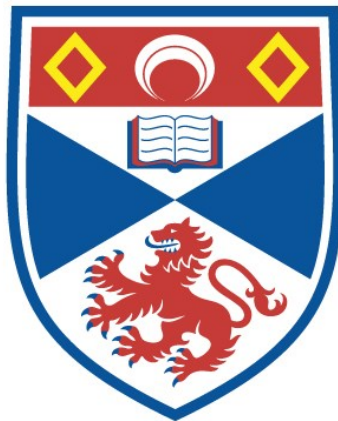


THE EXPRESSION OF NA, K-ATPASE IN THE MADIN-  
DARBY CANINE KIDNEY (MDCK) CELL LINE

Christopher Paul Cutler

A Thesis Submitted for the Degree of PhD  
at the  
University of St Andrews



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THE EXPRESSION OF Na,K-ATPase IN THE MADIN-DARBY CANINE KIDNEY (MDCK) CELL LINE.

A thesis submitted to the University  
of St. Andrews for the degree of  
Doctor of Philosophy.

by

CHRISTOPHER PAUL CUTLER.

Department of Biology and Preclinical Medicine,  
University of St. Andrews.

August 1990





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You see things, and say why?  
But I dream things that never were,  
and I say why not?

George Bernard Shaw

(cited by Ernst-L. Winnaker, 1987  
in "From Genes to Clones").

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ACADEMIC RECORD.

I graduated from the University of Birmingham in July 1985, with a B.Sc. (Hons.) in Biology (Genetics), 2<sup>nd</sup> class, 1<sup>st</sup> division.

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## ABSTRACT.

The efficiency of a number of experimental techniques for the extraction of total RNA from various cells and tissues (including MDCK strain I cells) was assessed, and the optimal conditions for hybridisation of Na,K-ATPase isoform-specific DNA probes to this RNA were determined.

The specificity of hybridisation of DNA probes for the Na,K-ATPase  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ , and  $\beta 1$  isoforms was assessed using RNA isolated from rat tissues. The relative abundance of isoform mRNA's in rat kidney, brain, lung, and myocardial tissues was determined by Northern blotting. The abundance of Na,K-ATPase isoforms was also determined in the myocardial tissues of the Milan rat, a hypertensive animal model. Significant differences between the abundance of Na,K-ATPase isoform mRNA's in hypertensive rats and their age and sex matched controls were found. The relative abundance (per  $\mu\text{g}$  of total RNA) of  $\alpha 1$ ,  $\alpha 3$ , and  $\beta 1$  mRNA's in left ventricle and, that of  $\alpha 1$ , and  $\beta 1$  mRNA's in right ventricle were significantly decreased in hypertensive rats. The relative abundance (per  $\mu\text{g}$  of total RNA) of  $\alpha 2$  and  $\beta 1$  mRNA's in atria was significantly increased in hypertensive rats. These differences found in ventricles and atria were further accentuated by expression of the results per gram wet weight of tissue. The results from ventricular tissues were in contrast to those previously reported by Herrera *et al.* (1988) who found either increases or no change in the abundance of  $\alpha 1$  and  $\beta 1$  mRNA's in hypertensive rat aorta, skeletal muscle and left ventricle. The differences between these results may be related to the deoxy-corticosterone treatment and high salt diet of the hypertensive rat model used by Herrera *et al.* (1988).

Na,K-ATPase isoform-specific DNA restriction endonuclease fragments were used to investigate the expression of the isoform mRNA's in MDCK strain I cells. Only  $\alpha 1$  and  $\beta 1$  mRNA's was detected on Northern blots, with no detectable  $\alpha 2$  or  $\alpha 3$  isoform mRNA signals being found in this cell line.

[ $^3\text{H}$ ]-ouabain binding to cells was used, as an estimate of the cell surface expression of Na,K-ATPase. Possible factors affecting the expression of Na,K-ATPase during the normal cell growth of MDCK strain I cells were investigated. Factors such as cell seeding density, cell growth substrate and the volume of growth medium used, were all found to affect both the level and pattern of expression of Na,K-ATPase during the normal cell growth or culturing cycle. After 2 days of culture the large increases in the expression of Na,K-ATPase assayed in low density compared to high density seeded cells, were not

correlated with concomitant changes in the relative abundance of Na,K-ATPase  $\alpha$  subunit mRNA. These results indicate that the large changes in cell surface expression of Na,K-ATPase found during cell growth are probably controlled by post transcriptional processes.

The effect of certain hormones or their agonists (aldosterone, deoxy-corticosterone, corticosterone, dexamethasone, and tri-iodo thyronine), on the expression of Na,K-ATPase in MDCK strain I cells was also briefly investigated. Under the conditions used, hormone treatment was not found to induce any measurable expression of  $\alpha 2$  or  $\alpha 3$  mRNA's. The mineralocorticoid aldosterone, and the glucocorticoid corticosterone, both produced small but significant increases in the level of Na,K-ATPase present on the cell membrane, however these increases were not correlated with similar increases in the abundance of both Na,K-ATPase  $\alpha 1$  and  $\beta 1$  mRNA's. The small size of increases in Na,K-ATPase enzyme abundance after hormone treatments and the inability of those treatments to induce consistent increases in Na,K-ATPase mRNA's further suggests that changes in the cell surface expression of Na,K-ATPase in MDCK cells is the result of regulation at a post transcriptional level.



## ABBREVIATIONS.

Abbreviations of measurements in the text follow the S.I. system of nomenclature.

