13.2      ATTCAACAGACAGCTGCATCTTCTCGTGCATTGCCAGCTGCATCCCTGAGACACCATGGT 76
BWB29074        ------------------------------------------------------------
HUMGAPDHG       GTGCAGCTGAGCTAGGCAGCAGCAAGCATTCCTGGGGTGGCATAGTGGGGTGGTGAATAC 2820
dJ871G17.5      ------------------------------------------------------------
dJ357D13.1      ------------------------------------------------------------
dJ871G17.3      ------------------------------------------------------------

dJ585I14b.1     ------------------------------------------------------------
bA378G13.2      GAAGGTGAAGGCTGGAGTCAACAGATTTGGTTGTATTGGCTGCCTGGTCACCAGGGCTGC 136
BWB29074        ------------------------------------------------------------
HUMGAPDHG       CATGTACAAAGCTTGTGCCCAGACTGTGGGTGGCAGTGCCCACATGGCCGCTTCTCCTGG 2880
dJ871G17.5      ------------------------------------------------------------
dJ357D13.1      ------------------------------------------------------------
dJ871G17.3      ------------------------------------------------------------

dJ585I14b.1     ------------------------------------------------------------
bA378G13.2      TTTAAACTCTGGTTAAGTCGATATTGTCGCCATCAATGACCCCTTCATTGACCTCAACAA 196
BWB29074        ------------------------------------------------------------
HUMGAPDHG       AA-GGGCTTCGTATGACTGGGGGTGTTGGGCAGCCCTGGAGCCTTCAGTTGCAGCCATGC 2940
dJ871G17.5      ------------------------------------------------------------
dJ357D13.1      ------------------------------------------------------------
dJ871G17.3      ------------------------------------------------------------

dJ585I14b.1     ------------------------------------------------------------
bA378G13.2      CACTGTCTACATGTTCC----AGTATAATTCTGCCCATGGCAAATTCCA----TGGCACC 248
BWB29074        ------------------------------------------------------------
HUMGAPDHG       CTTAAGCCAGGCCAGCCTGGCAGGGAAGCTCAAGGGAGATAAAATTCAACCTCTTGGGCC 3000
dJ871G17.5      ------------------------------------------------------------
dJ357D13.1      ------------------------------------------------------------
dJ871G17.3      ------------------------------------------------------------

dJ585I14b.1     ------------------------------------------------------------
bA378G13.2      GTCAAGGCTGAGAACAACAGCTGCATCTTCTCGTGCATTGCCA--GCTG-----CATCCC 301
BWB29074        ------GGCACGAGGGTCAGCCGCATCTTCTTTTGCGTCGCCA-GCCGAGCCACATCGC  52
HUMGAPDHG       CTCCTGGGGGTAAGGAGATGCTGCATTCGCCCTCTTAATGGGGAGGTGGCCTAGGGCTGC 3060
dJ871G17.5      ------------------------------------------------------------
dJ357D13.1      ------------------------------------------------------------
dJ871G17.3      ------------------------------------------------------------

dJ585I14b.1     ----------------AG------------GTGAAGGCCAAAGTAAA------AAGATTT 26
bA378G13.2      TGAGACACCATGGTGAAG------------GTGAAGGCTGGAGTCAA------CAGATTT 343
BWB29074        TGAGACACCATGGGGAAG------------GTGAAGGTCGGAGTCAA------CGGATTT 94
HUMGAPDHG       TCACATATTCTGGAGGAGCCTCCCCTCCTCATGCCTTCTTGCCTCTTGTCTCTTAGATTT 3120
dJ871G17.5      ------------------------------------------------------------
dJ357D13.1      ------------------------------------------------------------
dJ871G17.3      ------------------------------------------------------------

dJ585I14b.1     GGCAGTATCGGGCGC-TGGTCACCAGGGCTGCTTTTAACTCTGGTAAAGCAGATGTTCTC 85
bA378G13.2      GGTTGTATTGGCTGCCTGGTCACCAGGGCTGCTTTAAACTCTGGTTAAGTCGATATTGTC 403
BWB29074        GGTCGTATTGGGCGCCTGGTCACCAGGGCTGCTTTTAACTCTGGTAAAGTGGATATTGTT 154
HUMGAPDHG       GGTCGTATTGGGCGCCTGGTCACCAGGGCTGCTTTTAACTCTGGTAAAGTGGATATTGTT 3180
dJ871G17.5      ------------------------------------------------------------
dJ357D13.1      ------------------------------------------------------------
dJ871G17.3      ------------------------------------------------------------
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dJ585I14b.1     ACCATCAGTGACCCCTTCCTTGACCTCAACTACATAGT---------------------- 123
bA378G13.2      GCCATCAATGACCCCTTCATTGACCTCAACAACACTGT---------------------- 441
BWB29074        GCCATCAATGACCCCTTCATTGACCTCAACTACATGGT---------------------- 192
HUMGAPDHG       GCCATCAATGACCCCTTCATTGACCTCAACTACATGGTGAGTGCTACATGGTGAGCCCCA 3240
dJ871G17.5      ------------------------------------------------------------
dJ357D13.1      ------------------------------------------------------------
dJ871G17.3      ------------------------------------------------------------

dJ585I14b.1     ------------------------------------------------------------
bA378G13.2      ------------------------------------------------------------
BWB29074        ------------------------------------------------------------
HUMGAPDHG       AAGCTGGTGTGGGAGGAGCCACCTGGCTGATGGGCAGCCCCTTCATACCCTCACGTATTC 3300
dJ871G17.5      ----------------------------------------------TTACTCCTTGGAGGC 15
dJ357D13.1      ----------------------------------------------TTACTCCTTGGAGGC 15
dJ871G17.3      ------------------------------------------------------------

dJ585I14b.1     --------CTACATGTTCCCATATGATTCCACCCATGGCAAATCCCATGGCACCATCAAG 175
bA378G13.2      --------CTACATGTTCCAGTATAATTCCGCCCATGGCAAATTCCACGGCACCGTCAAG 493
BWB29074        --------TTACATGTTCCAATATGATTCCACCCATGGCAAATTCCATGGCACCGTCAAG 244
HUMGAPDHG       CCCCAGGTTTACATGTTCCAATATGATTCCACCCATGGCAAATTCCATGGCACCGTCAAG 3360
dJ871G17.5      CGTGTGGGCCATGAGGTTCACCACTTTGTTGCTATAACCAAATTCATTGTCATACCAGAA 75
dJ357D13.1      CATGTGGGCCATGAGGTCCACCACCCTGTTGTTGTAGCCAAATTCATTGTCATACCAGGA 75
dJ871G17.3      ------------------------------------------------------------

dJ585I14b.1     GCTGAGAATGGGAAGCTTGTCATCAATGGAAATCCCATCAGCATCTTTCAGGAG------ 229
bA378G13.2      GCTGAGAACGGGAAGCTTGTTATCAATGGAAATCTCATCACTATTTTCCAGGGG------ 547
BWB29074        GCTGAGAACGGGAAGCTTGTCATCAATGGAAATCCCATCACCATCTTCCAGGAG------ 298
HUMGAPDHG       GCTGAGAACGGGAAGCTTGTCATCAATGGAAATCCCATCACCATCTTCCAGGAGTGAGTG 3420
dJ871G17.5      AATGAGCTTGAGAAAGTGGTCACTGAGGGCAATGACAGCCCCAGGATCAAAGGTGGAAGA 135
dJ357D13.1      AATGAGCTTGACAAAGTGGTCGTTGAGGGCAGTGCCAGCCCCAGCATTGAAGGTGGAAGA 135
dJ871G17.3      ------------------------------------------------------------

dJ585I14b.1     ------------------------------------------------------------
bA378G13.2      ------------------------------------------------------------
BWB29074        ------------------------------------------------------------
HUMGAPDHG       GAAGACAGAATGGAAGAAATGTGCTTTGGGGAGGCAACTAGGATGGTGTGGCTCCCTTGG 3480
dJ871G17.5      GTGGGTGTCAATGGTTAAGCTGAAGTAGACAACCTAGTGCTCAGTGTAGCACAGGATGCC 195
dJ357D13.1      GTGGGTGTCACTGTTGAAGTCGGAGGAGACCACCTGGTGCCCAGTGTAGCCCAGGATGCC 195
dJ871G17.3      ------------------------------------------------------------

dJ585I14b.1     ------------------------------------------------------------
bA378G13.2      ------------------------------------------------------------
BWB29074        ------------------------------------------------------------
HUMGAPDHG       GTATATGGTAACCTTGTGTCCCTCAATATGGTCCTGTCCCCATCTCC---CCCCCACCCC 3537
dJ871G17.5      CTTGAGGGGATCCTCTGATGTCTGCTTCACCACCTTCTTGATGTTAT---ATTTGGCAGG 252
dJ357D13.1      TTTGAGGGGGCCCTCTGATGCCTGCTTCACCACCTTCTTGATGTCATCATATTTGGCAGG 255
dJ871G17.3      ------------------------------------------------------------

dJ585I14b.1     ------TGAGATCCCACCAAAATCAAATGGGGCCATGCTGGTGCTGAGTACATTATGGAG 283
bA378G13.2      ------CAAGATCTCACCAAAATCAAATGGGGCAATGCTGGCACTGAGTACATCATGGAG 601
BWB29074        ------CGAGATCCCTCCAAAATCAAGTGGGGCGATGCTGGCGCTGAGTACGTCGTGGAG 352
HUMGAPDHG       GGTAGGCGAGATCCCTCCAAAATCAAGTGGGGCGATGCTGGCGCTGAGTACGTCGTGGAG 3597
dJ871G17.5      TTTTTCCAGATGGCAGGTTAGGTCCATGACCAACATATTGGTGGTGGGTACATGGAAGGC 312
dJ357D13.1      TTTTTCCAGATGGTAGGTGAGGTCCACCACTGACACGTTGGCAGTGGGGACACGGAGGGC 315
dJ871G17.3      ----------------------------------------TTATTCCATTGGGGAT 16
                                                        *   *   *

dJ585I14b.1     CCCACCAGTATCTTCACGACCATGGAGAAGGCTGGAG---------------------- 320
bA378G13.2      TTCACCAGCATCTTCACCACCATGGAGAAGGCTGGGG---------------------- 638
BWB29074        TCCACTGGCGTCTTCACCACCATGGAGAAGGCTGGGG---------------------- 389
HUMGAPDHG       TCCACTGGCGTCTTCACCACCATGGAGAAGGCTGGGGTGAGTGCAGGAGGGCCCGCGGGA 3657
dJ871G17.5      CATGCCAGTGAGCTTCCCGTTCAGCCCATGGATCTCATTG------------------- 352
dJ357D13.1      CATGCCAGTGAGCTTACCATTCAGCTCAGGGATGACCTTC------------------- 355
dJ871G17.3      --TTATAGGGTCCTTGCATTTGGGC--AAGGATGACCTCA------------------- 52
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dJ585I14b.1     ------------------------------------------------------------
bA378G13.2      ------------------------------------------------------------
BWB29074        ------------------------------------------------------------
HUMGAPDHG       GGGGAAGCTGACTCAGCCCTGCAAAGGCAGGACCCGGGTTCATAACTGTCTGCTTCTCTG 3717
dJ871G17.5      ------------------------------------------------------------
dJ357D13.1      ------------------------------------------------------------
dJ871G17.3      ------------------------------------------------------------

dJ585I14b.1     -------CTCACTTGCAGCAGGGAGCCAAAAGGGTCATCATCTCTGCTGCCTCTGCTGAG 373
bA378G13.2      -------CTCACTTGGAGGGAGGAGCCAAAACGGTCATCATCTCTGCACCCTCTGCTGAT 691
BWB29074        -------CTCATTTGCAGGGGGGAGCCAAAAGGGTCATCATCTCTGCCCCCTCTGCTGAT 442
HUMGAPDHG       CTGTAGGCTCATTTGCAGGGGGGAGCCAAAAGGGTCATCATCTCTGCCCCCTCTGCTGAT 3777
dJ871G17.5      -------CCCACAGCCTTGACGGCGCCAGTAGATGCAAAGATGA--------------G 390
dJ357D13.1      -------CTCACAGCCTTGGCAGC-CCAGTAGTGGCAGGGATGATGT----TCTG-GAG 402
dJ871G17.3      -------CCCACAGCCTTGGCAGCACCAGTAGATGCGGGGATGATAT----TCTG-GAG 99
                       * **          *  ***  *    *

dJ585I14b.1     GCCCCCATGTTTAT-ATAGGCATGAACCATGAGAAGTATGACAACAGTCTCAAGATCGTC 433
bA378G13.2      GCCCCCATGTTCGTGATGGGTGTGAACCATGAGAAATATGACAACAG-CTCAAGATTATC 751
BWB29074        GCCCCCATGTTCGTCATGGGTGTGAACCATGAGAAGTATGACAACAGCCTCAAGATCATC 502
HUMGAPDHG       GCCCCCATGTTCGTCATGGGTGTGAACCATGAGAAGTATGACAACAGCCTCAAGATCATC 3837
dJ871G17.5      ATCTCTGCGGCCATGATGCCA--GTTTCACGAAGGGGC-ATTCACAGTCTTCTGGGTAGC 447
dJ357D13.1      AGCCCTGTGGCTGTCACGCCACAGTTTCCTGGAGGGGCCATTCACAGTCTTCTGGGTGGC 462
dJ871G17.3      AGACCTGTGATCGTCATGCCACAGCTTCCCAGAGGGGCTATCCAAAGTCTTCTGGCTGGC 159
                    *   *    * *       *   *                 * ** **   *     *

dJ585I14b.1     AG---------------------------------------------------------- 435
bA378G13.2      AG---------------------------------------------------------- 753
BWB29074        AG---------------------------------------------------------- 504
HUMGAPDHG       AGGTGAGGAAGGCAGGGCCCGTGGAGAAGCGGCCAGCCTGGCACCCTATGGACACGCTCC 3897
dJ871G17.5      AGCGATAG---------------------------------------------------- 455
dJ357D13.1      AGTGATGG---------------------------------------------------- 470
dJ871G17.3      AGTGATGG---------------------------------------------------- 167
                **

dJ585I14b.1     ---------------------------------CAATGCCTCCTGTACCACCAACTGCT 461
bA378G13.2      ---------------------------------CAATGCCTCCTGCACCACCAGCTGCT 779
BWB29074        ---------------------------------CAATGCCTCCTGCACCACCAACTGCT 530
HUMGAPDHG       CCTGACTTGCGCCCCGCTCCCTCTTTCTTTGCAGCAATGCCTCCTGCACCACCAACTGCT 3957
dJ871G17.5      -------CGCTGACTGTGGTCATGGGTCATTCCACTATTCCAAAGTTGTCATGAATCATC 508
dJ357D13.1      -------CGTGGACTGTGGTCATGAGTCCTTCCACGATACCAAAGTTGTCAGTGATGACT 526
dJ871G17.3      -------TGTGCACCATGATCACGAGTCCTTCCACGATACCAAAATTGTCATGGATGACC 220
                                                * ** **        **

dJ585I14b.1     TAGCACCCCTGGCCAAAGCCATCTATGACAACTTTGGTATCATGGAAGGATTCATG---- 517
bA378G13.2      TAACGCCCCTGGCCAAGGTCATCCATGACAACTTTGGTACCGTGGAAGGACTCATG---- 835
BWB29074        TAGCACCCCTGGCCAAGGTCATCCATGACAACTTTGGTATCGTGGAAGGACTCATG---- 586
HUMGAPDHG       TAGCACCCCTGGCCAAGGTCATCCATGACAACTTTGGTATCGTGGAAGGACTCATGGTAT 4017
dJ871G17.5      TTGGCTAGAGGTGCTATACAGTTGGTGGTACAGGAGGCATTGCTGATGTTCTTGAGGCTG 568
dJ357D13.1      TTGGCCAGGGGCGCTAGGCAGTTGGTGGTTCAGGTGGCATTGCTGATGATCTTGAGGCTG 586
dJ871G17.3      TTGGACAGGGGTGCTAAACAGTTGGTGGTACAGGAGTCATTGCTGATGATCTTGAACCTG 280
                *         *  * *     *   **        *  *     ** *   *

dJ585I14b.1     ------------------------------------------------------------
bA378G13.2      ------------------------------------------------------------
BWB29074        ------------------------------------------------------------
HUMGAPDHG       GAGAGCTGGGGAATGGGACTGAGGCTCCCACCTTTCTCATCCAAGACTGGCTCCTCCCTG 4077
dJ871G17.5      TT---------------------------------------------------------- 570
dJ357D13.1      TT---------------------------------------------------------- 588
dJ871G17.3      TT---------------------------------------------------------- 282

dJ585I14b.1     ------------------------------------------------------------
bA378G13.2      ------------------------------------------------------------
BWB29074        ------------------------------------------------------------
HUMGAPDHG       CTGGGGCTGCGTGCAACCCTGGGGTTGGGGGTTCTGGGGACTGGCTTTCCCATAATTTCC 4137
dJ871G17.5      ------------------------------------------------------------
dJ357D13.1      ------------------------------------------------------------
dJ871G17.3      ------------------------------------------------------------
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dJ585I14b.1     ------------------------------------------------------------
bA378G13.2      ------------------------------------------------------------
BWB29074        ------------------------------------------------------------
HUMGAPDHG       TTTCAAGGTGGGGAGGGAGGTAGAGGGGTGATGTGGGGAGTACGCTGCAGGGCCTCACTC 4197
dJ871G17.5      ------------------------------------------------------------
dJ357D13.1      ------------------------------------------------------------
dJ871G17.3      ------------------------------------------------------------