AII	Angiotensin II.
aa	Amino acid.
ADP	Adenosine di-phosphate.
Al	Aldosterone.
AMOG	Adhesion molecule on glia.
ANP	Atrial natriuretic peptide.
ARG	Arginine.
ASP	Apartic acid.
AT	Atria.
ATP	Adenosine tri-phosphate.
BHK	Baby hamster kidney.
BSA	Bovine serum albumin.
C1R-ATP	[ <sup>14</sup> C]-ATP- $\gamma$ -[4-(N-2-chloroethyl-N-methylamino)] benzylamid.
cDNA	Complementary DNA.
Co	Corticosterone.
Con	Control.
CPM	Counts per minute.
De	Dexamethasone.
DEP	Diethyl pyrocarbonate.
DMSO	Dimethyl sulphoxide.
DNA	Deoxy-ribonucleic acid.
Do or DOC	Deoxy-corticosterone.
DPM	Disintegrations per minute.
E.coli	Escherichia coli.
EBSS	Earle's balanced salt solution.
EDTA	Ethylenediamine tetra-acetic acid.
EGTA	1,2-Di(2-aminoethoxy)ethane-NNN'N'-tetra-acetic acid.
FCS	Foetal calf serum.
FITC	Fluorescein isothiocyanate.
FSBA	[ <sup>13</sup> C]-5-(p-fluorosulphonyl)-benzoyl-adenosine.

G+C	Guanine + cytosine.
GPI anchor	Glycoso phosphoinositol anchor.
Hp	17- $\alpha$ hydroxy progesterone.
K <sub>0.5</sub>	Concentration of half maximal binding.
Kb	Kilo base.
Kd	Dissociation constant.
kDa	Kilo daltons.
Ki	Inhibitory constant.
LEU	Leucine.
LV	Left ventricle.
MDCK	Madin Darby canine kidney.
MEM	Minimum essential medium.
MEME	Minimum essential medium eagle's.
MHS	Milan hypertensive strain.
MNS	Milan normotensive strain.
MOPS	3-(N-Morpholino)propanesulphonic acid.
mRNA	Messenger RNA.
MW	Molecular weight.
NBCS	New born calf serum.
nt	Nucleotide.
OD	Optical density.
PBS	Phosphate buffered saline.
PKC	Protein kinase C.
psi	Pounds per square inch.
RNA	Ribonucleic acid.
rpm	Revolutions per minute.
rRNA	Ribosomal RNA.
RV	Right ventricle.
SDS	Sodium dodecyl sulphate.
SEM	Standard error of the mean.
Sp	Spirolactone.
SS DNA	Salmon sperm DNA.
SSC	Standard saline citrate.
T0	Time 0.

T3	Tri-iodo thyronine.
T4	Thyroxine.
TBE	Tris-borate-EDTA-containing buffer.
TE	Tris-EDTA-containing buffer.
TGN	Trans-golgi network.
T <sub>hyb</sub>	Melting temperature under the conditions of hybridisation.
T <sub>m</sub>	Melting temperature.
TNESDS	Tris-NaCl-EDTA-SDS containing buffer.
tRNA	Transfer RNA.
T <sub>w</sub>	Melting temperature under the conditions of washing.
UV	Ultraviolet.
[ ] <sub>i</sub>	Intracellular concentration.
[ ] <sub>o</sub>	Extracellular concentration.

CHAPTER 1. GENERAL INTRODUCTION.

This general introduction, represents a wide ranging review of the structure, function, molecular biology, and regulation of the Na,K-ATPase enzyme. The text then goes on to discuss the currently available information on the regulation of Na,K-ATPase in Madin-Darby canine kidney (MDCK) cells, an epithelial cell line which shows variations in the expression of this protein during culture.

### **1.1. A historical perspective of Na,K-ATPase.**

The earliest discovery of information now known to be associated with Na,K-ATPase was in 1785 when Sir William Withering introduced an extract of the plant called "foxglove" (*Digitalis purpurea*) for the treatment of heart failure (Allen *et al.*, 1985; Torok, 1989). This extract contained the cardiac glycoside digitalis, which is a potent inhibitor of Na,K-ATPase. Early evidence for the existence of Na,K-ATPase started in the 1920's and 1930's with attempts to disprove apparent cases of active transport. At this time ion movements were thought to be passive and mediated by cellular colloids with a high affinity for K ions (Ussing 1988). In 1941, Dean referring to experiments done by others, concluded that "muscle can actively move potassium and sodium against concentration gradients". Further work done by Ussing and others conclusively demonstrated the existence of active sodium transport (Ussing, 1949). In 1953, the active transport of both Na and K was demonstrated to be inhibitable by cardiac glycosides (Schatzmann, 1953). Steinbach in 1954, demonstrated that in muscle tissue, not only were Na and K ions actively pumped in opposite directions across the cell membrane, but that these processes were linked (Steinbach, 1954). The discovery of the Na,K-ATPase enzyme has been credited to Skou who, while investigating the membranes of crab nerve cells, isolated a protein capable of hydrolysing ATP which required Mg, Na and K (Skou, 1957). Skou was the first person to propose the existence of a Mg-dependent Na,K-ATPase enzyme. Soon afterwards it was demonstrated that ouabain (a cardiac glycoside) specifically inhibited this enzyme (Post *et al.*, 1960), and that both ouabain and another cardiac glycoside digoxin (through the introduction of radiolabelled glycosides) bound directly to Na,K-ATPase (Albers *et al.*, 1968).

## 1.II. The function of Na,K-ATPase.

Na,K-ATPase (or the sodium pump) is a ubiquitous integral protein of the plasma membrane of all animal cells so far examined (Sweadner, 1989). As a consequence, the structure, function and regulation of the enzyme has been greatly studied. The primary function of the enzyme is to translocate Na and K ions across the impermeable plasma membrane of cells. The action of the enzyme maintains an intracellular environment of high K concentration (~140 mM) and low Na concentration (~5-15 mM) relative to the extracellular environment of low K concentration (~5 mM) and high Na concentration (~145 mM) (Alberts *et al.*, 1989). The enzyme acts in a cyclical fashion and during each cycle translocates 3 Na ions to the exterior of the cell for every 2 K ions to the interior of the cell (see figure 1.) (DeWeer, 1986). In common with other ion translocating ATPases this process requires energy, as ions are transported across the membrane against their concentration gradients. This energy is provided by the hydrolysis of one ATP molecule to ADP and inorganic phosphate for each cycle of the enzyme's action. The process of ion translocation, known as active transport is also electrogenic as a net amount of positively charged ions are transported out of the cell. The net transfer of charge (ions) out of the cell provides a small (~10%) component of the resting membrane potential. The other portion of the membrane potential (~90%) is provided indirectly by Na,K-ATPase due to the creation and maintenance of concentration gradients for Na and K ions. The resulting membrane potential is provided by the higher permeability of the membrane to K ions compared to Na ions. Potassium ions leave the cell through specific channels in the membrane down their concentration gradient. The process of positive ion loss is eventually balanced by the attraction of the charge differentially generated across the membrane (the membrane potential; Alberts *et al.*, 1989). Because of the essential role of Na,K-ATPase in the homeostasis of Na and K ions in the cell the enzyme represents a major metabolic load and is one of the major consumers of energy in the cell. In most cells, more than one third of all the ATP produced, is consumed by Na,K-ATPase in the maintenance of ion gradients. In electrically active nerve cells which constantly lose a significant portion of their Na and K ion gradients, Na,K-ATPase uses nearly two thirds of all the ATP produced. (Alberts *et al.*, 1989).