dJ585I14b.1     ---------ACCACAGTCCACGCTATCACTGCCACCCAGAAGACTGTGGATGGCCCCTCC 568
bA378G13.2      ---------ACCA------------TCGCTGCCACCCAGAAGACTATGGATGGCTCCTAT 874
BWB29074        ---------ACCACAGTCCATGCCATCACTGCCACCCAGAAGACTGTGGATGGCCCCTCC 637
HUMGAPDHG       CTTTTGCAGACCACACTCCATGCCATCACTGCCACCCAGAAGACTGTGGATGGCCCCTCC 4257
dJ871G17.5      ---------TTCATACTTCTCATGGTTCATACCCATTATAAACATGGGGGTATCAGCAGA 621
dJ357D13.1      ---------GTCATACTTCTCATGGTTCACACCCATCGTGAACATGGGGGGATCAGCAGA 639
dJ871G17.3      ---------GTCATACTTCTCATGGCTCACATCCATCAGAAACCTGGGGACGTTAGCAGC 333
                         **                  *       *  *  **       *


dJ585I14b.1     GGGAAACTGTGGCATGAGGGCTGTGTGGCTCTCCAGAACATCATCCCTGCATCTACTGGC 618
bA378G13.2      GGGAAACTGTGGGGTGACGGCCATGGGGCTCTCCAGAACATCCTCTCTGCCTCTACTGGT 934
BWB29074        GGGAAACTGTGGCGTGATGGCCGCGGGGCTCTCCAGAACATCATCCCTGCCTCTACTGGC 697
HUMGAPDHG       GGGAAACTGTGGCGTGATGGCCGCGGGGCTCTCCAGAACATCATCCCTGCCTCTACTGGC 4317
dJ871G17.5      GGGGGCAGAAATGATGACTCTTTTAGCTCAACCTTGCAAGTGATCTCCAGCCTTTTCCAT 681
dJ357D13.1      GGGGGCAGAGATGATGACCCTTTTGGCTCCCCACTGCAAGTGAGCCCCAGCATTCTCCAT 699
dJ871G17.3      TGGGGCAGAGATGATGGCCCTTTTGGCTCCCCTCTGAAAGCAAGCCCCAACCTTCTCCAT 393
                 **          **            *      * *      * *      *


dJ585I14b.1     ACTGCCAAGGCTGTGGGCAAGGTCATCCCCGAGCTGAACAGGAAGCTCAATG-GCATGGC 677
bA378G13.2      GCTGCCAAGGCTGTGAGGAAGGTCATCCCTGAGCTAAACGGGAAGCTCACTG-GCATGGC 993
BWB29074        GCTGCCAAGGCTGTGGGCAAGGTCATCCCTGAGCTGAACGGGAAGCTCACTG-GCATGGC 756
HUMGAPDHG       GCTGCCAAGGCTGTGGGCAAGGTCATCCCTGAGCTGAACGGGAAGCTCACTG-GCATGGC 4376
dJ871G17.5      GGTAGTGAAGACACCAGTGGACTCTATGATGTACTCAGTTCCAGAATCACCCTATTTGAT 741
dJ357D13.1      GGTGGTGAAGATGCCAGTGGACTCCATGACATACTCAGTGCCAGCATCACCCCACTTGAT 759
dJ871G17.3      GGTAGTGAAGACACTGATGGACTCCACAATGTACTCAGTGCCAGTATCGCCCTATTTGAT 453
                  *    * *          **         ** *     *   **        **


dJ585I14b.1     CTTCCATGTCCCCATTGCCAATGTGTCAGTTATGGACCTGACCTACCGTCTGGAGAAACC 737
bA378G13.2      CTTCCGTGTCCCCACTGCCAACATGTCAGTGGTGGACCTGACCTGCCGTCTGGAAAAACC 1053
BWB29074        CTTCCGTGTCCCCACTGCCAACGTGTCAGTGGTGGACCTGACCTGCCGTCTAGAAAAACC 816
HUMGAPDHG       CTTCCGTGTCCCCACTGCCAACGTGTCAGTGGTGGACCTGACCTGCCGTCTAGAAAAACC 4436
dJ871G17.5      TTTGGTGGGATCTAGCTTTTGGATGATGGTGGTG-----GGATTTCCACTGATGACAAGC 796
dJ357D13.1      TTTGGAGGGATCTGGCTCCTGGAAGATAGTGATG-----GGATTTCCATTGATGACAAGC 814
dJ871G17.3      TTTGGTAGGATCTAGCT-------------------------TTCCATTAATGACAAGC 487
                 **    *   *                                * **        ** *


dJ585I14b.1     TGCCAAATATGATGATATCAAGAAGGTGGTGAAGCAGGTGTCAGAGGGCCCCCTCAAGGG 797
bA378G13.2      TACCAAATATGATGACACCAAGAAGGTGGTGAAGCAGGCGTCAGAGGACCCCCTCAAAGG 1113
BWB29074        TGCCAAATATGATGACATCAAGAAGGTGGTGAAGCAGGCGTCGGAGGGCCCCCTCAAGGG 876
HUMGAPDHG       TGCCAAATATGATGACATCAAGAAGGTGGTGAAGCAGGCGTCGGAGGGCCCCCTCAAGGG 4496
dJ871G17.5      TTCTTGTTCTCA-GCCTTGATGGTGCCATGGAATTTGTCATAGGTGGACTCATACTGGAA 855
dJ357D13.1      TTCCCGTTCTCA-GCCTTGACGGTGCCATGGAATTTACCATGGGTGGGATCATACTGGAA 873
dJ871G17.3      TTCCCATTCTCA-GCCTTGACAGTGCCATGGAATTCGCCATGGGTGGAATCATACTGGAA 546
                * *   * * * *     *   *     ***       *  * **   *   *


dJ585I14b.1     CATCCTGGAC-TACA-CTGAGCACCAGATTGTCTCCTCCAAC------AGTGATACCCAC 849
bA378G13.2      CATCCTGGGC-TACT-CTGAGCACCAGGTGGTCTCCTCCAACTTCA--ACAGACACCCAC 1169
BWB29074        CATCCTGGGC-TACA-CTGAGCACCAGGTGGTCTCCTCTGACTTCAACAGCGACACCCAC 934
HUMGAPDHG       CATCCTGGGC-TACA-CTGAGCACCAGGTGGTCTCCTCTGACTTCAACAGCGACACCCAC 4554
dJ871G17.5      CATGTAGACCATGTAGTTGAGCTCTATGAAGGGGTGATTGAT---GGTGAGAATATTCAC 912
dJ357D13.1      CATGTAGACCATGTAGCTGAGGTCAATGAATGGGTCATTGAT---GGCGATAATATCTAC 930
dJ871G17.3      CATGTAGGCCTTGTAGTTGAGCTAAATGAAGGGGTCATCGAT---GGCGATAATATCCAC 603
                *** *  * *    ****   *                 *          * *  **


dJ585I14b.1     TCTTCCATCTTCCATGCTGGGGCTGGCAATGCCCTCAACAACCACCTT-GTCAAGCTCAT 908
bA378G13.2      TCTTCCACCTTCGATGCTGGGGCTGGCATTGCCCTCAACGACCACTTT-GTCAAGCTCAT 1228
BWB29074        TCCTCCACCTTTGACGCTGGGGCTGGCATTGCCCTCAACGACCACTTT-GTCAAGCTCAT 993
HUMGAPDHG       TCCTCCACCTTTGACGCTGGGGCTGGCATTGCCCTCAACGACCACTTT-GTCAAGCTCAT 4613
dJ871G17.5      TTTATCAGAGTTATAAGTAGCCCTGGTGA----CCCAGCACCCAATAAAGCCAAATCCAT 968
dJ357D13.1      TTTACCAGAATTACAAGCAGCCCTGATGA----CCAGGCGCCCAATACAACCAAATCCAT 986
dJ871G17.3      TTTACCAGAGTTAAAAGTAGCCCTGGTGA----CCAGACACCCAATACAGCCAAATCCGT 659
                *   **   *        *  ***       *    *  ***       ***   * *
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dJ585I14b.1     TTCCTGGTGTG---------------------------------------------------- 919
bA378G13.2      TTCCTGGTATG---------------------------------------------------- 1239
BWB29074        TTCCTGGTATG---------------------------------------------------- 1004
HUMGAPDHG       TTCCTGGTATGTGGCTGGGGCCAGAGACTGGCTCTTAAAAAGTGCAGGGTCTGGCGCCCT 4673
dJ871G17.5      TGACTTAGGCCTTCATTTTCACCAT-------------------------------------- 993
dJ357D13.1      TGACTCCAACCTTCACTTTCCCCAT-------------------------------------- 1011
dJ871G17.3      TGAC---------------------------------------------------------- 663
                *  *

dJ585I14b.1     ----------------------------------------------------------ACAAC 924
bA378G13.2      ----------------------------------------------------------ACAAT 1244
BWB29074        ----------------------------------------------------------ACAAC 1009
HUMGAPDHG       CTGGTGGCTGGCTCAGAAAAAGGGCCCTGACAACTCTTTTCATCTTCTAGGTATGACAAC 4733
dJ871G17.5      ------------------------------------------------------------
dJ357D13.1      ------------------------------------------------------------
dJ871G17.3      ------------------------------------------------------------

dJ585I14b.1     GAATTTGGCTACAGCAATATGGTGGTGGACCTCATGGCCCACA---TGGCCTCCAAGGAA 981
bA378G13.2      GAATTTGGCTGCAGCAACAGGGTGGTGGACCTCT--GCCCACAGTGTGGCTTCCAAGGAG 1302
BWB29074        GAATTTGGCTACAGCAACAGGGTGGTGGACCTCATGGCCCACA---TGGCCTCCAAGGAG 1066
HUMGAPDHG       GAATTTGGCTACAGCAACAGGGTGGTGGACCTCATGGCCCACA---TGGCCTCCAAGGAG 4790
dJ871G17.5      ------------------------------------------------------------
dJ357D13.1      ------------------------------------------------------------
dJ871G17.3      ------------------------------------------------------------

dJ585I14b.1     TAA----------------------------------------------------------- 984
bA378G13.2      TAAGACCCCCAGACCACCAGCCCCAGCGACAGCACGACGGGAAGAGAGCGGCCCTCACTG 1362
BWB29074        TAAGACCCCTGGACCACCAGCCCCAGCAAGAGCACAAGAGGAAGAGAGAGACCCTCACTG 1126
HUMGAPDHG       TAAGACCCCTGGACCACCAGCCCCAGCAAGAGCACAAGAGGAAGAGAGAGACCCTCACTG 4850
dJ871G17.5      ------------------------------------------------------------
dJ357D13.1      ------------------------------------------------------------
dJ871G17.3      ------------------------------------------------------------

dJ585I14b.1     ------------------------------------------------------------
bA378G13.2      CTGGAGAGTCCCTGCCACACTCAGTCTCCCACCACACTGAGAATCTCCCCTCCTCATAGT 1422
BWB29074        CTGGGGAGTCCCTGCCACACTCAGTCCCCCACCACACTG--AATCTCCCCTCCTCACAGT 1184
HUMGAPDHG       CTGGGGAGTCCCTGCCACACTCAGTCCCCCACCACACTG--AATCTCCCCTCCTCACAGT 4908
dJ871G17.5      ------------------------------------------------------------
dJ357D13.1      ------------------------------------------------------------
dJ871G17.3      ------------------------------------------------------------

dJ585I14b.1     ------------------------------------------------------------
bA378G13.2      TTCCATGCAGACCCCCTAAAA--GGGAGGAGCCGAGGGAGCCCCACCTTTTCATGTACCA 1480
BWB29074        TGCCATGTAGACCCCTTGAAGAGGGGAGGGGCCTAGGGAGCCGCACCTTGTCATGTACCA 1244
HUMGAPDHG       TGCCATGTAGACCCCTTGAAGAGGGGAGGGGCCTAGGGAGCCGCACCTTGTCATGTACCA 4968
dJ871G17.5      ------------------------------------------------------------
dJ357D13.1      ------------------------------------------------------------
dJ871G17.3      ------------------------------------------------------------