By virtue of Na,K-ATPase's control over the major ionic species of the cell (Na and K), the enzyme also provides a major contribution to the control of cell water content and consequently, cell osmolarity and volume. This regulation is achieved by the

## 1.11. The function of Na,K-ATPase.

Na,K-ATPase (or the sodium pump) is a ubiquitous integral protein of the plasma membrane of all animal cells so far examined (Sweadner, 1989). As a consequence, the structure, function and regulation of the enzyme has been greatly studied. The primary function of the enzyme is to translocate Na and K ions across the impermeable plasma membrane of cells. The action of the enzyme maintains an intracellular environment of high K concentration (~140 mM) and low Na concentration (~5-15 mM) relative to the extracellular environment of low K concentration (~5 mM) and high Na concentration (~145 mM) (Alberts *et al.*, 1989). The enzyme acts in a cyclical fashion and during each cycle translocates 3 Na ions to the exterior of the cell for every 2 K ions to the interior of the cell (see figure 1.) (DeWeer, 1986). In common with other ion translocating ATPases this process requires energy, as ions are transported across the membrane against their concentration gradients. This energy is provided by the hydrolysis of one ATP molecule to ADP and inorganic phosphate for each cycle of the enzyme's action. The process of ion translocation, known as active transport is also electrogenic as a net amount of positively charged ions are transported out of the cell. The net transfer of charge (ions) out of the cell provides a small (~10%) component of the resting membrane potential. The other portion of the membrane potential (~90%) is provided indirectly by Na,K-ATPase due to the creation and maintenance of concentration gradients for Na and K ions. The resulting membrane potential is provided by the higher permeability of the membrane to K ions compared to Na ions. Potassium ions leave the cell through specific channels in the membrane down their concentration gradient. The process of positive ion loss is eventually balanced by the attraction of the charge differentially generated across the membrane (the membrane potential; Alberts *et al.*, 1989). Because of the essential role of Na,K-ATPase in the homoeostasis of Na and K ions in the cell the enzyme represents a major metabolic load and is one of the major consumers of energy in the cell. In most cells, more than one third of all the ATP produced, is consumed by Na,K-ATPase in the maintenance of ion gradients. In electrically active nerve cells which constantly lose a significant portion of their Na and K ion gradients, Na,K-ATPase uses nearly two thirds of all the ATP produced. (Alberts *et al.*, 1989).

By virtue of Na,K-ATPase's control over the major ionic species of the cell (Na and K), the enzyme also provides a major contribution to the control of cell water content and consequently, cell osmolarity and volume. This regulation is achieved by the



translocation of Na ions to the exterior of the cell, which decreases the intracellular inorganic ion concentration, and counteracts the relatively high intracellular organic ion concentration. Other cellular functions in which the ion gradients generated by Na,K-ATPase are involved include control of the:- 1; intracellular pH (via Na-H exchange; Rindler and Saier, 1981), 2; free intracellular Ca concentration (in excitable cells via Na-Ca exchange; Philipson and Nishimoto, 1980), 3; uptake of sugars (Hilden and Sacktor, 1979), 4; uptake of certain amino acids (Hammermann and Sacktor, 1977), 5; uptake of inorganic phosphate (Cheng and Sacktor, 1981), and 6; uptake of iodide (in the thyroid gland; Vilijn and Carrasco, 1989).

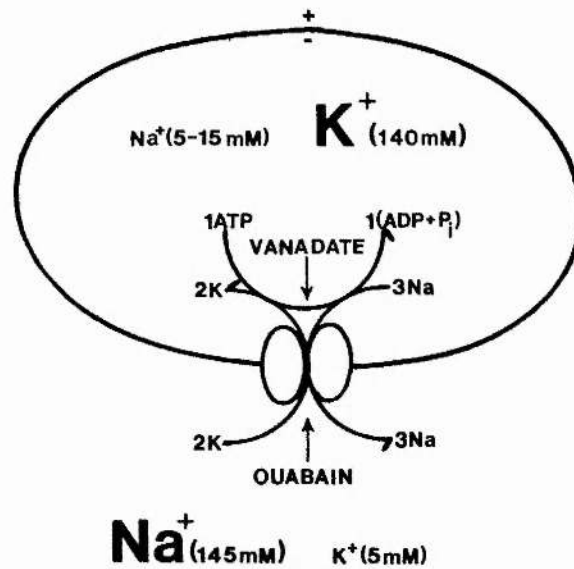


Figure 1. A schematic diagram of Na,K-ATPase and the transport and distribution of Na and K in a normal animal cell. Adapted from Norgaard, 1986.

The membrane potential generated indirectly by Na,K-ATPase enables nerve cells to perform their primary function of electrical conduction and synaptic transmission (excitation-secretion coupling). It also enables excitation-contraction coupling in muscle myofibrils. Other physiological processes in which Na,K-ATPase is involved include the: 1; fluid absorption in the gastrointestinal tract, 2; secretion of cerebrospinal fluid by the choroid plexus membrane, 3; formation and secretion of tears, 4; formation of inner ear endolymph, 5; aqueous humour formation and 6; production of urine (Lechne, 1988; Lindvall-Axelsson, 1989; Lingrel *et al.*, 1990; and Simon *et al.*, 1962).



The importance of Na,K-ATPase is paramount as correct functioning of the enzyme is required by all cells to maintain their viability. Without the maintenance of ion gradients cells cannot import the nutrients they need to survive. Abnormalities in the regulation of the activity of the Na,K-ATPase enzyme have been associated with a number of disease states including certain types of; hypertension (Hauptert, 1988), manic depressive psychosis (Naylor, 1986), diabetic neuropathy (Lattimer *et al.*, 1989), obesity and certain manifestations of uremic disorder (Katz, 1982).

### 1.III. Structure of the Na,K-ATPase enzyme.

The Na,K-ATPase enzyme was first purified to greater than 90% homogeneity from the outer medulla of pig and rabbit kidneys (Jorgenson, 1975). The enzyme is known to consist of 2 subunits,  $\alpha$  and  $\beta$ . A third proteolipid  $\gamma$  subunit was thought to exist for some time (Reeves *et al.*, 1980), but its existence has been disputed (Harris and Stahl, 1988). It has been reported that the  $\gamma$  subunit may represent a degradation product of the enzyme (Harris and Stahl, 1988) and it is not required for the normal functioning of Na,K-ATPase (Jorgenson, 1982; Sweadner, 1989). The structure of the active enzyme has been controversial for some time. During the purification and crystallisation of the enzyme, dimers of a minimal  $\alpha\beta$  monomeric form are produced (Maunsbach, 1988). However, it has since been determined that the minimal functioning unit of Na,K-ATPase is the  $\alpha\beta$  monomer (Jorgenson, 1988; Hayashi *et al.*, 1983; Hayashi *et al.*, 1989). It is not known whether the dimeric forms of Na,K-ATPase exist *in vivo*.

The Na,K-ATPase enzyme consists of two polypeptide subunits designated  $\alpha$  and  $\beta$ . The  $\alpha$  subunit is a protein containing approximately 1020 amino acids (aa's; depending on species) with a molecular weight (MW) of 112 kilo Daltons (kDa). It is known as the catalytic subunit and all known functions of the enzyme have been ascribed to it. The  $\beta$  subunit is a protein of approximately 300 aa's (depending on species) with a MW of approximately 35 kDa (Sweadner, 1989). The  $\beta$  subunit shows a larger apparent MW upon electrophoresis, due to the fact that it is glycosylated at up to three positions (Tankum and Fambrough, 1986). The glycosylated form of the  $\beta$  subunit has a MW of 40-60 kDa (Rossier *et al.*, 1987), and has no known involvement in the functional aspects of the enzyme's action. However, its association with the enzyme is essential as, it has never been separated from the  $\alpha$  subunit without irreversible loss of function (Sweadner, 1989).

### 1.III.i. The isoforms of Na,K-ATPase.

In several species so far examined in detail, isoforms of both the  $\alpha$  and  $\beta$  subunits have been found. The  $\alpha$  subunit has at least three isoforms, these have now been designated  $\alpha 1$ ,  $\alpha 2$  and  $\alpha 3$  (or  $\alpha$ ,  $\alpha+$  and  $\alpha III$  respectively) (Swadner, 1989). Initially, some confusion in the literature existed over the nomenclature. Different names for the isoforms were derived simultaneously, from work carried out at the protein and DNA levels by various groups. Emanuel *et al.*, (1987), Herrera *et al.*, (1987), and Kent *et al.*, (1987) have assigned the  $\alpha 2$  and  $\alpha 3$  isoform designations in reverse. Initially only one isoform of the  $\beta$  subunit was thought to exist (called  $\beta 1$  in this introduction), although recently a second isoform has been reported (called  $\beta 2$  in this introduction) (Martin-Vasallo *et al.*, 1989). The second protein has subsequently been shown to be identical to the adhesion molecule on glia (AMOG). It has therefore been suggested that one function of the  $\beta$  subunit might be as a recognition element for cell-cell adhesion (Gloor *et al.*, 1990). The presence of the  $\beta 2$  isoform was probably not detected in early nucleic acid hybridisation experiments because its DNA sequence is considerably diverged from that of the  $\beta 1$  isoform (Gick *et al.*, 1988a). Despite this divergence, many of the resultant aa changes are favoured substitutions (aa changes resulting in a chemically similar type of aa as replacement). Thus the  $\beta 2$  isoform aa sequence has a hydrophathy profile and probable structural organisation, similar to the  $\beta 1$  isoform (Martin-Vasallo *et al.*, 1989). More recently, another genomic sequence coding for the  $\beta$  subunit of Na,K-ATPase has been found, the sequence being most similar to that of the  $\beta 1$  isoform. This  $\beta 1$  isoform-like gene is however, thought to represent a pseudogene, as its sequence contains a number of mutations which would result in aa's of the coding sequence being replaced by stop codons. This would result in the premature termination of translation of the polypeptide product (Georgiou *et al.*, 1989; Ushkaryov *et al.*, 1989).

### 1.III.ii. A model for the action of Na,K-ATPase.

As stated previously the Na,K-ATPase enzyme translocates ions in a cyclical fashion. The original model for the action of this enzyme was that proposed by Post and Albers (Albers, 1967; Post *et al.*, 1969), this model has since been modified (Jorgensen, 1986; see figure 2.) and has 2 major conformational states known as  $E_1$  and  $E_2$ , in which ion binding sites are either exposed to the intra- or extra-cellular environments respectively.

The  $E_1$  form is initiated after the binding of ATP at the cytoplasmic face of the enzyme. Two K ions occluded in the previous enzyme cycle are then de-occluded and released into the cytoplasm. This action is followed by the binding of 3 Na ions to the enzyme intracellularly. The bound ATP is then hydrolysed, the  $\gamma$ -phosphate group of the ATP is transferred to an Asp residue of the enzyme (aa 369 of the sheep Na,K-ATPase  $\alpha$  subunit sequence), and the ADP generated is released. The phosphorylated enzyme, occludes and then translocates the bound Na ions to the exterior surface of the membrane. After translocation, the Na ions are de-occluded and released, and the enzyme's conformation changes to the  $E_2$ -P conformation. The binding of 2K ions extracellularly is followed by dephosphorylation of the enzyme. The bound K ions are then occluded, and with the binding of ATP at an intracellular site, are translocated to and released at the intracellular surface of enzyme. The binding of ATP results in a conformational change in the enzyme, which returns to its  $E_1$  form. This action completes the reaction cycle of the enzyme.