dJ585I14b.1     ------------------------------------------------
bA378G13.2      TCAATAAAATCCCCTGTGCTTGCCAAAAAAAAAAAAA---------- 1517
BWB29074        TCAATAAAGTACCCTGTGCTCAACCAAAAAAAAAAAAAAAAAAAAAAAAAAA 1304
HUMGAPDHG       TCA------------------------------------------------ 4971
dJ871G17.5      ------------------------------------------------
dJ357D13.1      ------------------------------------------------
dJ871G17.3      ------------------------------------------------
```

nnnnn = Forward gapd primer; nnnnn = Reverse gapd primer; nnnnn = Forward gapd 'pseudoegene-binding'; nnnnn = Reverse gapd 'pseudogene-binding' primer; dJ585I14b.1, bA378G13.2, dJ871G17.5, dJ357D13.1, dJ871G17.3 = gapd pseudogene sequences; BWB29074 = gapd cDNA sequence; HUMGAPDHG = gapd genomic DNA sequence; * nnnnn = 100% homology between all 7 sequences.

5.4 Discussion

Reverse-transcription polymerase chain reaction is a widely used technique for the analysis of gene expression as it enables increased sensitivity when the source of message is limited. This is often the case in analysis of gene expression in archive paraffin embedded tissue samples, from which extraction of RNA is notoriously difficult. Use of a comparator gene is essential for both quantitative and semi-quantitative analyses as it provides a means of accounting for variability in experimental applications such as sample handling, pipetting and gel loading. Housekeeping genes were originally considered to be expressed constitutively in all cells and it was initially thought they provide the qualities necessary for the comparison of signal intensity between samples.

The gene for glyceraldehyde-3-phosphate dehydrogenase is the most widely used internal control gene in expression studies. It has been reported however, that the expression of *gapd* is altered under particular experimental conditions and in certain disease states (table 5a, page 134). The characterisation of the pseudogenes of *gapd* is an additional factor which brings into question the use of *gapd* as a comparator gene for RT-PCR. The gene for glyceraldehyde-3-phosphate dehydrogenase is not the only internal control gene for which pseudogene sequences exist (Raff *et al.*, 1997). The existence of pseudogene sequences is often overlooked in gene expression analyses leading to erroneous results due to the incorporation of the signal generated from contaminating genomic DNA. Primers which have been designed without consideration of this phenomenon, will not permit normalisation against a signal obtained from both the message and contaminating genomic DNA. This will result in the incorrect ratios of target:control signal, and may cause a target gene which is up-regulated, to appear to be down-regulated or to remain unchanged. Interference by contaminating genomic DNA may be eradicated by DNase treatment of the original RNA.

However, the yield of RNA from some sources can be too low to permit DNase digestion without complete loss of signal. Paraffin-embedded tissue can be used as a valuable source of genetic material in retrospective gene expression analyses. Often the amount of RNA obtained from such material may be too low to allow DNase digestion prior to reverse transcription. Furthermore, even with DNase digestion, the complete eradication of contaminating genomic DNA from RNA samples cannot be guaranteed. DNase digestion typically removes 99% of contaminating DNA and the remaining 1% is sufficient to be detected by PCR amplification.

Expression analyses which employ internal control genes for which no pseudogene sequences exist may still be affected by the incorporation of genomic DNA during the PCR stage of RT-PCR analysis. If primers are designed such that they amplify a product which does not span an intronic region of the gene, then genomic DNA may be amplified without detection. This results in interference with expression analyses as described above. However, primers which span an intron will allow the detection of contaminating genomic DNA at the PCR stage. This could eliminate the possibility of genomic DNA interference in expression analyses, but the effect of genomic DNA contamination on the stoichiometry of the PCR in RT-PCR must be investigated.

Numerous genes can be used as controls in expression studies, namely the genes which code for cytoplasmic β-Actin, 18S rRNA, and to a lesser extent, cyclophilin. However, one important incentive for the use of *gapd* in preference to other internal control genes is that the overall consensus sequence of *gapd* is sufficiently conserved to allow the isolation of genes from unconventional or rare species with relative ease.

Despite the numerous disadvantages of the use of *gapd* as an internal control gene, employing primers which will not bind to the pseudogenes of *gapd* further validates its use in expression studies.

5.5 Conclusion

A set of primers which will amplify *gapd* RNA only have been designed. They do not bind to the pseudogene sequences of *gapd* and so eliminate the possibility of amplification of any contaminating genomic DNA, which may not be eradicated entirely by treatment with DNase. This prevents interference of genomic DNA both within the cDNA PCR product, and through sequestering of reagents resulting in an unknown effect on the stoichiometry of PCR.

The use of primers which will not bind to the pseudogene sequences of housekeeping genes is imperative in ensuring that any signal obtained in expression studies is genuine, and is not derived through the amplification of contaminating genomic DNA. The primers designed here are candidates for the employment of *gapd* as an internal control for the analysis of *arsa* expression (Chapter 6).

CHAPTER 6

6. EXPRESSION OF ARYLSULPHATASE A

6.1 Introduction

Urinary arylsulphatase A (ASA) activity is increased in subjects with diabetes mellitus when compared to a normal control group (Chapter 3). This increased activity is not due to the existence of an isoform of ASA which has a higher affinity for dopamine sulphate and this lack of heterogeneity has been illustrated by kinetics analysis of arylsulphatase A (Chapter 3) together with stuctural analysis of the gene for ASA (*arsa*). Furthermore, an increased enzyme activity is unlikely to be due to structural alterations in the coding region of its gene and these findings have been confirmed in studies which demonstrate no difference in the sequence of *arsa* in normal subjects as compared with a diabetic population (Chapter 4). Taking these observations into consideration it is apparent that the expression of *arsa* may be altered in patients with diabetes mellitus. The increased activity of ASA in patients with diabetes mellitus was not observed in the serum or white blood cells (previous in-house investigations, results not shown), indicating that *arsa* expression may be elevated in the kidney of such patients to compensate for the diminished dopamine formation from L-dopa, sequestering of dopamine by autoxidation, and salt-retention associated with diabetes mellitus.

To assess the renal expression of human *arsa*, use of archival paraffin-embedded (P-E) renal samples was proposed. Archive samples provide a valuable source of easily accessible tissue for retrospective analyses and indeed RNA has been successfully extracted from archive samples up to 80yrs old (Krafft *et al.*, 1997). Such samples are invaluable particularly when morphologically intact cell populations are required. These can be precisely removed and

analysed using laser capture microdissection techniques (Goldsworthy *et al.*, 1999; Specht *et al.*, 2001). One disadvantage when analysing nucleic acids derived from P-E tissue is the degradation which may occur during tissue handling and fixation prior to paraffin embedding. This necessitates analysis of relatively small nucleic acid fragments (Coombs *et al.*, 1999), often below 300 base pairs in length. The extraction of nucleic acids from P-E tissue samples has been demonstrated previously; the influenza virus of the 1918 pandemic has been retrospectively characterised from fixed lung tissue (Taubenberger *et al.*, 2000). Furthermore, DNA fragments >600bp have been successfully extracted from paraffin-embedded bone marrow trephine biopsies for the diagnosis of bone marrow disease including non-Hodgkin's lymphoma (Wickham *et al.*, 2000). Extraction of DNA from P-E tissue is less problematic than RNA extraction, due to the comparative instability of DNases. Conversely, the ubiquity and stability of RNases means RNA extraction is inherently complicated by degradation of the nucleic acid. The extraction of RNA from P-E tissue is notoriously difficult due to the extensive manipulation that the tissue undergoes both post-extraction, and during fixation. The exposure of archive tissue RNA to RNAses together with the storage time of such samples (months – years) prior to analysis, results in a relatively low yield of RNA from paraffin-embedded tissue compared to that obtained from blood or fresh/frozen tissue samples (Goldsworthy *et al.*, 1999). Previous literature has demonstrated the successful extraction of RNA from P-E tissue (Chomczynski & Sacchi, 1987; Rupp and Locker, 1988; Stanta & Schneider, 1991; Weisacker *et al.*, 1991; Koopmans *et al.*, 1993; Foss *et al.*, 1994; Martin *et al.*, 1994; Sorg & Metzler, 1995; Turbett *et al.*, 1995; Tyrrell *et al.*, 1995; O'Driscoll *et al.*, 1996; Krafft *et al.*, 1997; Svoboda-Newman *et al.*, 1997; Coombs *et al.*, 1999; Specht *et al.*, 2000; Specht *et al.*, 2001). In many cases the tissue was fixed with consideration for the subsequent analysis of RNA however, in this study tissue was fixed and paraffin-embedded without implementation of any methods to inhibit RNA degradation. An alternative source of renal material is biopsy tissue obtained for diagnostic purposes (Miyazaki *et al.*,1994; Del

Prete, 1998), however tissue from renal biopsies may be unsuitable since it is almost inevitable that these patients will have renal disease. Furthermore, the amount of tissue taken at biopsy is small and may not provide sufficient yield of nucleic acid for expression analysis.

6.1.1 Multiplex PCR for Analysis of Gene Expression

Gene expression analyses can be carried out using numerous molecular biological techniques including northern blotting, *in-situ* hybridisation, and reverse transcription polymerase chain reaction (RT-PCR). Due to the increased sensitivity of RT-PCR, this technique is favoured in circumstances where the message is in low abundance and the signal requires amplification. Furthermore, where P-E tissue is used as the source of nucleic acid, the RNA may be too degraded for downstream applications requiring long sequences, such as northern blotting (Krafft *et al.*, 1997). Reverse transcriptase polymerase chain reaction can be adapted for the purpose of gene expression analysis in several ways.

6.1.1.1 Standard RT-PCR

Reverse transcription polymerase chain reaction allows the detection of the presence or absence of gene expression in the cell or tissue under investigation, however this technique provides no information regarding the relative level of gene expression. To allow some comparative indication of the expression level modifications of the standard RT-PCR method have been described.

6.1.1.2 Semi-quantitative RT-PCR

In this application amplification of the gene under investigation remains in the exponential phase. Relative expression levels of one gene are compared in identical tissues / cells subjected to different external stimuli. This technique does not produce quantitative results, and may require southern blotting to detect very small differences in expression between samples. Any discrepancy between signal intensities due to sample handling, pipetting and gel loading is not accounted for using RT-PCR of the target gene alone.

6.1.1.3 Quantitative RT-PCR

Quantitative RT-PCR involves amplification using primer pairs which will bind the cDNA transcript of the target gene and a standard RNA (Loitsch *et al.*, 1999). The standard RNA is usually synthetic and is designed to contain a mutation (either an insertion or deletion) such that PCR exhibits bands of different lengths corresponding to the standard RNA and target RNA transcripts. Alternatively the standard may contain restriction sites which are not present in the target gene sequence. During PCR, the standard RNA-derived cDNA will compete with the target mRNA-derived cDNA for primers and enzyme, hence this technique is often referred to as competitive RT-PCR. The concentration of the target mRNA is equivalent to that of the standard mRNA where there is a 1:1 ratio of each product.

It is critical to determine the concentration of the standard RNA accurately prior to RT-PCR to enable the calculation of the concentration of target RNA. This is an accurate way of measuring the concentration and therefore the expression level of a target gene, however quantification of the standard RNA can be problematic due to the relative instability of RNA.

Furthermore, if a large sample number require analysis, quantitative RT-PCR can be time-consuming and expensive (McPherson & Moller, 2000).

6.1.1.4 Multiplex RT-PCR

In this method comparisons of the expression of a target gene from identical cells or tissues are required and the use of a control gene may be employed to account for experimental error derived from sample handling (Chapter 5). Multiplex RT-PCR defines standard or semi-quantitative RT-PCR using more than one primer set at the PCR stage (O'Driscoll *et al.*, 1996; Spencer & Christensen, 1999). One of the applications of multiplex RT-PCR is the amplification of the target gene and a 'control' housekeeping gene concurrently. The ratio of signal intensity of the target gene to that of the control gene is calculated whilst the PCR remains in the exponential phase, and a comparison of this ratio made to detect any changes in target gene expression between samples.

Each primer set must be designed carefully to ensure no primer-primer interaction occurs as this may interfere with the stoichiometry of the PCR reaction resulting in decreased yield or even total loss of product formation. Furthermore, the optimal PCR conditions must be appropriate for both primer sets. The choice of control gene for such analysis is therefore critical (Chapter 5).

6.1.2 β-Actin gene (*actb*) Primers versus Glyceraldehyde-3-phosphate Dehydrogenase Gene (*gapd*) Primers as Controls in Expression Analyses

To normalise the expression of *arsa* by multiplex RT-PCR, an appropriate control gene must be chosen. The choice of housekeeping gene depends upon numerous factors which are discussed for general applications in section 5.1. Both *gapd* and *actb* were proposed as candidate housekeeping genes in the multiplex RT-PCR analysis of the expression of *arsa*. The use of *gapd* as a housekeeping gene in expression studies must be approached with caution due to its variable expression in numerous disease states and experimental conditions. The presence of pseudogenes for *gapd* is an additional consideration when assessing the use of housekeeping genes in such analyses. This phenomenon was not considered at the initial stages of this investigation, and consequently false positive results were obtained (section 6.3.3). The gene for *actb* also possesses pseudogene sequences (Dakhama *et al.*, 1996), however there are numerous factors which favour the use of *actb* in preference to *gapd* as a control gene in this analysis: the amplification of *actb* RNA has been successfully achieved from formalin fixed P-E lung tissue (Dakhama *et al.*, 1996), and *actb* is expressed in renal tissue (Hoey *et al.*, 1997). The remaining characteristics of *actb*, which support its use as the internal control, are discussed below.

6.1.2.1 Presence of Pseudogenes for the Candidate Housekeeping Gene

Pseudogenes are non-functional transcripts of an original gene which have been incorporated into genomic DNA sequence. They lack intronic sequence and often differ from the coding regions of the functional gene by several base pairs. The amplification of contaminating genomic DNA may therefore occur at the PCR stage of RT-PCR (Foss *et al.*, 1994; Krafft *et*

al., 1997), which may be misinterpreted as amplification of mRNA from such housekeeping genes leading to erroneous results with respect to changes in expression of the target gene. The design of PCR primers which will amplify only genuine genomic DNA sequence overcomes this poorly considered phenomenon. A primer set which will not bind the pseudogene sequences of *actb* has previously been designed (Raff *et al.*, 1997, table 6a.). Furthermore, primers which will not bind *gapd* pseudogene sequence have also been designed as described in Chapter 5.