### 1.III.iii. The amino acid sequence of Na,K-ATPase and ion translocation.

Whether the enzyme has two separate binding sites for Na and K or one site occupied alternately is not known (Forbrush III, 1988). Evidence suggests that the conformational change from the  $E_1$  form to the  $E_2$  form is associated with the movement of an extra 10-25% of the polypeptide into the membrane (Karlsh et al., 1977). In the case of the Ca-

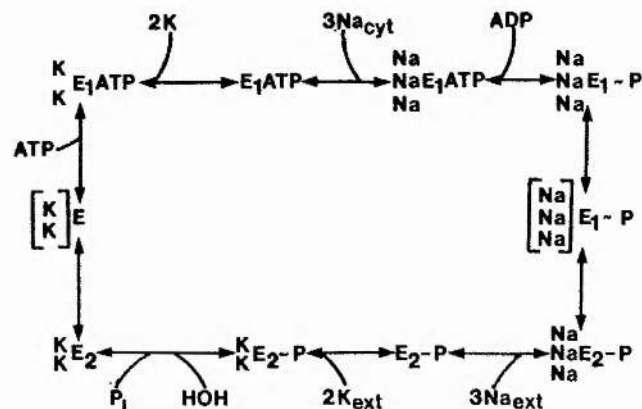


Figure 2.  $E_1$ - $E_2$  reaction cycle of Na,K-ATPase with ping-pong sequential cation translocation. The scheme is based on four major occluded conformations but is otherwise similar to the Post-Albers scheme (see text). Na and K indicate which cations are bound to the 'open' enzyme form whereas, [Na] and [K] indicate occluded forms of the enzyme, which are prevented from exchanging with cations in the medium. P designates enzyme phospho-forms. Adapted from Jorgenson, 1986.

ATPase (a protein which is structurally related to Na,K-ATPase), this extra membranous material is thought to be located within a region equivalent to aa's 200-500 of the Na,K-ATPase  $\alpha$  subunit sequence and involves a movement of a minimum of 38 aa's into the bilayer (Anderson *et al.*, 1986). The disappearance of tryptic and chymotryptic cleavage sites at Arg-262 and Leu-266 (of the sheep Na,K-ATPase  $\alpha$  subunits aa sequence) in the E<sub>2</sub> conformation and Arg-438 in the E<sub>1</sub> conformation, suggests that the region between these residues, including the phosphorylation site at Asp-369 is involved in the E<sub>1</sub>-E<sub>2</sub> transition.(Jorgenson and Collins, 1988; Jorgenson and Andersen, 1988).

#### 1.III.iv. The phosphorylation and nucleotide binding sites of Na,K-ATPase.

Other functional regions of the  $\alpha$  subunit of Na,K-ATPase have also been determined. Regions of the aa sequence associated with nucleotide binding and phosphorylation have been determined. These regions are highly conserved and show a high degree of homology between; 1, isoforms of the  $\alpha$  subunit, 2,  $\alpha$  subunit sequences from different species and 3, other ion transporting ATPase's, such as H,K-ATPase and Ca-ATPase. The aa phosphorylated during the reaction cycle of the enzyme has been shown to be the aspartic acid residue located at position 369 of the aa sequence (sheep  $\alpha$  subunit; Shull *et al.*, 1985). The nucleotide binding site involves a number of distinct regions of the proteins primary aa sequence, the main site being at position 543-561 of the aa sequence (Jorgenson, 1988). The presence and location of the other regions has been determined by the binding of various ATP antagonists. Fluorescein isothiocyanate (FITC) an inhibitor, binds to the lysine residue at position 501 (Karlsh, 1980), [<sup>14</sup>C]-ATP- $\gamma$ [4-(N-2-chloroethyl-N-methylamino)] benzylamid (C1R-ATP) binds to aspartic acid residues at positions 710 and 714 (Dzhandzhugazyan *et al.*, 1988) and [<sup>13</sup>C]-5-(p-fluorosulphonyl)-benzoyl-adenosine (FSBA) radiolabels two regions of the aa sequence, at positions 655-664 and 704-722 (Ohta *et al.*, 1986a). A third possible FSBA labelling site may exist near the phosphorylation site at position 386-402 (Herrera *et al.*, 1987). The region of the  $\alpha$  subunit between aa's 596-641 shares significant homology to the aa sequence encompassing the C1R-ATP binding sites and the second FSBA labelling site (aa residues 695-741) (Shull *et al.*, 1988). Both the homologous region at aa's 596-641 and the FITC binding region are thought to be exposed to the cytoplasm at some time during the enzymes cycle (Ohta *et al.*, 1988). These ATP analogues or binding inhibitors are thought to act by occupying or interfering with the ATP binding site. The regions



involved in ATP binding are dispersed along the primary structure of the protein. This evidence yields information on the tertiary structure of Na,K-ATPase because the regions involved in nucleotide binding must be in close proximity to each other, in order that they can interact with ATP (Dzhandzhugazyan et al., 1988).