6.1.2.2 Size of PCR Products

When using multiplex PCR it is recommended that the size of the PCR product obtained from the gene under investigation differs from that of the housekeeping gene sufficiently to ensure detectable resolution by agarose gel electrophoresis to allow any subsequent densitometric analysis (Henegariu *et al.*, 1997), however the PCR should not produce amplicons which are excessively disparate in size as this may lead to errors in comparative analyses. This can be overcome in multiplex RT-PCR by comparing the ratio of control:target gene signal intensities.

6.1.2.3 Theoretical Melting Temperature of Gene Primers

Primer design requires that the theoretical melting temperature of each primer corresponds with that of its partner. This temperature is then used as a guide for the empirical elucidation of the optimal annealing temperature at which any non-specific binding is eradicated. For this

analysis the *gapd* primers were unsuitable due to the relatively high annealing temperature of this primer set (71°C) compared to that of the primer set for *arsa* (61°C, section 6.2.2).

Taking the aforementioned parameters into consideration, the gene for *actb* was the housekeeping gene of choice for the analysis of the expression of *arsa* in patients with diabetes mellitus and normal control subjects, by non-competitive multiplex RT-PCR.

6.2 Materials and Methods

6.2.1 RNA Handling

The analysis of RNA requires meticulous care due to ubiquitous RNAses which may contaminate apparatus or reagents leading to the degradation of RNA. The maintenance of an RNase-free environment is therefore essential when working with RNA. To prevent the degradation of RNA by RNAses the following procedures were carried out:

6.2.1.1 Treatment of Glassware

All glassware was baked at 180°C for at least eight hours prior to use with RNA.

6.2.1.2 Treatment of Plasticware

All non-disposable solvent-resistant plasticware was rinsed with chloroform and autoclaved before use with RNA. All sterile disposable plasticware was sufficiently RNase-free to render prior treatment with chloroform unnecessary.

6.2.1.3 Treatment of Solutions

Solutions were made up using solid reagents designated for RNA work and the H_2O used for solutions was pre-treated with dimethyl pyrocarbonate (DMPC). The effect of DMPC is to inhibit RNase and DNase leaving the nucleic acid intact. Diethyl pyrocarbonate (DEPC) has

the same effect, however it is more toxic than DMPC thus utilisation of DMPC was favoured in this investigation.

6.2.1.3.1 DMPC Treated H_2O

A 50% ($^{v}/_{v}$) ethanol solution was made using double-distilled H_2O. This was used to make up a 1% ($^{v}/_{v}$) DMPC solution which was diluted 1:10 in double-distilled H_2O. This 0.1% ($^{v}/_{v}$) DMPC solution was incubated at room temperature for 30 mins to allow the decomposition of DMPC, and subsequently autoclaved.

6.2.1.4 Treatment of Surfaces

Prior to working with RNA all surfaces were treated with 0.1M NaOH /1mM EDTA solution, and then rinsed with DMPC treated water.

6.2.2 *arsa, gapd* and *actb* Primer Design

The factors considered when designing primers for this multiplex PCR included the general criteria for PCR primer design as described in Chapter 1. The existence of pseudogenes for *actb* (Chapter 5), the size of the amplicons produced, and the theoretical melting temperature and therefore the annealing temperature of each primer set were considered.

The genomic DNA sequences (including both intronic and exonic regions) of human *gapd* (Accession No. J04038, Ercolani *et al.*, 1988), *actb* (Accession No. M10277, Nakajima-Iijima *et al.*, 1985), and *arsa* (Accession No. X52150, Kreysing *et al.*, 1990) were obtained from the 'National Centre for Biotechnology Information' (NCBI) (http://www.ncbi.nlm.nih.gov). The corresponding cDNA sequences of human *gapd* (Accession No. NM002046, Tso *et al.*, 1985), *actb* (Accession No. NM001101, Nakajima-Iijima *et al.*, 1985) and *arsa* (Accession No. X52151, Stein *et al.*, 1989) were also obtained from NCBI to assess each cDNA PCR product size. Primers for the amplification of *gapd* and *arsa* were designed using the Primer 3 (release 6.0) tool within 'Biology Workbench' (http://workbench.sdsc.edu). The properties of the primers designed for the amplification of *arsa* and the housekeeping gene *gapd* are described in table 6a. The properties of primers designed for the amplification of the gene for *actb* as designed by Raff *et al.* (1997), are also described.

Theoretical melting temperatures of each individual primer are illustrated in table 6a. These values were calculated via the MWG Biotech website (wwww.mwg-biotech.com) using the following equation:

$$T_m = 69.3 + 0.41 \times \%GC - \left[\frac{650}{\text{sequence length}} \right]$$

Table 6a. Properties of All Primer Pairs Designed for the Analysis of *arsa*

			Start Position				Size of Product	
Primer	Sequence	Size	cDNA	gDNA	T_m	AT	cDNA	gDNA
arsa F1 Forward	5' GCGCCCGCAGCCCGGTAC 3'	18	N/A	261	67.4	64	540	540
arsa F1 Reverse	5' CAGCCCTCCCGCCGCCAG 3'	18	171	800	67.4			
arsa F2 Forward	5' CGGCAAGTGGCACCTTGGGGTG 3'	22	372	1141	67.7	65	382	570
arsa F2 Reverse	5' CCCAAATGGCCCGCGGCCTG 3'	20	738	1710	67.6			
arsa F3 Forward	5' GGCGGCTGCTCCGGTCTCTTG 3'	21	873	2152	67.6	66	218	308
arsa F3 Reverse	5' CCCAGCAGCAGGGGGCTGAG 3'	20	1091	2459	67.6			
arsa F4 Forward	5' GCCCTCGGCAGTCTCTCTTC 3'	20	1102	2721	63.5	61	408	523
arsa F4 Reverse	5' CTGGGCAATGGCAGCAAGCTG 3'	21	1510	3243	63.7			
actb Forward	5' CCTCGCCTTTGCCGATCC 3'	18	N/A	-8	58.2	60	626	1966#
actb Reverse	5' GCATCTTCATGAGGTAGTCAGTC 3'	23	618	1958	60.6			
gapd P* Forward	5' ACATCAAGAAGGTGGTGAAGC 3'	21	830	4451	57.9	61	239	343
gapd P* Reverse	5' TTACTCCTTGGAGGCCATGTG 3'	21	1069	4793	59.8			
gapd N~ Forward	5' GTCCACTGGCGTGTTCACCA 3'	20	352	3597	61.4	71	260	635
gapd N~ Reverse	5' GTGGCAGTGATGGCATGGAC 3'	20	612	4232	61.4			

gDNA = genomic DNA; T_m = theoretical melting temperature (°C); AT = optimal annealing temperature (°C); # = product not detectable using standard agarose gel elecrophoresis; * = pseudogene binding primer; ~ = non-pseudogene binding primer.

6.2.3 Extraction, Fixation and Paraffin-Embedding of Archive Renal Samples

Renal tissue was excised and trimmed before being placed into a sample cassette. Each sample was subject to fixation and paraffin-embedding using the Pathcentre (Thermo Shandon Ltd, UK), as is routinely performed at the Department of Histopathology, Birmingham Heartlands Hospital. Briefly, samples were perfused with 10% ($^{v}/_{v}$) Formal saline at 45°C for 45 minutes and the sample drained. This process was then repeated for two hours. The samples were then subject to six alcohol treatments at 40°C. The first of these was for 40 minutes and the remaining five for 45 minutes each. Two xylene washes at 40°C followed, each for 1 hour. The samples were then perfused with wax at 60°C, three times each for 30 minutes, and an additional wax perfusion for 45 minutes followed. Samples were then stored at room temperature for at least five years prior to sectioning. Paraffin-embedded renal tissue was sectioned using a base sledge microtome (Thermo Shandon Ltd, UK) into 10µm or 15µm sections. A fresh blade was used and the microtome cleaned between samples to maintain an RNase-free environment. Sectioned samples were stored at room temperature prior to RNA extraction.

6.2.4 RNA Extraction from Paraffin –Embedded Renal Tissue

A comprehensive literature search revealed numerous methods for the extraction of RNA from P-E tissue samples.

6.2.4.1 RNeasy Mini Protocol

A modification of the RNeasy Mini protocol for the extraction of RNA from animal tissue (QIAGEN Ltd, UK) was used to extract RNA from P-E renal tissue. Briefly sections were deparaffinised with xylene, washed using ethanol and the tissue pellet homogenised in a buffer containing β-mercaptoethanol. RNA was then extracted using the RNeasy Mini Protocol for the extraction of RNA from animal tissue. This method employs guanidium thiocyanate and binding of RNA to a silica membrane. The RNA was washed and eluted with RNase-free H_2O in which it was stored at -20°C prior to reverse transcription.

6.2.4.2. TRI Reagent

TRI reagent is an acid guanidium thiocyanate-phenol-chloroform based reagent for the one-step isolation of RNA (Chomczynski & Sacchi, 1987). Renal P-E tissue was weighed and 107.5mg placed into a sterile 1.5mL microfuge tube. A 1200µL volume of xylene was added, the sample mixed by vortexing, and centrifuged at 14,000 rpm for five minutes. The xylene was removed and the deparaffinised tissue washed twice with 100% ($^v/_v$) ethanol. The pellet was then dried, the sample snap frozen in liquid nitrogen and homogenised using an RNase-free, 1000µL pipette tip (Axygen Scientific Inc., USA). The homogenate was then resuspended in 100µL of DMPC-treated H_2O, and 500µL TRI reagent (Sigma, UK) and 100µL bromochloropropane (Sigma, UK) were added. From here the protocol for the extraction of RNA from faecal samples, Bristol PHL, SOP SRSVPCR 1.2, was followed.

6.2.4.3 Paraffin Block RNA Isolation Kit (PBRIK)

This method is based upon proteinase K digestion followed by guanidium isothiocyanate-phenol-chloroform extraction of RNA. Three 10μm sections of P-E renal tissue per sample were subject to RNA extraction using the Paraffin Block RNA Isolation kit (PBRIK, Ambion, UK). The protocol suggests one-to-two 20μm sections per sample. Reverse transcription was carried out as described in section 6.2.6.1 and PCR using *gapd* P* primers was performed (Chapter 5).

6.2.4.3.1 Variation of Parameters of PBRIK

Previous investigations have employed techniques which demonstrate variations upon the protocol described in section 6.2.4.3. The following parameters were adjusted.

6.2.4.3.1.1 Proteinase K Digestion

Incomplete digestion of deparaffinised tissue may result in loss of yield of RNA. The PBRIK protocol recommends proteinase K digestion for between ten minutes and two hours. However, previous studies have incubated deparaffinised tissue for between five minutes and 36 hours (Rupp & Locker, 1988; Stanta & Schneider, 1991; Weisacker *et al.*, 1991; Koopmans *et al.*, 1993; Martin *et al.*, 1994; Sorg & Metzler, 1995; Turbett *et al.*, 1995; Tyrell *et al.*, 1995; O'Driscoll *et al.*, 1996; Krafft *et al.*, 1997; Stanta *et al.*, 1998; Specht *et al.*, 2001). The temperature of proteinase K digestion varies between previous analyses. The Ambion kit recommends proteinase K digestion at 45°C, whereas previously successful RNA

extraction from paraffin-embedded tissue has been achieved using proteinase K digestion at between 45°C and 60°C (Rupp & Locker, 1988; Stanta & Schneider, 1991; Weisacker *et al.*, 1991; Koopmans *et al.*, 1993; Martin *et al.*, 1994; Sorg & Metzler, 1995; Turbett *et al.*, 1995; Tyrell *et al.*, 1995; O'Driscoll *et al.*, 1996; Krafft *et al.*, 1997; Stanta *et al.*, 1998; Specht *et al.*, 2001).

6.2.4.3.1.2 Amount of Starting Material

In an attempt to aid proteinase K digestion, and therefore RNA extraction, a smaller amount of starting material was used. One 10µm section per sample was subject to RNA extraction using PBRIK.

6.2.4.3.1.3 Fragmenting Samples Prior to Xylene Treatment

To aid proteinase K digestion, 1x10µm section was finely chopped using a sterile scalpel prior to the extraction of RNA using PBRIK.

6.2.4.3.1.4 mRNA Isolation

The extraction of RNA may have been successful however the yield of mRNA may have been too low to visualise against total RNA background as reverse transcription uses random priming for first strand cDNA synthesis. To enrich the mRNA content within each sample the Oligotex kit (QIAGEN Ltd, UK) was employed.

6.2.4.3.1.5 Analysis of Alternative Tissue Types

Ribonucleic acid was extracted from samples of paraffin-embedded spleen, tonsil, liver, and kidney tissue using PBRIK. Reverse transcription was performed as described in section 6.2.6.

All the experiments described in section 6.2.4.3. were analysed alongside a positive control mouse liver tissue section supplied with the PBRIK.

Optimisation of a multiplex RT-PCR was carried out using blood-derived RNA with a view to the application of this method to RNA derived from a renal cell line. The alteration in gene expression of *arsa* may be measured as a ratio of the intensities of the signals obtained from *arsa*:*actb*.

6.2.5 RNA Extraction from Blood

Total RNA was extracted from fresh blood samples taken into 4mL EDTA vacuette tubes (Greiner Labortechnik, UK) as described in section 5.2.2.2. using the RNeasy kit for the extraction of total RNA from animal cells (QIAGEN Ltd, UK).

6.2.6. Reverse Transcription

A 4μL aliquot of each total RNA was added to 5.6μL nulease-free H_2O and 2.4μL random decamer primers (6μM final concentration, Ambion, UK) and any secondary RNA structure

denatured by heating to 70°C for 5 mins using a PTC-100 thermal cycler (MJ Research, Watertown, USA). Samples were immediately placed on ice. First strand synthesis buffer (5x, Promega, UK) was then added along with dNTP mix (0.5mM final concentration, ABgene, UK), and M-MLV reverse transcriptase (200U/reaction, Promega UK). Reverse transcription occurred at 42°C for one hour and the reaction stopped by heating to 75°C for five minutes. The use of random decamer primers in this reaction resulted in the formation of total cDNA which was stored at -20°C until use as template for PCR.

6.2.7 Optimisation of Multiplex PCR using *arsa* and *actb* Primers with Blood-derived RNA as cDNA Template

The optimum number of cycles and annealing temperature of this multiplex PCR were based upon those required for the successful amplification of PCR product using each primer set individually. The *actb* primers required 27 cycles at an annealing temperature of 60°C, whereas the primers for *arsa* required 30 cycles at an annealing temperature of 61°C. Each set of primers was manufactured by Alta Biosciences (University of Birmingham, UK), reconstituted in DEPC treated water to a stock concentration of 100mM and stored at -20°C until required. Working solutions were made using a 1:10 dilution of the stock.

A master mix containing the following components was prepared: 1 x PCR buffer containing 1.5mM $MgCl_2$ (final concentration), HotstarTaq DNA polymerase (0.625units per reaction, QIAGEN Ltd, UK), dNTP mix (0.2mM each final concentration, Amersham Biosciences UK Ltd), forward and reverse *actb* primers (0.4μM final concentration), forward and reverse *arsa* primers (1.0μM final concentration). A 0.5μl sample of either human genomic DNA or total cDNA reverse transcription mix was used as template and each reaction made up to a final

volume of 25µl per reaction with double distilled filter-sterilised water. Temperature cycling was performed in a PTC-100 Thermal cycler (MJ Research, Watertown, MA, USA) as follows: 95°C for 15mins, then 30 cycles of 94°C for 30s, 61°C for 60s, 72°C for 60s, followed by a final elongation step at 72°C for 10mins.

A positive PCR control using genomic DNA as template, and a negative PCR control replacing any template with H_2O were analysed concurrently to assess the validity of each PCR.

6.3 Results

In each experiment the positive control sample for RT-PCR and the positive control for the PCR alone, provided a signal with all primers used.

6.3.1 RNeasy Mini Protocol

The extraction of RNA using this method was deemed unsuccessful using reverse transcription as described in section 6.2.6. and *gapd* P* primers illustrated in table 6a. To ensure that this result was not due to inefficient reverse transciption or failure of PCR, a sample of blood-derived RNA (section 6.2.5) was reverse transcribed in parallel with the renal P-E tissue-derived RNA samples as a positive control for reverse transcription. Moreover, a sample of genomic DNA was amplified alongside the aforementioned samples as a positive control for the PCR. Both positive control samples produced the expected PCR products.

6.3.2 TRI Reagent

No PCR products were observed using the *gapd* P* primers described in table 6a. The positive control samples described in section 6.3.1 produced the expected PCR products indicating the failure was due to unsuccessful RNA extraction using this technique.

6.3.3 Paraffin Block RNA Isolation it (PBRIK)

Initially it was thought that the RNA extraction from P-E tissue had been successful using this method. A cDNA sized PCR product was obtained, however further investigation revealed that this positive signal originated from the amplification of *gapd* pseudogene sequence from contaminating DNA within this sample (Chapter 5). The use of *gapd* N~ primers (table 6a) was used at the PCR stage to prevent further false positive results.

6.3.3.1 Variations of Parameters of PBRIK (Ambion, UK)

6.3.3.1 1 Proteinase K Digestion

As proteinase K digestion buffers generally contain RNAse inhibitors, the increased proteinase K digestion time did not have a detrimental effect upon the stability of RNA, however, increasing the time of proteinase K digestion from the recommended 10 minutes to 1 hour, 2 hours and overnight did not result in successful RNA extraction from P-E renal tissue. This may be due to incomplete digestion of sample. Increasing the proteinase K digestion to 60 C for one hour did not result in successful RNA extraction. This may be due to the incomplete digestion of tissue despite increasing the temperature of proteinase K digestion. Increasing the amount of proteinase K in the sample to double that recommended in the PBRIK protocol had no effect upon the proteinase K digestion and therefore RNA extraction.

6.3.3.1.2 Amount of Starting Material

Reducing the amount of starting material applied to each sample, and performing a proteinase K digestion at 60 C for one hour produced no PCR products using *gapd* N~ primers (table 6a).

6.3.3.1.3 Fragmenting Samples Prior to Xylene Treatment

Fragmenting each sample prior to RNA extraction, and subsequent proteinase K digestion at 60 C for one hour, gave no PCR products using *gapd* N~ primers.

6.3.3.1.4 mRNA Isolation

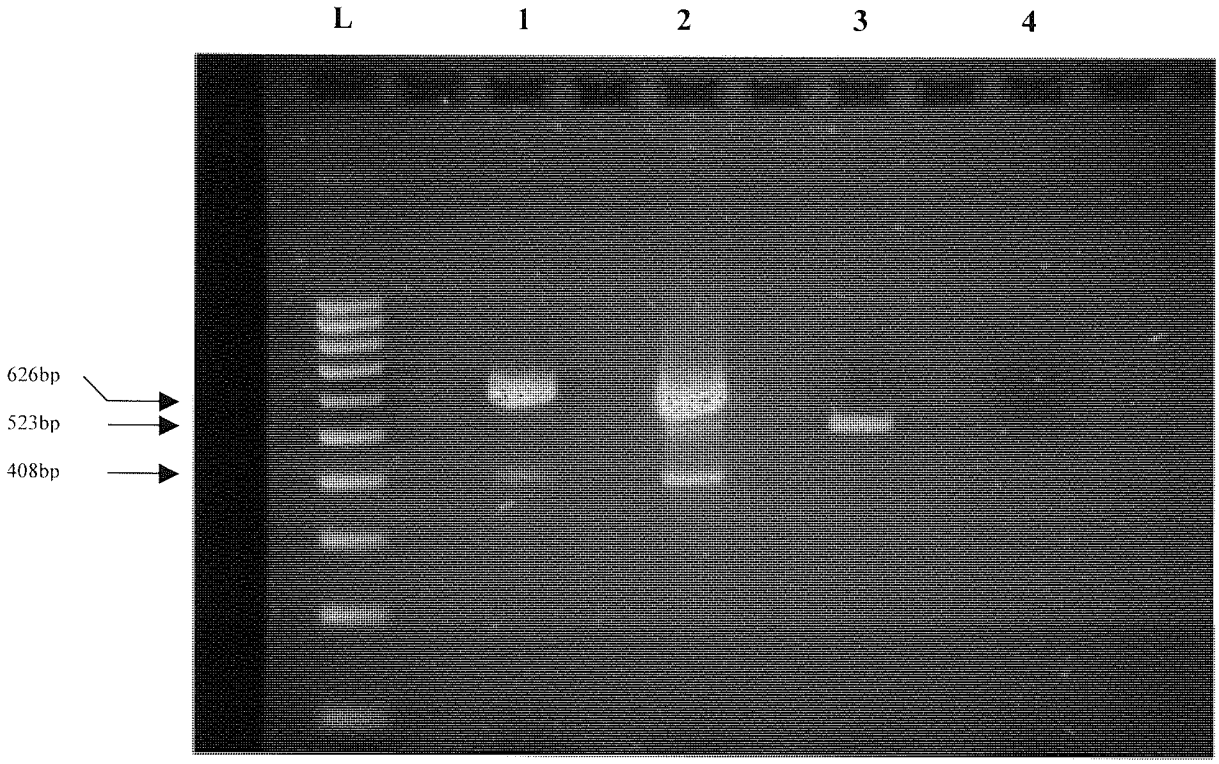
Sample enrichment for mRNA with subsequent PCR using *gapd* N~ primers gave no products.

6.3.3.1.5 Analysis of Alternative Tissue Types

The extraction of RNA from spleen, tonsil, liver, and kidney and subsequent PCR using *gapd* N~ primers produced no amplicons.

The extraction of RNA from paraffin-embedded renal samples was unsuccessful in this instance, however the RT-PCR of blood-derived RNA was successfully optimised (as illustrated in figure 6a), with a view to the use of cell culture as a candidate source of diabetic and normal renal material.

Fig. 6a. Multiplex RT-PCR Using Primers for the Amplification of both *arsa* and *actb*



L = 100bp DNA ladder (Sigma UK); 1 = Total cDNA template for PCR (with DNase treatment prior to RT-PCR); 2 = Total cDNA template for PCR (without DNase treatment prior to RT-PCR); 3 = Genomic DNA template for PCR; 4 = No template for PCR. Bands at 408bp correspond with the amplification of ARSA cDNA, Bands at 523bp correspond to the amplification of ARSA genomic DNA, and bands at 626bp correspond to the amplification of *actb* cDNA (*actb* genomic DNA is not visible using agarose gel electrophoresis with ethidium bromide staining).

6.4 Discussion

Ribonucleic acid has previously been successfully extracted from P-E tissue sections varying in size from 5-100μm. They were deparaffinised using xylene washes (Stanta & Schneider, 1991; Weisacker *et al.*, 1991; Foss *et al.*, 1994; Martin *et al.*, 1994; Sorg & Metzler, 1995; Turbett *et al.*, 1995; Tyrell *et al.*, 1995; Stanta *et al.*, 1998; Specht *et al.*, 2001), or an equivalent solvent such as Pro-par (Koopmans *et al.*, 1993), octane (O'Driscoll *et al.*, 1996) or Homo De (Krafft *et al.*, 1997). The deparaffinised tissue was then washed with ethanol to remove any traces of xylene which may interfere with subsequent enzymatic steps (Stanta *et al.*,1998). Several methods involved the digestion of the deparaffinised tissue with the proteolytic enzyme proteinase K (Rupp & Locker, 1988; Stanta & Schneider, 1991; Weisacker *et al.*, 1991; Koopmans *et al.*, 1993; Martin *et al.*, 1994; Sorg & Metzler, 1995; Turbett *et al.*, 1995; Tyrell *et al.*, 1995; O'Driscoll *et al.*, 1996; Krafft *et al.*, 1997; Stanta *et al.*, 1998; Specht *et al.*, 2001). Either a simple phenol-chloroform extraction method (Rupp and Locker, 1988; Weisacker *et al.*, 1991; Koopmans *et al.*, 1993; Stanta *et al.*, 1998; Specht *et al.*, 2001), or the use of the chaotrope guanidium (iso)thiocyanate coupled with extraction with phenol-chloroform (Chomczynski & Sacchi, 1987; Stanta & Schneider, 1991; Koopmans *et al.*, 1993; Martin *et al.*, 1994; Tyrrell *et al.*, 1995) was then used to extract RNA. Sorg & metzler (1995) used a guanidium thiocyanate RNA extraction method coupled with phenol-chloroform-isoamylalcohol. Whereas O'Driscoll *et al* (1996) extracted RNA successfully using phenol-chloroform-isoamylalcohol alone. Ribonucleic acid was then precipitated using either ethanol (Koopmans *et al.*, 1993; Martin *et al.*, 1994), isopropanol (Tyrrell *et al.*, 1995; Svoboda-Newman *et al.*, 1997) or both (Sorg & Metzler, 1995). Precipitation has been facilitated by the use of sodium acetate (Weisacker *et al.*, 1991; Foss *et al.*, 1994; O'Driscoll *et al.*, 1996; Specht *et al.*, 2001) and / or a carrier molecule such as glycogen (Stanta & Schneider, 1991; Krafft *et al.*, 1997; Stanta *et al*, 1998). Lithium chloride has also been used

to precipitate RNA (Rupp & Locker, 1988). This method is unsuitable for use with reverse transcription as a downstream application, since lithium salts can inhibit subsequent cDNA synthesis (Sambrook *et al.*, 1989). Once precipitated, the sample was then centrifuged (preferably at 4°C) to pellet the precipitated RNA, washed in ethanol and once dried it was resuspended in RNase-free (usually DEPC-treated) H_2O (Weisacker *et al.*, 1991; Koopmans *et al.*, 1993; Foss *et al.*, 1994; Martin *et al.*, 1994; Sorg & Metzler, 1995; O'Driscoll *et al.*, 1996; Krafft *et al.*, 1997; Svoboda-Newman *et al.*, 1997; Stanta *et al.*, 1998; Specht *et al.*, 2001). Often the RNA was used immediately for an appropriate downstream application (Stanta & Schneider, 1991; Tyrrell *et al.*, 1995) thus preventing any possible degradation during storage. However RNA should remain stable when stored at –20°C to -80°C. The Paraffin Block RNA Isolation Kit (Ambion, UK) was used in this study as it is based upon RNA extraction using the aforementioned method. Alterations were made to the PBRIK protocol as described without success. Due to time limitations it was not possible to investigate this method further. One may consider increasing the time of centrifugation to pellet precipitated RNA and repeating the acid phenol chloroform extraction. The use of alternative methods of RNA extraction failed in this instance, however the methods of Coombs *et al* (1999) or Turbett *et al* (1995) were not attempted. No single technique has been confirmed as the optimal RNA extraction method, as the success of RNA extraction is multifactorial and may depend upon the RNA together with the tissue type under investigation (Krafft *et al.*, 1997). This study illustrates that the tissue type is not responsible for the failure of RNA extraction. In this case no RT-PCR products were observed when RNA extraction from spleen, tonsil, or liver samples was employed. However, in all cases the extraction of RNA from control mouse liver sections was successful indicating the importance of sample handling and maintenance of an RNAse-free environment. All samples were obtained from the Histopathology laboratory archive at Birmingham Heartlands Hospital. As such they were subject to a routine fixation and embedding process for which an RNase-free

environment is not necessary. This may have exposed all tissues to extensive RNA degradation such that the nucleic acid was too fragmented to allow detection using the method employed.

The effect of a variety of fixatives upon the yield of RNA obtained from paraffin-embedded tissue has been investigated (Koopmans *et al.*, 1993; Foss *et al.*, 1994; Tyrrell *et al.*, 1995;). Tissue which had been fixed using acetone gave a high yield of RNA, however samples also exhibited relatively high levels of contaminating DNA. A consensus has not yet been reached as to the optimum fixative for use alongside RNA extraction. Although previous studies have successfully extracted RNA from P-E tissue which has been fixed using formalin (O'Driscoll *et al.*, 1996; Sorg & Metzler, 1995; Weizsacker *et al.*, 1991; Turbett *et al.*, 1996; Svoboda-Newman *et al.*, 1997) and subsequently visualised using agarose gel electrophoresis with ethidium bromide staining. It should be noted however that formalin is known to covalently modify RNA (Masuda *et al.*, 1999). In this study tissue was fixed using formal saline (1:10 dilution of formalin in 0.9% ($^{w}/_{v}$) saline), indicating the fixative itself may not be responsible for the failure of RNA extraction experienced here.

The use of primers which will not bind pseudogene sequences circumvents the amplification of contaminating genomic DNA. This phenomenon has been considered in the literature (Foss *et al.*, 1994), however numerous studies have not accounted for the existence of pseudogene sequences of several housekeeping genes and have demonstrated false positive results (Tyrrell *et al.*, 1995; Dakhama *et al.*, 1996). In this analysis a false positive result was observed but was prevented in further studies by the use of primers which will not bind the pseudogenes of β-actin (Raff *et al.*, 1997). The development of primers which will not bind the pseudogenes of *gapd* (Chapter 5) will allow the accurate use of *gapd* as an internal control in future expression analyses.

The detection of RNA can be achieved quantitatively or semi-quantitatively using RT-PCR as described in section 6.1.1. The most efficient and widely employed detection method is agarose gel electrophoresis with ethidium bromide staining (Chomczynski & Sacchi, 1987; Weisacker *et al.*, 1991; Koopmans *et al.*, 1993; Sorg & Metzler, 1995; Turbett *et al.*, 1995; Tyrrell *et al.*, 1995; O'Driscoll *et al.*, 1996; Svoboda-Newman *et al.*, 1997; Specht *et al.*, 2001). Southern blotting using a radiolabeled probe (Stanta & Schneider, 1991; Weisacker *et al.*, 1991; Koopmans *et al.*, 1993; Foss *et al.*, 1994; Krafft *et al.*, 1997) or one which can be indirectly detected using chemiluminescence (e.g. Digoxigenin) (Martin *et al.*, 1994) has been employed to detect RT-PCR products of paraffin-embedded tissue derived RNA. The increased sensitivity of this method of detection may be outweighed by the increased cost, and in the case of use of a radioactive probe, the risk associated with the use of radioactivity. Southern blotting is more time-consuming than visualisation using agarose gel electrophoresis combined with ethidium bromide staining. Furthermore, the signal may be less defined making subsequent densitometry problematic. An alternative method of visualisation is silver staining following polyacrylamide gel electrophoresis (Del Prete *et al.*, 1998).

One way to overcome the detection problems associated with agarose gel electrophoresis and Southern blotting, is the use of real-time RT-PCR (Specht *et al.*, 2001). Here fluorescent probes are employed in a one-step RT-PCR. Excitation of hybridisation, hydrolysis, or molecular beacons probes, or DNA binding dyes which are specific for the target gene, is relative to the amount of PCR product. Measurement of the fluorescence of such probes is relative to the amount of starting material and therefore the level of expression of the target gene. This methodology can be employed to analyse multiple PCR products, as with traditional multiplex RT-PCR, to allow correction for any pre-RT-PCR experimental variation. This comparative technique can also be applied quantitatively (Bustin, 2000).

Nested PCR may also be employed to increase the sensitivity of traditional RT-PCR (Sorg & Metzler, 1995; Svoboda-Newman *et al.*,1997). Here a second 'round' of PCR with the original gene-specific primers using the original PCR product as template, results in an increased yield of PCR product which may be visible using agarose gel electrophoresis with ethidium bromide staining. However, the specificity of this analysis may be compromised due to increased amplification of any non-specific PCR products.

In this study the amount of RNA used for RT-PCR was not quantified as the analysis was comparative and any discrepancy between the amount of starting material in each sample is corrected for by the calculation of the ratio of signal intensities of test and control genes. Since RNA extraction from renal tissue samples proved unsuccessful, an alternative source of renal material may be cell culture. Ribonucleic acid can be extracted relatively easily from cells which are grown with a view to RNA extraction. The use of the 'Cells to cDNA' kit (Ambion, UK) eliminates isolated RNA extraction and reverse transcription steps, decreasing the likelihood of RNA degradation through exposure of samples to potential contaminants.

A multiplex RT-PCR assay has been developed to analyse the expression of *arsa* alongside that of *actb* with a view to its application to renal cells, however further optimisation is required to apply this reaction to expression analysis as each gene has reached the plateau phase of PCR. The optimum number of cycles of PCR needs to be assessed to ensure production of visible amplicons from both genes, with the maintenance of each gene in the exponential phase of PCR.

6.5 Conclusion

A multiplex PCR using *actb* and *arsa* primers has been successfully optimised using blood-derived RNA as template. In this study P-E tissue proved an unsuitable source of renal material due the difficulties experienced with RNA extraction. The most likely contributing factor is that the tissue used was extracted, fixed and paraffin-embedded without consideration for subsequent RNA analysis. The extraction of RNA from P-E tissue which has been handled with appropriate care may be a candidate for future investigations, however the scarcity of such material prevents its use as an appropriate source of renal tissue. The method described may be applied to cell-culture derived RNA where the expression of *arsa* is measured using RNA derived from a renal cell line which is grown under conditions of increased glucose concentration to mimic uncontrolled diabetes mellitus. The expression of *arsa* may then be analysed using the multiplex RT-PCR developed in this investigation. Diabetes mellitus may be a salt-retaining state for which increased urinary activity of ASA is a compensatory mechanism. Therefore, renal cells which are subject to increased sodium could be an alternative model with which to compare the expression of *arsa* to that found in a 'normal' renal cell line.

CHAPTER 7

7. FINAL DISCUSSION

The role of peripheral (renal) dopamine as a natriuretic hormone is well established and dopamine and its precursor L-dopa have been extensively analysed in salt retaining states. Peripheral dopamine is also formed from dopamine sulphate via arylsulphatase A (ASA), however despite the development of techniques for the measurement of urinary catecholamines and their sulphate conjugates, research into the role of dopamine sulphate and ASA in sodium homeostasis is lacking.

The formation of dopamine from L-dopa is catalysed by the enzyme L-aromatic amino acid decarboxylase (L-AAAD). Although the mRNA sequence encoding L-AAAD is known, the genomic DNA sequence has not yet been elucidated, limiting the study of this pathway of peripheral dopamine formation at the molecular level. However, the analysis of the genetics of dopamine formation via ASA is facilitated by the availability of both the genomic and coding DNA sequences of the gene for ASA (*arsa*).

An important finding emanating from this study was that dopamine sulphate is a physiological reservoir of active urinary free dopamine which is utilised with the requirement for natriuresis. However, despite a collection of evidence, including results from this investigation (Chapter 3), suggesting that dopamine excretion is blunted in states which can be sodium retaining, the activity of ASA is increased in diabetes mellitus. This complex disease can be sodium-retaining, but also has many chronic complications associated with it. Of these, diabetic nephropathy associated with the accumulation of advanced glycated end-products (AGE), may affect the site of peripheral dopamine formation. Whether the development of nephropathy is caused by poor glycaemic control is not fully understood,