### 1.III.v. Membrane spanning regions of the $\alpha$ subunit of Na,K-ATPase.

With the availability of the aa sequences, derived from the nucleotide (nt) sequences of Na,K-ATPase genes, information regarding the structure of the protein has been determined (see figure 3.). The putative membrane spanning regions of each subunit have been determined by hydropathy analysis. Hydropathy analysis involves the estimation of the average hydrophobicity of groups of aa's, determined sequentially along the length of the polypeptide. This analysis has been performed primarily by the method of Kyte and Doolittle (Kyte and Doolittle, 1982) although other methodologies are available (Eisenberg, 1984; Engelmann *et al.*, 1986; Cornette et al., 1987). Based on this type of analysis the  $\alpha$  subunit is thought to have between 6 and 11 membrane spanning regions (Lingrel et al., 1990; Arzamazova *et al.*, 1988), whereas the  $\beta$  subunit is thought to have only 1 transmembrane domain (Shull *et al.*, 1986a; Farley *et al.*, 1986; Ohta *et al.*, 1986b). Thermodynamic principles indicate that the membrane spanning regions of both the  $\alpha$  and  $\beta$  subunits are likely to be  $\alpha$ -helical structures (Arzamazova *et al.*, 1988).

Specific antibodies to regions of the  $\alpha$  and  $\beta$  subunit aa chains have been used to determine the cellular location of these regions of the proteins. Results from these studies

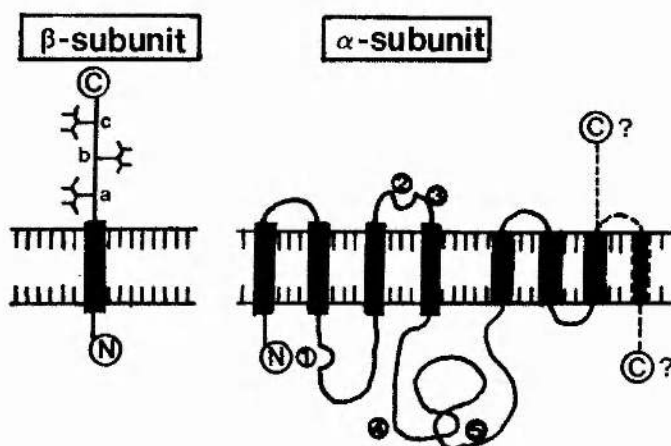


Figure 3. Model for the membrane insertion of the  $\alpha$  and  $\beta$  subunit of Na,K-ATPase. This is schematically represented with five binding sites: the Na<sup>+</sup> binding site (1), the K<sup>+</sup> binding site (2), the ouabain binding site (3), the phosphorylation site (4), and the ATP binding site (5). the a, b, and c represent glycosylation sites on the  $\beta$  subunits. Adapted from Rossier *et al.*, 1987.

suggests that the N terminus of the  $\alpha$  subunit is located in the cytoplasm and the C terminus is located extracellularly (Arzmazova *et al.*, 1988; Bayer, 1990). If the C terminal end of Na,K-ATPase is located extracellularly, then there must be an uneven number of membrane spanning regions (see figure 3). This result together with other evidence from proteolysis suggests that the  $\alpha$  subunit has 7 transmembrane domains (Arzmazova *et al.*, 1988). This would allow only 3 short aa loop segments and the C terminal end of the polypeptide to be located extracellularly. Evidence from both X-ray crystallography and protease digestion indicate that 25-50% of the enzyme resides in the membrane (Karlisch *et al.*, 1977; Maunsbach *et al.*, 1988; and Demin *et al.*, 1988). However, even using a model of the Na,K-ATPase with the  $\alpha$  subunit having 8 transmembrane domains, only 15% of the mass of the protein would be predicted to be within the membrane (Jorgenson, 1988). This is clearly at odds with the idea that only the proposed transmembrane domains reside in the membrane. It has been suggested (Lodish, 1988) that discrepancies, associated with multiple membrane spanning proteins such as Na,K-ATPase, may be explained by the presence of other membrane spanning regions. These regions, which would not necessarily be hydrophobic or  $\alpha$ -helical, could be stabilized in the membrane by being surrounded by the other hydrophobic membrane spanning domains. This would increase the mass of polypeptide within the membrane.

#### 1.III.vi. The structural organisation of the $\beta$ subunit of Na,K-ATPase.

The N terminal of the  $\beta$  subunit has been located within the cytoplasm whereas, the C terminal has been located extracellularly (Farley *et al.*, 1986). The membrane spanning domain of the  $\beta$  subunit is located near the N terminal of the protein, which leaves the bulk of the  $\beta$  subunit on the extracellular side of the membrane. The  $\beta$  subunit has three (or four in fish species; Noguchi *et al.*, 1987) potential glycosylation sites, which are all thought to be glycosylated in the mature  $\beta$  subunit polypeptide (Geering, 1990). The positions of the glycosylation sites are such that they are all located on the extracellular portion of the  $\beta$  subunit (Tankum and Fambrough, 1986). Up to 3 possible cysteine-cysteine disulphide bonds have also been reported within the extracellular domain of the  $\beta$  subunit but their significance is still unknown (Kirley, 1989; Gevondyan *et al.*, 1989; Miller and Farley, 1990). The oxidative state of the numerous cysteine residues within the aa chain of the  $\alpha$  subunit is uncertain, however, recent studies by Gevondyan *et al.*

(1989) and Millar and Farley (1990) suggest that up to two disulphide bonds probably exist.