however the formation of AGE increases the oxidative stress to which the kidney is subjected. Dopamine is known to autoxidise to 6-hydroxydopamine (6-OHDA) and in doing so releases H_2O_2. Whether this autoxidation is a result of the increased oxidative stress caused by AGE accumulation, or whether it is one of the mechanisms which itself promotes oxidative stress is at present unknown. Although excretion of 6-OHDA is significantly higher in patients with diabetic nephropathy, the establishment of this phenomenon as a cause or effect of this complication is unclear.

The expectation that ASA activity would be decreased in diabetes and other sodium-retaining states thereby supporting the observation of diminished excretion of dopamine was not fulfilled; rather an increase in activity was observed. This paradoxical situation may be explained not only by the blunted conversion of L-dopa to dopamine, but the sequestering of dopamine by autoxidation to 6-OHDA, resulting in a compensatory increase in ASA activity.

Numerous isoforms of ASA have been reported in patients with schizophrenia and alcoholism, however it was established that the increased activity of ASA in patients with diabetes mellitus was not due to the existence of an isoform of ASA which has a higher affinity for dopamine sulphate. The increased activity of ASA was subsequently hypothesised to be as a consequence of increased renal expression of *arsa*.

Expression analyses were complicated in this study due to the use of archival P-E tissue which had been handled without consideration for its use in expression analyses. These studies would have been viable were diabetic renal tissue more abundant, and / or the tissue handled from excision to paraffin-embedding with a view to RNA analysis. Unfortunately neither of these factors applied and an alternative source of renal material is required for further investigations. The use of renal cell lines which are grown in media containing an

elevated glucose concentration and subsequent analysis of *arsa* expression within these cells, may provide information regarding the expression of *arsa* in cases of poor glyceamic control. Although a diabetic cell line is currently unavailable, it should be possible to maintain an appropriate renal cell culture in a pseudo diabetic state by careful maintenance of the glucose levels in the growth medium. Comparing *arsa* expression in cells maintained at physiological glucose levels, with that observed in cells grown with glucose in excess, would provide an important insight into the role of ASA in diabetes mellitus and funding is available to support this line of investigation further. Expression analysis has been facilitated by the development and optimisation of a multiplex RT-PCR assay for the analysis of *arsa* expression alongside that of the housekeeping gene for β-Actin (*actb*). Here the primers used for PCR of *actb* amplified only the functional gene and not any of its associated pseudogenes. In addition, this investigation has demonstrated the design of primers and optimisation of a PCR for the amplification of *gapd* without concurrent amplification of pseudogenes for *gapd*. This is a valuable tool for future expression analyses in which a 'housekeeping' gene is used as an internal control and will prevent erroneous analyses resulting from the amplification of pseudogene sequences.

In conclusion, this study has described for the first time the role of dopamine sulphate as a reservoir of active free urinary dopamine, and its role in sodium homeostasis. The enzyme ASA is an integral component of dopamine sulphate metabolism, the activity of which was found to be increased in diabetes mellitus, an example of a salt retaining state. Molecular and kinetic analyses of *arsa* and ASA showed that this observed activity increase was not due to functional or genetic sequence heterogeneity. A RT-PCR assay was developed to study the expression of *arsa* as a candidate cause of the increased activity observed. Although a validated comparative RT-PCR assay was developed for amplification of *arsa* transcripts

from fresh blood samples, expression analysis from archived paraffin-embedded renal tissue was complicated by the low yield and degradation of unprotected RNA.

PUBLICATIONS ARISING FROM THIS WORK

The Effect of Salt Restriction on Excretion of Dopamine and Dopamine Sulphate

Harper LV, Bayly GR, Bartlett WA, Jones AF

(Presented as a poster at FOCUS, the Annual National Meeting of the Association of Clinical Biochemists 1998, the abstract is published in the Proceedings)

The Effect of Sodium Restriction on Urinary Free Dopamine and Dopamine Sulphate Concentration

Harper LV, Bayly GR, Bartlett WA, Jones AF

(Presented as oral presentation for the Bayer award at Pathology 2000, ICC, Birmingham UK, the abstract is published in the Proceedings)

The Effect of Dietary Sodium Restriction on Urinary Free Dopamine and Dopamine Sulphate Concentration

Harper LV, Bayly GR, Bartlett WA, Hilton AC, Jones AF

(In preparation for submission to Annals of Clinical Biochemistry)

Urinary Arylsulphatase A Activity in Patients with Diabetes Mellitus

Moore AJ, Harper LV, Jones AF

(In preparation for submission to Diabetes Research and Clinical Practice)

Use of *gapd* as a Transcriptional Benchmark in RT-PCR: Design of Primers Which do not Bind to *gapd* Pseudogene Sequences

Harper LV, Hilton AC, Jones AF

(In preparation for submission to Biotechniques)

REFERENCES

Abenhaim, L., Romain, Y. and Kuchel, O. (1980). "Platelet Phenolsulphotransferase and Catecholamine: Physiological and Pathological Variations in Humans" *Canadian Journal of Physiology and Pharmacology* **59**: 300 – 306.

Alberti, K. G. M. M. and Zimmet, P. Z. (1999). "Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications." http://www.diabetes.org.uk/infocentre/carerec/diagnosi.doc

Aledo, J. C., Segura, J. A., Barbero, L. G. and Marquez, J. (1999). "Upregulation of Glyceraldehyde-3-phosphate Dehydrogenase mRNA in the Spleen of Tumor-Bearing Mice." *Biochimie* **81**: 1109 - 1113.

Alexander, R. W., Gill, J. R., Yamabe, H, Lovenberg, W. and Keiser, H. R. (1974). "Effects of Dietary Sodium and of Acute Saline Infusion on the Interrelationship Between Dopamine Excretion and Adrenergic Activity in Man." *Journal of Clinical Investigation* **54**: 194 - 200.

Alexander, M., Curtis, G., Avruch, J. and Goodman, H. M. (1985). "Insulin Regulation of Protein Biosynthesis in Differentiated 3T3 Adipocytes." *The Journal of Biological Chemistry* **260**(22): 11978 - 11985.

Andreeva, L., Motterlini, R. and Green, C. J. (1997). "Cyclophilins are Induced by Hypoxia and Heat Stress." *Biochemical and Biophysical Research Communications* **237**: 6 - 9.

Andrew, R., Watson, D. G., Best, S. A., Midgley, J. M., Wenlong, H. and Petty, R. K. H. (1993). "The Determination of Hydroxydopamines and Other Trace Amines in the Urine of Parkinsonian Patients and Normal Controls." *Neurochemical Research* **18**(11): 1175 - 1177.

Aoki, K., Kikuchi, K., Yamaji, I., Nishimura, M., Honma, C., Kobayakawa, H., Yamamoto, M., Kudoh, C., Shimazaki, M., Sakamoto, T., Wada, A. and Iimura, O. (1989). "Attenuated Renal Production of Dopamine in Patients with Low Renin Essential Hypertension." *Clinical and Experimental Hypertension - Theory and Practice* **A 11** – Suppl. 1: 403 - 409.

Aperia, A., Holtback, U., Syren, M. L., Svensson, L. B., Frycksted, J. and Greengard, P. (1994). "Activation / Deactivation of Renal Na^+ / K^+ ATPase: A Final Common Pathway for Regulation of Natriuresis." *The FASEB journal* **8**: 436 - 439.

Arakawa, K., Miura, S., Koga, M., Kinoshita, A., Urata, H. and Kiyonaga A. (1995). "Activation of Renal Dopamine System by Physical Exercise." *Hypertension Research* **18** Suppl. 1: S73 - S77.

Arcari, P., Martinelli, R. and Salvatore, F. (1989). "Human Glyceraldehyde-3-Phosphate Dehydrogenase Pseudogenes: Molecular Evolution and a Possible Mechanism for Amplification." *Biochemical Genetics* **27**(7 - 8): 439 - 450.

Baines, A. D. and Chan, W. (1980). "Production of Free Dopamine From Dopa; a Micropuncture Study." *Life Sciences* **26**: 253 - 259.

Ball S. G. and Lee, M. R. (1977). "The Effect of Carbidopa Administration on Urinary Sodium Excretion in Man. Is Dopamine An Intrarenal Natriuretic Hormone?" *British Journal of Clinial Pharmacology* **4**: 115 - 119.

Barisani, D., Meneveri, R., Ginelli, E. Cassani, C. and Conte, D. (2000). "Iron Overload and Gene Expression in HepG2 Cells: Analysis by Differential Display" *FEBS Letters* **469**: 208 - -212.

Barroso, I., Benito, B. Garcia-Jimenez, C., Hernandez, A., Obregon, M. J. and Santisteban, P. (1999). "Norepinephrine Tri-iodothyronine and Insulin Upregulate Glyceraldehyde-3-phosphate Dehydrogenase mRNA During Brown Adipocyte Differentiation" *European Journal of Endocrinology* **141**:169-179.

Bartlett, W. A. " Utility and Measurement of Urinary Free Catecholamines" *personal communication*, Birmingham Heartlands Hospital.

Baum, H., Dodgson, K. S. and Spencer, B. (1959) " The Assay of Arylsulphatases A and B in Human Urine" *Clin. Chem. Acta* **4**: 453 – 455.

Bayly, G. R., Bartlett, W. A., Harper, L. V. and Jones, A. F. J. (1999). "A Novel Fully Automated Method for the Measurement of Sulphoconjugated Catecholamines in Urine Using the Gilson ASTED-XL Sample Preparation System and High-Performance Liquid Chromatography." *Biomedical Chromatography* **13**: 1 - 5.

Benham, F. J., Hodgkinson, S. and Davies, K. E. (1984). "A Glyceraldehyde-3-phosphate Dehydrogenase Pseudogene on the Short Arm of the Human X Chromosomes Defines a Multigene Family." *EMBO Journal* **3**(11): 2635 - 2640.

Benham, F. J. and Povey, S. (1989). "Members of the Human Glyceraldehyde-3-Phosphate Dehydrogenase-Related Gene Family Map to Dispersed Chromosomal Locations." *Genomics* **5**: 209 - 214.

Ben-Yoseph, Y. and Mitchell, D. A. (1995). "Discrimination Between Metachromatic Leukodystrophy and Pseudo-deficiency of Arylsulfatase A by Restriction Digest of Amplified Gene Fragments." *The American Journal of Medical Sciences* **309**(2): 88 - 91.

Berger, J., Gmach, M., Fae, I., Molzer, B. and Bernheimer, H. A. (1996). "A New Polymorphism of Arylsulfatase A Within the Coding Region." *Human Genetics* **98**: 348 - 350.

Bergquist, J. Sciubisz, A., Kaczor, A. and Silberring, J. (2002). "Catecholamines and Methods for their Identification and Quantitation in Biological Tissues and Fluids" *Journal of Neuroscience Methods* **113**: 1 – 13.

Bertorello, A., Hokfelt, T., Goldstein, M. and Aperia, A. (1988). "Proximal Tubule Na^+-K^+-ATPase Activity is Inhibited During High-Salt Diet: Evidence for DA-Mediated Effect." *American Journal of Physiology* **254**: F795 - F801.

Bhatia, P., Taylor, W. R., Greenberg, A. H. and Wright, J. A. (1994). "Comparison of Glyceraldehyde-3-Phosphate Dehydrogenase and 28S-Ribosomal RNA Gene Expression as RNA Loading Controls for Northern Blot Analysis of Cell Lines of Varying Malignant Potential." *Analytical Biochemistry* **216**: 223 - 226.

Bindoli, A., Rigobello, M. P. and Deeble, D. J. (1992). "Biochemical and Toxicological Properties of the Oxidation Products of Catecholamines" *Free Radical Biology and Medicine* **13**: 391 – 405

Bird, I. (1989). "High Performance Liquid Chromatography: Principles and Clinical Applications." *British Medical Journal* **299**: 783 - 787.

Boomsma, F., van der Hoorn, F. A. J., Man in't Veld, A. J. and Schalekamp M. A. D. H. (1988). "Determination of 3,4-dihydroxyphenylalanine (Dopa) in Plasma and Cerebrospinal Fluid by High Performance Liquid Chromatography with Electrochemical Detection." *Clinical Chimica Acta* **178**: 59 - 70.

Brown, M. J. and Allison, D. J. (1981). "Renal Conversion of Plasma Dopa to Urine Dopamine." *Br. J. Clin. Pharmac.* **12**: 251 - 253.

Bustin, S. A. (2000). "Absolute Quantification of mRNA Using Real-Time Reverse Transcription Polymerase Chain Reaction Assays." *Journal of Molecular Endocrinology* **25**: 169 – 193.

Calvo, E. L., Boucher, C., Coulombe, Z. and Morisset, J. (1997). "Pancreatic GAPDH Gene Expression During Ontogeny and Acute Pancreatitis Induced by Caerulein." *Biochemical and Biophysical Research Communications* **235**: 636 - 640.

Carey, R. M., Van Loon, G. R., Baines, A. D. and Ortt, E. M. (1981). "Decreased Plasma and Urinary Dopamine During Dietary Sodium Depletion in Man." *Journal of Clinical Endocrinology and Metabolism* **52**(5): 903 - 909.

Casson, I. F., Lee, M. R., Brownjohn, A. M., Parsons, F. M., Davison, A. M., Will, E. J. and Clayden, A. D. (1983). "Failure of Renal Dopamine Response to Salt Loading in Chronic Renal Disease." *British Medical Journal* **286**: 503 – 506.

Chan, J. C. N., Critchley, J. A. J. H., Ho, C. S., Nicholls, M. G., Cockram, C. S. and Swaminathan, R. (1992). "Atrial Natriuretic Peptide and Urinary Dopamine Output in Non-insulin-dependent Diabetes Mellitus." *Clinical Science* **83**: 247 - 253.

Chetty, R. K., Bartlett, W. A., Jones A. F. (2002). "Elevated Urinary 6-hydroxydopamine, but not N-Acetyl Dopamine, in Diabetic Patients with Nephropathy." *in press*

Chin, K. V., Seifer, D. B., Feng, B., Lin, Y. and Shih, W. C. (2002). "DNA Microarray Analysis of the Expression Profiles of Luteinized Granulosa Cells as a Function of Ovarian Reserve." *Fertility and Sterility* **77**(6): 1214 – 1218.

Chiueh, C. C., Miyake, H. and Peng, M. T. (1993). "Role of Dopamine Autoxidation, Hydroxyl Radical Generation, and Calcium Overload in Underlying Mechanisms Involved in MPTP-induced Parkinsonism." *Advances in Neurology* **60**: 251 - 258.

Chomczynski, P. and Sacci, N. (1987). "Single-Step Method of RNA Isolation by Acid Guanidium Thiocyanate-Phenol-Chloroform Extraction." *Analytical Biochemistry* **162**: 156 - 159.

Clark, B. A., Rosa, R. M., Epstein, F. H., Young, J. B. and Landsberg, L. (1992). "Altered Dopaminergic Responses in Hypertension." *Hypertension* **19**(6 pt 1): 589 - 594.

Coombs, N. J., Gough, A. C. and Primrose, J. N. (1999). "Optimisation of DNA and RNA Extraction from Archival Formalin-fixed Tissue." *Nucleic Acids Research* **27**(16): e12.

Cosentino, M., Raffaella, B., Ferrari, M., Marino, F., Rasini, E., Maestroni, G. J. M., Conti, A., Boveri, M., Lecchini, S. and Frigo, G. (2000). "HPLC-ED Measurement of Endogenous Catecholamines in Human Immune Cells and Hematopoietic Cell Lines." *Life Sciences* **68**: 283 – 295.

Coulter-Mackie M. and Gagnier, L. (1997). "Two New Polymorphisms in the Arylsulphatase A Gene and Their Haplotype Associations with Normal, Metachromatic Leukodystrophy and Pseudo-deficiency Alleles." *American Journal of Medical Genetics* **73**: 32 - 35.

Cummings, J., Matheson, L. M. and Smythe, J. F. (1990). "Method for the Determination of γ-L-glutamyl-L-dihydroxyphenylalanine and its Major Metabolites L-dihydroxyphenylalanine, Dopamine and 3,4-dihydroxyphenylacetic acid by High Performance Liquid Chromatography with Electrochemical Detection." *Journal of Chromatography* **528**: 43 - 53.

Dakhama, A., Macek, V., Hogg, J. C. and Hegele, R, G. (1996). "Amplification of Human β-Actin Gene by the Reverse Transriptase-Polymerase Chain Reaction: Implications for Assessment of RNA from Formalin-Fixed Paraffin-Embedded Material." *The Journal of Histochemistry and Cytochemistry* **44**(10): 1205 - 1207.

de Leeuw, W. J. F., Slagboom, P. E. and Vijg, J. (1989). "Quantitative Comparison of mRNA Levels in Mammalian Tissues: 28S Ribosomal RNA Level as an Accurate Internal Control." *Nucleic Acids Research* **17**(23): 10137 - 10138.

Del Prete, M. D., Forino, M., Gambaro, G., D'Angelo, A., Baggio, B. and Anglani, F. (1998). "A Comparative Kinetic RT/PCR Strategy for the Quantitation of mRNAs in Microdissected Human Renal Biopsy Specimens." *Experimental Nephrology* **6**: 563 - 567.

Demassieux, S., Bordeleau, L., Gravel, D. and Carriere, S. (1986). "Catecholamine Sulfates: End Products or Metabolic Intermediates?" *Life Sciences* **40**: 183 - 191.

Devereux, J., Haeberli, P. and Smithies, O. (1984). "A Comprehensive Set of Sequence-Analysis Programs for the VAX." *Nucleic Acids Research* **12**: 387 – 395.

Drayer, J. I. M., Weber, M. A., Sealey, J. E. and Laragh, J. H. (1981). "Low and High Renin Essential Hypertension: a Comparison of Clinical and Biochemical Characteristics." *The American Journal of Medical Sciences* **281**(3): 135 - 142.

Dutton, J., Hodgkinson, A. J., Hutchinson, G. and Roberts, N. B. (1999). "Evaluation of a New Method for the Analysis of Free Catecholamines in Plasma Using Automated Sample Trace Enrichment with Dialysis and HPLC." *Clinical Chemistry* **45**(3): 394 - 399.