### 1.III.vii. Cardiac glycoside binding to Na,K-ATPase.

Before the determination of the nucleotide and aa sequences of Na,K-ATPase, the site of cardiac glycoside binding to the enzyme was thought to involve 3 separate regions. These 3 regions were thought to interact with the sugar, steroid and lactone ring moieties of the glycoside molecule (Schwartz *et al.*, 1988). Now that aa sequences of the  $\alpha$  subunit are available, extracellular regions including the M3-M4 (M = membrane spanning region, numbered from the N terminal of the polypeptide) and M5-M6 extracellular loops (Herrera *et al.*, 1987), as well as a region near the C terminal end of the  $\alpha$  subunit (Shull *et al.*, 1988) have been suggested to be involved in glycoside binding (see figure 3). The M3-M4 loop has a region of aa sequence which shares homology with the superfamily of steroid binding proteins and may bind the steroid ring portion of cardiac glycosides (Price and Lingrel, 1988; Baker, 1986). Differences in the affinity of cardiac glycosides binding to Na,K-ATPase exist, both within and between species. The binding affinity of the  $\alpha 1$  isoform of the mouse and rat (with a Kd of 28-110  $\mu$ M; Erdmann *et al.*, 1980; Schwartz *et al.*, 1988; and Shyjan *et al.*, 1990a) is much lower than other species (such as in HeLa cells from humans where the Kd is  $\sim$ 5 nM; Aiton, 1976). The ouabain binding affinity of the rat or mouse  $\alpha 1$  isoforms are also much lower than that of their  $\alpha 2$  isoforms (Kd = 16-160 nM; Orłowski and Lingrel, 1988; Schwartz *et al.*, 1988; and Berrebebi-Bertrand *et al.*, 1990) or  $\alpha 3$  isoforms (Kd = 10-160 nM; Schwartz *et al.*, 1988; Hara *et al.*, 1988; Hara *et al.*, 1989; Urayama and Sweadner, 1988; and Shyjan *et al.*, 1990a). The diversity of the Kd's determined for the ouabain binding to the isoforms may be due to the possible existence of other  $\alpha$  isoforms, the presence of which is at present indeterminable. Comparison of the aa sequences of the extracellular regions of the  $\alpha$  subunit, between the ouabain insensitive rat  $\alpha 1$  isoform, and other sensitive  $\alpha$  sequences, shows that most of the aa changes reside in the M1-M2 loop. Two aa changes have been shown by site directed mutagenesis to be responsible for the difference in ouabain binding affinity (Price and Lingrel, 1988; Emanuel *et al.*, 1989; and Canfield *et al.*, 1990). The two aa's altered were the glutamine and asparagine (at positions 111 and 122 of the sequence, within the M1-M2 loop) of the human  $\alpha 1$  isoform. These aa's were changed to those present at the same location in the

rat  $\alpha 1$  isoform sequence (arginine and aspartic acid respectively). The expression of the altered sequence in ouabain sensitive cultured cells conferred ouabain resistance to the cells. The asparagine at position 121 of the rat  $\alpha 1$  sequence (which is highly conserved) has also been shown to be important for the binding of ouabain to the enzyme (Price *et al.*, 1989). The M1-M2 extracellular loop is clearly very important for the binding of cardiac glycosides to Na,K-ATPase.

#### 1.III.viii. Possible regulatory regions of the $\alpha$ subunit of Na,K-ATPase.

The N terminus of the  $\alpha$  subunit shows the greatest divergence of aa sequence homology for any specific isoform between species, and between isoforms within one species. Likewise, the sequence of the N terminus of the  $\alpha$  subunit of Na,K-ATPase and other related ion transporting ATPase's are also heterogeneous (Shull *et al.*, 1988; Jorgenson and Anderson, 1988). This domain does however, have a common feature within Na,K-ATPase  $\alpha$  isoforms, that being, a short relatively conserved region with a high proportion of lysine residues (Shull *et al.*, 1988; Shull *et al.*, 1986b). The N terminal region has been postulated to be involved in determining the rate of ion transport, as tryptic cleavage of this region (including the conserved lysines), results in an increase in the amount of the enzyme in the  $E_1$  conformation and, reduced enzyme activity (Jorgenson and Collins, 1988). This suggests that the rate of  $E_1$ - $E_2$  conformational transition is reduced (and consequently Na ion translocation across the membrane) by tryptic cleavage. However, the large differences between the N terminal regions of the isoforms, suggest that this region may also be involved in the regulatory and functional differences that have been demonstrated to exist between isoforms (Lytton *et al.*, 1985; Lytton, 1985). Similarly, another highly diverged and possible regulatory site is situated in the central portion of the  $\alpha$  subunit. This region (designated the I box) is located at aa's 494-503 of the sequence and is situated next to the FITC binding site (Herrera *et al.*, 1987).

The C terminal aa sequence is also more diverged than the average between Na,K-ATPase and other phosphorylated, ion transporting ATPase's (Jorgenson and Anderson, 1988). The Na,K-ATPase is the only member of the family of related proteins known to have a  $\beta$  subunit (Sweadner, 1989). If the C terminus of the  $\alpha$  subunit proves to be extracellularly located, as is most of the  $\beta$  subunit, then this may be a major site of



interaction between the  $\alpha$  and  $\beta$  subunits. However, this at present is speculation as no information on the site(s) of interaction between the subunits exists.

#### 1.III.ix. Future directions of studies on the structure of Na,K-ATPase.

In the future, experiments will be needed to try and resolve the following problems or questions :- 1, Proof of the existence or otherwise of the  $\gamma$  subunit of Na,K-ATPase. If it does exist, its structure, function and regulation need to be investigated. 2, The tertiary and quaternary structure of the enzyme still has to be determined. This includes the relative locations of all the regions of the primary aa sequences of both the  $\alpha$  and  $\beta$  subunits, during each conformation of the enzyme's reaction cycle. Also, information is required regarding the location of segments of aa's involved in the interaction between the  $\alpha$  and  $\beta$  subunits. 3, The number of hydrophobic membrane spanning domains, and the intra- or extra- cellular location of the C terminal end of the  $\alpha$  subunit needs to be determined. 4, The regions of the  $\alpha$  or  $\beta$  subunit (besides the hydrophobic membrane spanning domains), located within the plasma membrane need to be established. 5, Information is required on both the nature and location of the Na and K binding sites. Is there only one site, or two separate and distinct sites for the binding of Na and K ions? 6, What is the exact mechanism by which ions are translocated across the membrane and how the energy is transduced from the location of the hydrolysis of ATP? 7, What role does the  $\beta$  subunit play in the action of the enzyme, and what is the function of the glycosylation sites and disulphide bonds within the  $\beta$  subunit? 8, What is the exact location of the cardiac glycoside binding site in relation to the quaternary structure of the enzyme and, by what mechanism do cardiac glycosides bind to and inhibit the enzyme? 9, What properties of the enzyme are different between the isoforms of the  $\alpha$  and  $\beta$  subunits, and how do these relate to differences in the primary amino acid sequences?