Eisenhofer, G., Goldstein, D. S., Stull, R., Keiser, H. R., Sunderland, T., Murphy, D.L. and Kopin, I. J. (1986). "Simultaneous Liquid-chromatographic Determination of 3,4-dihydroxyphenlylglycol, Catecholamines, and 3,4-dihydroxyphenylalanine in Plasma, and their responses to Inhibition of Monoamine Oxidase." *Clinical Chemistry* **32**(11): 2030 - 2033.

Elchisak, M. A. (1983)a. "Determination of Dopamine-O-Sulfate and Norepinephrine-O-Sulfate Isomers and Serotonin-O-Sulfate by High Performance Liquid Chromatography Using Dual-electrode Electrochemical Detection." *Journal of Chromatography* **264**: 119 – 127.

Elchisak, M. A. (1983)b. "Determination of Conjugated Compounds by Liquid Chromatography with Electrochemical Detection using Post-Column Hydrolysis: Application to Dopamine Sulfate Isomers." *The Journal of Chromatography* **255**: 475 - 482.

Elchisak, M. A. (1986). "Determination of Sulfated Biogenic Amines: Emphasis on Dopamine Sulfate Isomers." *Federation Proc* **45**: 2241 - 2246.

Elder, P. K., Schmidt, L. J., Ono, T. and Getz, M. J. (1984). "Specific Stimulation of Actin Gene Transcription by Epidermal Growth Factor and Cycloheximide." *Proc. Natl. Accad. Sci. USA* **81**: 7476 – 7480.

Engler-Blum, G., Meier, M., Frank, J. and Muller, G. A. (1993). "Reduction of Background Problems in Nonradioactive Northern and Southern Blot Analyses Enables Higher Sensitivity than ^{32}P-based Hybridisations." *Analytical Biochemistry* **210**: 235 – 244.

Ercolani, L., Florence, B., Denaro, M., Alexander, M. (1988). "Isolation and Complete Sequence of a Functional Human Glyceraldehyde-3-Phosphate Dehydrogensase Gene." *The Journal of Biological Chemistry* **263**(30): 15335 - 15341.

Fedorcsak, I. And Ehrenberg, L. (1966). "Effects of Diethyl Pyrocarbonate and Methyl Methanesulfonate on Nucleic Acids and Nucleases." *Acta Chemica Scandanavica* **20**: 107 – 112.

Feldt-Rasmussen, B., Mathiesen, E. R., Deckert, T., Gise, J., Christensen, N. J., Bent-Hansen, L. and Nielsen, M. D. (1987). "Central Role for Sodium in the Pathogenesis of Blood Pressure Changes Independent of Angiotensin, Aldosterone and Catecholamines in Type 1 (Insulin-dependent) Diabetes Mellitus." *Diabetologia* **30**: 610 - 617.

Finke, J., Fritzen, R., Ternes, P., Lange, W. and Dolken, G. (1993). "An Improved Strategy and a Useful Housekeeping Gene for RNA Analysis from Formalin-Fixed, Paraffin-Embedded Tissues by PCR." *Biotechniques* 14(3): 448 - 453.

Flack, J. M., Ensrud, K. E., Mascioli, S., Launcer, C. A., Svendsen, K., Elmer, P. J. and Grimm, R. H. (1991). "Racial and Ethnic Modifiers of the Salt-Blood Pressure Response." *Hypertension* **17**(Suppl 1): I115 - I121.

Foss, R. D., Thakurta, N., Conran, R. M. and Gulman, P. (1994). "Effects of Fixative and Fixation Time on the Extraction and Polymerase Chain Reaction Amplification of RNA from Paraffin-Embedded Tissue." *Diagnostic and Molecular Pathology* **3**: 148 - 155.

Fowler, L. R. and Rammler, D. H. (1963). "Sulfur Metabolism of *Aerobacter Aerogenes* II. The Purification and Some Properties of a Sulfatase." *Biochemistry* **3**(2): 230 – 237.

Francavilla, A., Carr, B. I., Starzl, T. E., Azzarone, A., Carrieri, G. and Zeng, Q. H. (1992). "Effects of Rapamycin on Cultured Hepatocyte Proliferation and Gene Expression." *Hepatology* **15**: 871 – 877.

Ganong, W. F. (1991). "Review of Medical Physiology." 15th Edn. Appleton and Lange, Conneticut, USA.

Garbay, B., Boue-Grabot, E. and Garrett, M. (1996). "Processd Pseudogenes Interfere with Reverse-Transcriptase-Polymerase Chain Reaction Controls." *Analytical Biochemistry* **237**: 157 - 159.

Garmyn, M., Yaar, M., Holbrook, N. and Gilchrest, B. A. (1991). "Immediate and Delayed Molecular Response of Human Keratinocytes to Solar-Simulated Irradiation." *Laboratory Investigation* **65**(4): 471 – 478.

Georgopolous, D. E. and Manowitz, P. (1982). "Arylsulfatase A Activity and Electrophoretic Banding Patterns from Isolated Human Blood Fractions." *Biochemical Genetics* **20**(9/10): 907 - 918.

Gerdts, E. Lund-Johansen, P. and Omvik, P. (1999). "Reproducibility of Salt Sensitivity Testing Using Dietary Approach in Essential Hypertension." *Journal of Human Hypertension* **13**: 375 - 384.

Gieselmann, V., Polten, A. P., Kreysing, J and von Figura, K. (1989). "Arylsulfatase A Pseudodeficiency: Loss of Polyadenylation Signal and N-Glycosylation Site." *Proc. Natl. Acad. Sci. USA* **86**: 9436 – 9440.

Gieslemann, V., Kreysing, J. and von Figura, K.(1994) "Genetics of Metachromatic Leukodystrophy." *Gene Therapy* **19**(2): S87.

Gill, J. R., Grossman, E. and Goldstein, D. S. (1991). "High Urinary Dopa and Low Urinary Dopamine-to-Dopa Ratio in Salt-sensitive Hypertension." *Hypertension* **18**(5): 614 - 621.

Goldstein, D. (1984). "Simultaneous Measurement of Dopa, Dopac, and Catecholamines in Plasma by Liquid Chromatography with Electrochemical Detection." *Clinical Chemistry* **30**(5): 815 - 816.

Goldstein, D. S., Mezey, E., Yamamoto, T., Anceman, A., Friberg, P. and Eisenhofer, G. (1995). "Is There a Third Peripheral Catecholaminergic System? Endogenous Dopamine as an Autocrine / Paracrine Substance Derived from Plasma Dopa and Activated by Conjugation." *Hypertension Research* **18** Suppl 1: S93 - S99.

Goldstein, D. S., Swoboda, K. J., Miles, J. M., Coppack, S. W., Aneman, A. Holmes, C., Lamensdorf, I. and Eisenhofer, G. (1999). "Sources and Physiological Significance of Plasma Dopamine Sulphate." *Journal of Clinical Endocrinology and Metabolism* **84**(7): 2523 - 2531.

Goldsworthy, S. M., Goldsworthy, T. L., Sprankle, C. S. and Butterworth, B. E. (1993). "Variation in Expression of Genes used for Normalisation of Northern Blots After Induction of Cell Proliferation." *Cell Prolif.* **26**: 511 – 517.

Goldsworthy, S. M., Stockton, P. S., Trempus, C. S., Foley, J. F. and Maronpot, R. R. (1999). "Effects of Fixation on RNA Extraction and Amplification from Laser Capture Microdissected Tissue." *Molecular Carcinogenesis* **25**: 86-91.

Gong, Y., Cui, L. and Minuk, G. Y. (1996). "Comparison of Glyceraldehyde-3-phosphate Dehydrogenase and 28S-Ribosomal RNA Gene Expression in Human Hepatocellular Carcinoma." *Hepatology* **23**: 734 – 737.

Graham, D. (1978) a. "Oxidative Pathways for Catecholamines in the Genesis of Neuromelanin and Cytotoxic Quinones." *Molecular Pharmacology* **14**: 633 - 643.

Graham, D. G., Tiffany, S. M., Bell, W. R., Gutnecht, W. F. (1978) b. "Autoxidation Versus Covalent Bonding of Quinones as the Mechanism of Toxicity of Dopamine, 6-Hydroxydopamine, and Related Compounds Toward C1300 Neuroblastoma Cells *in Vitro*." *Molecular Pathology* **14**: 644 - 653.

Graven, K. K. and Farber, H. W. (1998). "Endothelial Cell Hypoxic Stress Proteins." *J. Lab. Clin. Med.* **132**: 456 –463.

Green, B., Cooper, J. D. H., Turnell, D. C. (1989). "An Automated Method for the Analysis of Urinary Free Catecholamines using the ASTED and High Performance Liquid Chromatography." *Ann. Clin. Biochem* **26**: 361 - 367.

Guiot, Y. and Rahier, J. (1997). "Validation of Nonradioactive In Situ Hybridisation as a Quantitative Approach of Messenger RNA Variation." *Diagnostic Molecular Pathology* **6**(5): 261 - 266.

Hayashi, M., Yamaji, Y., Kitajima, W., Saruta, T. (1990). "Aromatic L-Amino Acid Decarboxylase Activity Along the Rat Nephron." *American Journal of Physiology* **258**(1 Pt 2): F28 - F33.

Hazell, A. S., Desjardins, P. and Butterworth, R. F. (1999). "Increased Expression of Glyceraldehyde-3-phosphate Dehydrogenase in Cultured Astrocytes Following Exposure to Manganese." *Neurochemistry International* **35** 11 – 17.

Henegariu, O., Heerema, N. A., Dlouhy, S. R., Vance, G. H. and Vogt, P. H. (1997). "Multiplex PCR: Critical Parameters and Step-by-step Protocol."*Biotechniques* **23**: 504 – 511.

Heppner, K. J., Matrisian, L. M., Jensen, R. A. and Rodgers, W. H. (1996). "Expression of Most Matrix Metalloproteinase Family Members in Breast Cancer Represents a Tumour-Induced Host Response." *American Journal of Pathology* **149**(1): 273 - 282.

Hoey, J. G., Garrett, S. H., Sens, M. A., Todd, J. H. and Sens, D. (1997). "Expression of MT-3 mRNA in Human Kidney, Proximal Tubule Cell Cultures, and Renal Cell Carcinoma." *Toxicology Letters* **92**: 149 - 160.

Hubbard, P. C. and Henderson, I. W. (1995). "Renal Dopamine and the Tubular Handling of Sodium." *Journal of Molecular Endocrinology* **14**: 139 - 155.

Hulyakar, A. R., Nora, R. and Manowitz, P. (1984). "Arylsulfatase A Variants in Patients with Alcoholism." *Clinical and Experimental Research* **8**(3): 337 - 341.

Ibarra, F., Aperia, A., Svensson, L. B., Elkof, A.C.and Greengard, P. (1993). "Bidirectional Regulation of Na^+/K^+-ATPase Activity by Dopamine and an α-adrenergic Agonist." *Proc. Natl. Acad. Sci (USA)* **90**: 21 - 24.

Iimura, O. (1996). "The role of Renal Dopaminergic Activity in the Pathophysiology of Essential Hypertension." *Japanese Heart Journal* **37**: 815 - 828.

Imai, K., Sugiura, M. and Tamura, Z. (1970). "The Presence of Conjugated Dopamine in Normal Human Plasma." *Chem Pharm. Bull* **18**(10): 2134.

Ito, Y., Pagano, P. J., Tornheim, K., Brecher, P. and Cohen, R. A. (1996). "Oxidative Stress Increases Glyceraldehyde-3-phosphate Dehydrogenase mRNA Levels in Isolated Rabbit Aorta." *American Journal of Physiology* **270**: H81 - H87.

Jellinger, K., Linert, L., Kienzl, E., Herlinger, E. and Youdim, M. B. H. (1995). "Chemical Evidence for 6-hydroxydopamine to be an Endogenous Toxic Factor in the Pathogenesis of Parkinson's Disease." *J. Neural. Transm.* **46**(Suppl): 297 – 314.

Kagedal, B. and Goldstein, D. S. (1988). "Catecholamines and their Metabolites."*Journal of Chromatography* **429**: 177 – 233.

Knaust, A., Schmidt, B., Dierks, T., von Bulow, R., von Figura, K. (1998). "Residues Critical for Formylglycine Formation and/or Catalytic Activity of Arylsulphatase A." *Biochemistry* **37**: 13941 - 13946.

Koller, M. (1988). "Results for 74 Substances Tested for Interference with Determination of Plasma Catecholamines by High Performance Liquid Chromatography with Electrochemical Detection." *Clinical Chemistry* **34**(5): 947 - 949.

Koopmans, M., Monroe, S. S, Coffield, L. M. and Zaki, S. (1993). "Optimisation of Extraction and PCR Amplification of RNA Extracts from Paraffin-embedded Tissue in Different Fixatives." *Journal of Virological Methods* **43**: 189 - 204.

Kostrzewa, R. M. and Jacobwitz, D. M. (1974). "Pharmocological Actions of 6-Hydroxydopamine" *Pharmacological Reviews* **26**(3): 199 – 279.

Krafft, A. E., Duncan, B. W., Bijwaard, K. E., Taubenberger, J. K. and Lichy, J. H (1997). "Optimization of the Isolation and Amplification of RNA from Formalin-Fixed, Paraffin-Embedded Tissue: The Armed Forces Institute of Pathology Experience and Literature Review." *Molecular Diagnosis* **2**(3): 217-230.

Kreysing J, von Figura, K., Gieslemann, V. (1990). "Structure of the Arylsulphatase A Gene." *Eur. J. Biochem.* **191**: 627 - 631.

Kuchel, O., Buu, N. T., Unger, T., Lis, M. and Genest, J. (1979). "Free and Conjugated Plasma and Urinary Dopamine in Human Hypertension." *Journal of Clinical Endocrinology and Metabolism* **48**(3): 425 - 429.

Kuchel, O., Buu, N. T., Hamet, P., Larochelle, P., Bourque, M. and Genest, J. (1984)a. "Catecholamine Sulfates and Platelet Phenolsulfotransferase Activity in Essential Hypertension." *J. Lab. Clin. Med* **104**(2): 238 - 244.

Kuchel, O., Buu. N. T., Roy, P., Hamet, P., Larochelle, P. and Genest, J. (1984)b. "Regional Sources of Free and Sulphoconjugated Catecholamines in Hypertension." *Hypertension* **6** (Suppl. 1): I51 - I55.

Kuchel, O. and Buu, N. T (1985). "Circadian Variations of Free and Sulphoconjugated Catecholamines in Normal Subjects." *Endocrine Research* **11**(1 - 2): 17 - 25.

Kuchel, O., Buu. N. T., Racz, K., de Lean, A., Serri. O. and Kyncl, J. (1986). "Role of Sulfate Conjugation of Catecholmaines in Blood Pressure Regulation." *Federation Proc.* **45**: 2254 - 2259.

Kuchel, O. and Shigetomi, S. (1992). "Defective Dopamine Generation From Dihydroxyphenylalanine in Stable Essential Hypertensive Patients." *Hypertension* **19**: 634 - 638.

Lemay, S., Mao, C. and Singh, A. K. (1996). "Cytokine Gene Expression in the MRL/*lpr* Model of Lupus Nephritis." *Kidney International* **50**: 85 – 93.

Leznicki, A. J. and Rozanska, M. (1991). "Localisation of Arylsulphatase A and B Activities in Rat Kidney." *Acta Biochimica Polonica* **38**(1): 151 – 156.

Linert, W., Herlinger, E., Jameson, R. F., Kienzl, E., Jellinger, K and Youdim, M. B. H. (1996). "Dopamine, 6-Hydroxydopamine, Iron, and Dioxygen – Their Mutual Interactions and Possible Implication in the Development of Parkinson's Disease." *Biochimica et Biophysica Acta* **1316**: 160 – 168.

Lodish, H., Berk, A., Zipursky, L. S., Matsudaira, P., Baltimore, D. and Darnell, J (2000). "Molecular Cell Biology." 4th Edn. W. H. Freeman, New York.

Loitsch, S. M., Kippenberger, S., Dauletbaev, N., Wagner, T. O. F.and Bargon, J. (1999). "Reverse-Transcription-Competitive Multiplex PCR Improves Quantification of mRNA in Clinical Samples - Application to the Low Abundance *CFTR* mRNA." *Clinical Chemistry* **45**(5): 619-624.

Lokhandwala, M. F., Kansra, V.and Chen, C. (1995). "Dopaminergic Modulation of Na^+/K^+-ATPase Activity in the Proximal Tubules of Normotensive and Hypertensive Rats." *Hypertension Research* **18**(Suppl 1): S43 - S46.

Leutjens, C. M., Lankiewicz, S., Bui, N. T., Krohn, A. J., Poppe, M. and Prehn, J. H. M. (2001). "Up-regulation of BCL-XL in Response to Subtoxic β-Amyloid: Role in Neuronal Resistance Against Apoptotic and Oxidative Injury" *Neuroscience* **102**(1): 139 – 150.

Luketala, G., Krauss, N., Theis, K., Selmer, T., Gieselmann, V., von Figura, K. and Saenger, W. (1998). "Crystal Structure of Human Arylsulfatase A: The Aldehyde Function and the Metal Ion at the Active Site Suggest a Novel Mechnism for Sulfate Ester Hydrolysis." *Biochemistry* **37**: 3654 – 3664.

Lundstrom, J., Ong, H. and Creveling, C. R. (1973). "Isomers of 2,4,5-Trihydroxyphenethylamine (6-Hydroxydopamine): Long Term Effects on the Accumulation of [^{3}H]-Norepinephrine in Mouse Heart *in Vivo*."*Molecular Pharmacology* **9**: 505 – 513.

Manowitz, P., Goldstein, L. and Bellomo, F. (1978). "An Improved Method for Arylsulphatase A Detection on Polyacrylamide Slab Gels." *Analytical Biochemistry* **89**: 423 - 429.

Manowitz, P., Goldstein, L. and Nora, R. (1981). "An Arylsulfatase A Variant in Schizophrenic Patients: Preliminary Report." *Biological Psychiatry* **16**(11): 1107 - 1113.

Martin, R. K., Archer, K. T. and Tuttle, M. R. (1994). "Detection of *ptc* in Archival Formalin-Fixed, Paraffin-Embedded Tissues." *Diagnostic Molecular Pathology* **3**(4): 233-239.

Masuda, N., Ohnishi, T., Kawamoto, S., Monden, M. and Okubo, K. (1999). "Analysis of Chemical Modification of RNA from Formalin-Fixed Samples and Optimisation of Molecular Biology Applications for Such Samples." *Nucleic Acids Research* **27**(22): 4436 – 4443.

Mattes, R. D. and Falkner, B. (1999). "Salt Sensitivity Classification in Normotensive Adults." *Clincal Science* **96**: 449 - 459.

Mayer, G. S. and Shoup, R. E. (1983). "Simultaneaous Multiple Electrode Liquid Chromatographic Electrochemical Assay for Catecholamines, Indolamines, and Metabolites in Brain Tissue." *Journal of Chromatography* **255**: 533 - 544.

McNulty, S. E. and Toscano, W. A. (1995). "Transcriptional Regulation of Glyceraldehyde-3-Phosphate Dehydrogenase by 2,3,7,8-Tetrachlorodibenzo-p-dioxin." *Biochemical and Biophysical Reseacrh Communications* **212**(1): 165 - 171.

McPherson, M. J and Moller, S. G. (2000). "PCR – The Basics." 1st Edn. BIOS Scientific Publishers Ltd, Oxford, UK.

Meister, B. Fried, G., Holgert, H., Aperia, A. and Hokfelt, T. (1992). "Ontogeny of Aromatic L-aromatic-amino Acid Decarboxylase-Containing Tubular Cells in Rat Kidney." *Kidney International* **42**: 617 - 623.

Meister, B. and Aperia, A. (1993). "Molecular Mechanisms Involved in Catecholamine Regulation of Sodium Transport." *Seminars in Nephrology* **13**(1): 41 - 49.

Miura, Y., Takahashi, M., Sano, N., Ohzeki, T., Meguro, Y., Sugawara, T., Noshiro, T., Watanabe, H., Shimizu, K., Abe, K.and Yoshinaga, K. (1989). "Plasma Free Dopamine in Human Hypertension." *Clin and Exper. - Theory and Practice* **A11**(Suppl 1): 227 - 236.

Miura, Y., Watanabe, T., Noshiro, T., Shimizu, K., Kusakari, T., Akama, H., Shibukawa, S., Miura, W., Ohzheki, T., Takahashi, M.and Sano, N. (1995). "Plasma Free Dopamine: Physiological Variability and Pathophysiological Significance." *Hypertension Research* **18**(Suppl 1): S65 - S72.

Mizayaki, M., Nikolic-Paterson, D. J., Endoh, M., Nomoto, Y., Sakai, H., Atkins, R. C.and Koji, T. (1994). "A Sensitive Method of Non-Radioactive In Situ Hybridisation for mRNA Localisation within Human Renal Biopsy Specimens: Use of Digoxigenin Labeled Oligonucleotides." *Internal Medicine* **33**(2): 87 - 91.

Molnar, I. and Horvath, C. (1976). "Reverse-phase Chromatography of Polar Biological Substances: Separation of Catechol Compounds by High Performance Liquid Chromatography." *Clinical Chemistry* **22**: 1497 - 1502.

Morris, R. C., Sebastian, A., Forman, A., Tanaka, M. and Schmidlin, O. (1999). "Normotensive Salt-Sensitivity: Effects of Race and Dietary Potassium." *Hypertension* **33**: 18 - 23.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G. and Erlich, H. (1986). "Specific Amplification of DNA *in Vitro*: The Polymerase Chain Reaction." *Cold Spring Harb. Symp. Quant. Biol.* **51**(1): 263 – 273.

Murabayashi, S., Baba, T., Tomiyana, T. and Takebe, K. (1989). "Urinary Dopamine, Noradrenaline, and Adrenaline in Type 2 Diabetic Patients With and Without Nephropathy." *Horm. Metab. Res* **21**: 27 - 32.

Nakai, A., Satoh, M., Hirayoshi, K. and Nagata, K. (1991). "Identification of the ATP-Binding Heat-Inducible Protein of Mr=37,000 as Glyceraldehyde-3-Phosphate Dehydrogenase." *Biochemical and Biophysical Research Communications* **176**(1): 59 - 64.

Nakajim-Iijima, S., Hamada, H., Reddy, P. and Kakunaga, T. (1985). "Molecular Structure of the Human Cytoplasmic β-Actin Gene: Interspecies Homology of the Sequences in the Introns." *Proc. Natl. Acad. Sci. USA* **82**: 6133 – 6137.

Nakaya, Y. Ktayama, T., Nomura, M., Ishimura, Y., Ohuchi, T., Oka, M., Yamamoto, M. and Mizobuchi S (1994). "Pathophysiologic Significance of Free and Conjugated Dopamine in Congestive Heart Failure." *American Heart Journal* **127**(3): 613 - 317.

Nishi, A., Celsi, G.and Aperia, A. "High Salt Diet Upregulates Activity and mRNA of Renal Na^+/K^+-ATPase in Dahl Salt-sensitive Rats." *American Journal of Physiology*.**264**(3 Pt 2): F448 – F452.

O'Driscoll, L., Kennedy, S., McDermott, E., Kelehan, P. and Clynes, M. (1996). "Multiple Drug-Resistance-Related Messenger RNA Expression in Archival Formalin-Fixed Paraffin-Embedded Human Breast Tumour Tissue." *European Journal of Cancer* **32A**(1): 128 - 133.

Oates, N. S., Ball, N., Perkins, C. M.and Lee, M. R. (1979). "Plasma and Urine Dopamine in Man Given Sodium Chloride in the Diet." *Clinical Science* **56**: 261 - 264.

Park, D. S., Pretz, R. D., Ricketts, M. H. and Manowitz, P. (1996). "Arylsulphatase A: Relationship of Genotype to Variant Electrophoretic Properties." *Biochemical Genetics* **34**(3/4): 149 – 161.

Pastoris, A., Cerutti, L., Sacco, R., De Vecchi, L. and Sbaffi, A. (1995). "Automated Analysis of Urinary Catecholamines by High Performance Liquid Chromatography and On-line Sample Pretreatment." *Journal of Chromatography* **664**: 287 – 293.

Patrick, A. W., Jeffrey, R. F., Collier, A., Clarke, B. F. and Lee, M. R. (1990). "Relationship Between Urinary Excretion of Sodium and Dopamine in Type 1 Diabetic Patients with and without Microalbuminuria." *Diabetic Medicine* **7**: 53 – 56.

Raff, T., van der Giet, M., Endemann, D., Wiederholt, T. and Paul, M. (1997). "Design and Testing of Beta Actin Primers for RT-PCR that Do Not Co-amplify Processed Pseudogenes." *Biotechniques* **23**: 456 - 460.

Re, R. N., Sancho, J., Kliman, B. and Haber, E. (1978). "The Characterisation of Low-renin Hypertension by Plasma Renin Activity and Plasma Aldosterone Concentration." *Journal of Clinical Endocrinology and Metabolism* **46**(2): 189 - 195.

Revillion, F., Pawlowski, V., Hornez, L. and Peyrat, J. P. (2000). "Glyceraldehyde-3-Phosphate Dehydrogenase Gene Expression in Human Breast Cancer." *European Journal of Cancer* **36**: 1038 - 1042.

Roche, E., Assimacopoulos-Jeannet, F., Witters, L. A., Perruchoud, B., Yaney, G., Corkey, B., Asfari, M. and Prentki, M. (1997), "Induction by Glucose of Genes Coding for Glycolytic Enzymes in a Pancreatic β-Cell Line (INS-1)." *The Journal of Biological Chemistry* **272**(5): 3091 – 3098.

Rodriguez-Soriano, J., Rivera, J. M., Vallo, A., Prats-Vinas, J. M. and Castillo G (1978). "Proximal Renal Tubular Acidosis in Metachromatic Leukodystrophy." *Helv Paediat. Acta* **33**: 45 - 52.

Routh, J. I., Bannow, R. E, Fincham, R. W., Stool, J. L. (1971). "Excretion of L-dopa and its Metabolites in Urine of Patients Receiving L-dopa Therapy." *Clinical Chemistry* **17**(19): 867 - 871.

Rudberg, S., Lenne, C., Persson, B., Krekula, A., de Faire, U.and Aperia, A. (1997). "The Dopaminuric Response to High Salt Diet in Insulin-Dependent Diabetes Mellitus in Family History of Hypertension." *Pediatric Nephrology* **11**(2): 169 - 173.

Rupp, G. M.and Locker, J. (1988). "Purification and Analysis of RNA from Paraffin-Embedded Tissues." *Biotechniques* **6**(1): 56-60.

Sakai, T., Maeda, H., Matsumoto, N., Miura, S., Kinoshita, A,. Sasaguri, M., Ideishi, M., Tanaka, H., Shindo, M. and Arakawa, K. (1995). "Plasma Free and Sulphoconjugated Dopamine Before and After a Half-Marathon." *Hypertension Research* **18**(Suppl 1): S161 - S163.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual." 2nd Edn. Cold Spring Harbour. Laboratory Press, New York.

Satoh, T., Ominato, M.and Katz, A. I. (1995). "Different Mechanisms of Renal Na^+/K^+-ATPase Regulation by Dopamine in the Proximal and Distal Nephron." *Hypertension Research* **18**(Suppl 1): S137 - S140.

Schoors, D. F., Velkeniers, B. and Dupont, A. G. (1990). "A Case of Hyperdopaminuria Due to Increased Renal Dopamine Production." *Nephron* **56**: 329 – 331.

Segers, O., Gerlo, E., Dupont, A. G. and Somers, G. (1995). "Decreased Urinary Dopamine Excretion and Disturbed Dopamine / Sodium Relationship in Type-1-Diabetes Mellitus." *Diabetic Medicine* **12**(3): 229 - 234.

Selvey, S., Thompson, E. W., Matthaci, K., Lea, R. A., Irving, M. G. and Griffiths, L. R. (2001). "Beta Actin - An Unsuitable Control for RT-PCR." *Molecular and Cellular Probes* **15**: 307 - 311.

Senard, J. M., Verwaerde, P., Rascol, O. and Montastruc, J. L. (1995). "Effects of Acute Levodopa Administration on Blood Pressure and Heart Variability in Never-Treated Parkinsonians." *Hypertension Research* **18**(Suppl 1): S175 - S177.

Sherwood, L. (1993). "Human Physiology from Cells to Systems." 2nd Edn. West Publishing Company, Minneapolis, USA.

Shibutani, M., Uneyama, C., Mizaki, K., Toyoda, K. and Hirose, M. (2000). "Methacarn Fixation: A Novel Tool for Analysis of Gene Expressions in Paraffin-Embedded Tissue Specimens." *Laboratory Investigation* **80**(2): 199-208.

Shigetomi, S., Yamada, Z., Ishii, H., Sanada, H., Watanbe, H. and Fukuchi, S. (1995). "Dopaminergic Activity and Endorenal Dopamine Synthesis in Non-insulin Dependent Diabetes Mellitus." *Hypertension Research* **18**(Suppl 1): S125 - S130.

Shimokawa, T., Kato, M., Shioduka, K., Irie, J.and Ezaki, O. (1997). "Effect of Triiodothyronine on Muscle Cell Differentiation and Blood Glucose Level in Hyperglycemic KK Mice." *Biochemical and Biophysical Research Communications* **235**: 790 - 793.

Shum, A., Van Loon, G. R. and Sole, M. J. (1982). "Measurement of L-dihydroxyphenylalanine in Plasma and Other Biological Fluids by High Pressure Liquid Chromatography with Electrochemical Detection." *Life Sciences* **31**: 1541 - 1545.

Smedes, F., Kraak, J. C.and Poppe, H. (1982). "Simple and Fast Solvent Extraction System for Selective and Quantitative Isolation of Adrenaline, Noradrenaline, and Dopamine from Plasma and Urine." *Journal of Chromatography* **231**: 25 - 39.

Smit, A. J., Lieverse, A. G., van Veldhuisen, D. J. and Girbes. A. R. J. (1995). "Dopaminergic Modulation of Physiological and Pathological Neurohumoral Activation in Man." *Hypertension Research* **18**(Suppl 1): S107 - S111.

Sorg, I. and Metzler, A. (1995). "Detection of Borna Disease Virus in Formalin-Fixed, Paraffin-Embedded Brain Tissues by Nested PCR." *Journal of Clinical Microbiology* **33**(4): 821-823.

Sowers, J. R., Zemel, M. B, Zemel, P., Beck, F. W. J., Walsh, M. F. and Zawada, E. T. (1988). "Salt-Sensitivity in Blacks - Salt Intake and Natriuretic Substances." *Hypertension* **12**(5): 485 - 490.

Specht, K., Richter, T., Muller, U., Walch, A., Werner, M. and Hofler, H. (2000). "Quantitative Gene Expression Analysis in Microdissected Archival Tissue by Real-time RT-PCR." *Journal of Molecular Medicine* **78**: B27

Specht, K., Richter, T., Muller, U., Walch, A., Werner, M. and Hofler, H. (2001). "Quantitative Gene Expression Analysis in Microdissected Archival Formalin-Fixed and Paraffin-Embedded Tumor Tissue." *American Journal of Pathology* **158**(2): 419 - 429.

Spencer, W. E. and Christensen, M. (1999). "Multiplex Relative RT-PCR Method for Verification of Differential Gene Expression." *Biotechniques* **27**: 1044-1052.

Stanta, G. and Schneider, C. (1991). "RNA Extracted from Paraffin-embedded Human Tissues is Amenable to Analysis by PCR Amplification." *Biotechniques* **11**(3): 304 - 308.

Stanta, G., Bonin, S. and Perin R. (1998). "Methods in Molecular Biology." Humana Press Totawa, NJ, USA.

Stein, C., Gieselmann, V., Kreysing, J., Schmidt, B., Pohlman, R., Waheed, A., Meyer, H. E., O'Brien, J. S. and von-Figura, K. (1989). "Cloning and Expression of Human Arylsulfatase A." *Journal of Biological Chemistry* **164**(2): 1252 - 1259.

Stenvinkel, P., Saggar-Malik, A. K, Warenberg, H., Dicfalusy, U., Bolinder, J. and Alvestrand A (1991). "Impaired Intrarenal Dopamine Production Following Intravenous Sodium Chloride Infusion in Type-1-(Insulin Dependent) Diabetes Mellitus." *Diabetologia* **34**: 114 - 118.

Stinsoff, K. (1972). "Kinetic Properties of the Arylsulphatase A from Human Kidneys." *Biochimica et Biophysica Acta* **276**: 475 – 490

Strobel, G., Werle, E. and Weiker, H. (1990). "Isomer Specific Kinetics of Dopamine β-Hydroxylase and Arylsulfatase Towards Catecholamine Sulfates." *Biochemistry International* **20**(2): 343 – 351.

Sturzenbaum, S. R. and Kille, P. (2001). "Control Genes in Quantitative Molecular Biological Techniques: The Variability of Invariance." *Comparative Biochemistry and Physiology Part B* **130**: 281 - 289.

Suzuki, T., Higgins, P. J. and Crawford, D. R. (2000). "Control Selection for mRNA Quantitation." *Biotechniques* **29**: 332 - 337.

Svetkey, L. P., McKeown, S. P., Wilson, A. F. (1996). "Heritability of Salt-Sensitivity in Black Americans." *Hypertension* **28**: 854 - 858.

Svoboda-Newman, S. M., Greenson, J. K., Singleton, T. P., Sun, R. and Frank, T. S. (1997). "Detection of Hepatitis C by RT-PCR on Formalin-Fixed Paraffin-Embedded Tissue from Liver Transplant Patients." *Diagnostic Molecular Pathology* **6**(2): 123-129.

Takemoto, F., Satoh, T., Cohen, H. T. and Katz, I. (1991). "Localization of Dopamine-1 Receptors along the Microdissected Rat Nephron." *Plugers Arch* **419**(3 – 4) 243 – 248.

Taubenberger, J. K., Reid, A. H. and Fanning, T. G. (2000). "The 1918 Influenza Virus: A Killer Comes Into View." *Virology* **274**: 241-245.

Tong, J. and Baines, A. D. (1993). "In Patients Receiving Infusions for Treatment of Shock do Free Radicals Convert Dopamine to 6-hydroxydopamine?" *Clinical Biochem* **26**: 199 - 205.

Tso, J. Y., Sun, X. H., Kao, T. H., Reece, K. S. and Wu, R. (1985). "Isolation and Characterisation of Rat and Human Glyceraldehyde-3-Phosphate Dehydrogenase cDNAs: Genomic Complexity and Molecular Evolution of the Gene." *Nucleic Acids Research* **13**(7): 2485 – 2502.

Turbett, G. R., Barnett, T. C., Dillon, E. K.and Sellner, L. N. (1995). "Single-Tube Protocol for the Extraction of DNA or RNA from Paraffin-Embedded Tissues Using a Starch-Based Adhesive." *Biotechniques* **20**: 846-853.

Tyrrell, L., Elias, J. and Longley, J. (1995). "Detection of Specific mRNAs in Routinely Processed Dermatopathology Specimens." *The American Journal of Dermatopathology* **17**(5): 476-483.

Waldow, A., Scmidt, B., Dierks, T., von Bulow, R. and von Figura, K. (1999). "Amino Acid Residues Forming the Active Site of Arylsulfatase A."*The Journal of Biological Chemistry* **274**(18): 12284 – 12288.

Weiker, H. (1988). "Determination of Free and Sulfoconjugated Catecholamines in Plasma and Urine by High Performance Liquid Chromatography." *Int. J. Sports. Med.* **9**: 68 – 74.

Weizsacker, F. V., Labeit, S., Koch, H. K., Oehlert, W., Gerok, W. and Blum, H. E. (1991). "A Simple and Rapid Method for the Detection of RNA in Formalin-Fixed, Paraffin-Embedded Tissues by PCR Amplification." *Biochemical and Biophysical Research Communications* **174**(1): 176-180.

Wickham, C. L., Boyce, M., Joyner, M. V., Sarsfield, P., Wilkins, B. S., Jones, D. B. and Ellard, S. (2000). "Amplification of PCR Products in Excess of 600 Base Pairs Using DNA Extracted From Decalcified, Paraffin Wax Embedded Bone Marrow Trephine Biopsies." *Journal of Clinical Pathology: Molecular Pathology* **53**: 19 - 23.

Williams, M., Young, J. B., Rosa, R. M., Gunn, S., Epstein, F. H.and Landsberg, L. (1986). "Effect of Protein Ingestion on Urinary Dopamine Excretion." *J. Clin. Invest* **78**: 1687 - 1693.

Williams, C. M. and Coleman, J. W. (1995). "Induced Expression of mRNA for IL-5, IL-6, TNF-α, MIP-2 and IFN –γ in Immunologically Activated Rat Peritoneal Mast Cells: Inhibition by Dexamethsone and Cyclosporin A." *Immunology* **86**: 244 – 249.

Witsell, A. L. and Shook, L. B. (1990). "Clonal Analysis of Gene Expression by PCR." *Biotechniques* **9**(3): 318 - 322.

Wolfovitz, E., Grossman, E., Folio, C. J., Keiser, H. R., Kopin, I. J. and Goldstein, D. S. (1993). "Derivation of Urinary Dopamine from Plasma Dihydroxyphenylalanine in Humans." *Clinical Science* **84**: 549 - 557.

Wu, A. H. B.and Gornet, T. (1985). "Preparations of Urine Samples for Liquid Chromatographic Determination of Catecholamines: Bonded-Phase Phenylboronic Acid, Cation-Exchange Resin, and Alumina Adsorbents Compared." *Clinical Chemistry* **31**(2): 298 - 302.

Yamada, H., Chen, D., Monstein, H. J. and Hakanson, R. (1997). "Effects of Fasting on the Expression of Gastrin, Cholecystokinin, and Somatostatin Genes in the Pancreas and Upper Digestive Tract." *Biochemical and Biophysical Research Communications* **231**: 835 - 838.

Yan, S. D., Schmidt, A. M., Anderson, G. M., Zhang, J., Brett, J.,Zou, Y. S., Pinsky, D. and Stern, D. (1994). "Enhanced Cellular Oxidant Stress by the Interaction of Advanced Glycation End Products with Their Receptors / Binding Proteins." *The Journal of Biological Chemistry* **269**(13): 9889 – 9897.

Yan, Q. Briehl, M., Crowley, C. L., Payne, C. M., Bernstein, H. and Bernstein, C. (1999). "The NAD^+ Precursors, Nicotinic Acid and Nicotinamide Upregulate Glyceraldehyde-3-Phosphate Dehydrogenase and Glucose-6-Phosphate Dehydrogenase mRNA in Jurkat Cells." *Biochemical and Biophysical Research Communications* **255**: 133 - 136.

Yoneda, S., Alexander, N. and Vlachakis, N. D. (1983). "Enzymatic Deconjugation of Catecholamines in Human and Rat Plasma and Red Blood Cell Lysate." *Life Sciences* **33**: 935 – 942.

Yoshimura, M., Komori, T., Nakanishi, T.and Takahashi, H. (1993). "Estimation of Sulphoconjugated Catecholamine Concentrations in Plasma by High Performance Liquid Chromatography." *Ann Clin Biochem* **30**: 135 - 141.

Yoshizumi, M. (1992)a. "Deconjugating Activity for Sulphoconjugated Dopamine in Homogenates of Organs from Dogs." *Biochemical Pharmacology* **44**(11): 2263-2265.

Yoshizumi, M., Nakaya, Y., Hibino, T., Nomura, M., Minakuchi, K., Kitagawa, T., Katoh, I., Ohuchi, T. and Oka M. (1992)b. "Changes in Plasma Free and Sulfoconjugated Catecholamines Before and After Acute Physical Exrecise: Experimental and Clinical Studies." *Life Sciences* **51**: 227 – 234.

Yoshizumi, M., Ishimura, Y., Masuda, Y., Ohuchi, T., Katoh, I. and Oka, M. (1995). "Physiological Significance of Plasma Sulfoconjugated Dopamine: Experimental and Clinical Studies." *Hypertension Research* **18**(Suppl 1): S101 - S106.

Yoshizumi, M., Kitagawa, T., Hori, T., Katoh, H., Ohuchi, T. and Oka, M. (1996). "Physiological Significance of Plasma Sulfoconjugated Dopamine in Patients with Hypertension." **59**(4): 323 - 330.

Zar, J. H. (1996). "Biostatistical Analysis." 3rd Edn. Prentice Hall, Inc, USA.

Zhong, H. and Simons, J. W. (1999). "Direct Comparison of GAPDH, β-Actin, Cyclophilin, and 28S rRNA as Internal Standards for Quantifying RNA Levels under Hypoxia." *Biochemical and Biophysical Research Communications* **259**: 523 – 526.

Zimlichman, R., Levinson, P. D., Kelly, G., Stull, R., Keiser, H. R. and Goldstein, D. (1988). "Derivation of Urinary Dopamine from Plasma Dopa." *Clinical Science* **75**: 515 - 520.