#### 1.IV. The molecular biology of Na,K-ATPase.

##### 1.IV.i. Isoforms of the subunits of Na,K-ATPase.

The first cloning of the genes encoding the  $\alpha$  and  $\beta$  subunits of Na,K-ATPase was performed by the screening of a cDNA library using the technique of nucleic acid hybridization. In initial experiments, a mixture of synthetic oligonucleotide probe

sequences, derived from the N-terminal aa sequences of tryptic digestion fragments, obtained from the  $\alpha$  subunit of Na,K-ATPase, were used (Kawakami *et al.*, 1985; Shull *et al.*, 1985). The first report of cDNA sequences was in August 1985, when the entire sequences of the Na,K-ATPase  $\alpha 1$  subunit from sheep kidney (Shull *et al.*, 1985) and *Torpedo californica* electric organ (Kawakami *et al.*, 1985) were cloned. Progress occurred rapidly following this, and the first cloning of  $\alpha 1$ ,  $\alpha 2$  and  $\alpha 3$  isoforms from rat was reported in December 1986 (Shull *et al.*, 1986b). To date, 10  $\alpha 1$ -like DNA sequences with varying homologies have been reported from a number of species including :- rat (100%) (Shull *et al.*, 1986b; Hara *et al.*, 1987; and Herrera *et al.*, 1987), human (97%) (Kawakami *et al.*, 1986a; Chehab *et al.*, 1987), sheep (97%) (Shull *et al.*, 1985), pig (97%) (Ovchinnikov *et al.*, 1986), horse (96%) (Kano *et al.*, 1989), chicken (93%) (Takeyasu *et al.*, 1988), frog (*Xenopus laevis*) (91%) (Verrey *et al.*, 1989), electric ray (*Torpedo californica*) (87%) (Kawakami *et al.*, 1985), fruit fly (*Drosophila melanogaster*) (80%) (Lebovitz *et al.*, 1989) and brine shrimp (*Artemia salina*) (69%) (Baxter-Lowe *et al.*, 1989) (figures in parentheses refer to % aa homology compared to the rat  $\alpha 1$  sequence). The *Drosophila* and *Artemia* genes display equal homology to all three  $\alpha$  isoforms from rat species. The *Drosophila* gene is also thought to be the only Na,K-ATPase  $\alpha$  'subunit-like' gene in this species (Lebovitz *et al.*, 1989; Varadi *et al.*, 1989). The complete aa sequences of the  $\alpha 2$  and  $\alpha 3$  isoforms have so far only been reported for 2 species. In the rat, the aa homology of the  $\alpha 1$  and  $\alpha 2$  isoforms is 86%, and the  $\alpha 1$  and  $\alpha 3$  isoforms is 85% (Shull *et al.*, 1986b; Hara *et al.*, 1987; and Herrera *et al.*, 1987). The other reported aa sequence is from humans where the aa homology shared by the  $\alpha 1$  and  $\alpha 2$  isoforms is 87% and the  $\alpha 1$  and  $\alpha 3$  isoforms is 86% (Kawakami *et al.*, 1986a; Shull *et al.*, 1989; Ovchinnikov *et al.*, 1987; Ovchinnikov *et al.*, 1988; Svedlov *et al.*, 1987; and Svedlov *et al.*, 1989). Partial aa sequences have also been reported for the  $\alpha 1$ ,  $\alpha 2$ , and,  $\alpha 3$  isoforms of chicken (Takeyasu *et al.*, 1989). These sequences are also highly homologous to their counterparts in humans and rats. Famborough *et al.* (1987) have suggested that at least 4 and possibly 5 different isoforms are expressed in chickens. The possibility of the existence of isoforms in brine shrimp has also been suggested (Morohashi and Kawamura, 1984), although so far no conclusive evidence has been produced. The nucleotide sequence homologies of the coding regions of the three rat  $\alpha$  isoforms have been determined (Shull *et al.* 1986b). The homology shared between the nucleic acid sequences is less than that between the comparable aa sequences ( $\alpha 1$  vs  $\alpha 2$  = 77%,  $\alpha 1$  vs  $\alpha 3$  = 76% and  $\alpha 2$  vs  $\alpha 3$  = 79%).

In the case of the  $\beta$  subunit, the  $\beta 1$  isoform has now been cloned and sequenced in 9 species including; human (100%) (Kawakami *et al.*, 1986b), rat (95%) (Mercer *et al.*, 1986; Young *et al.*, 1987), sheep (92%) (Shull *et al.*, 1986c), pig (92%) (Ovchinnikov *et al.*, 1986), dog (91%) (Brown *et al.*, 1987), mouse (89%) (Gloor 1989), chicken (68%) (Takeyasu *et al.*, 1987), frog (*Xenopus Laevis*) (68%) (Verry *et al.*, 1989) and electric ray (*Torpedo californica*) (61%) (Noguchi *et al.*, 1986) (figures in parentheses refer to the % aa sequence homology compared to human  $\beta 1$ ). A second isoform of the  $\beta$  subunit exists, the  $\beta 2$  isoform, the aa sequence of which shows only 35% aa homology with that of the  $\beta 1$  isoform. Sequences for the  $\beta 2$  isoform are currently only available for the human, rat and, mouse (Martin-Vasallo *et al.*, 1989; Gloor *et al.*, 1990). The aa homology of these compared to rat species are human (98%) and mouse (94%). The nt homology of the  $\beta 2$  compared to the  $\beta 1$  subunit is 53%. This is greater than the homology of the aa sequence and reflects the large number of favoured or conserved aa substitutions (24%) (aa substitutions where the replacing aa is chemically similar to the original one, the chemical nature of the primary aa sequence is therefore conserved) (Martin-Vasallo *et al.*, 1989).

#### 1.IV.ii. The genomic location and structural organisation of Na,K-ATPase genes.

Much of the information available on genomic DNA sequences of the Na,K-ATPase genes has been obtained from work on humans, and so far five  $\alpha$  subunit-like genes have been detected. The genomic DNA sequences of the  $\alpha 1$ ,  $\alpha 2$  and  $\alpha 3$  isoforms have been designated ATP1A1, ATP1A2 and ATP1A3 respectively (Yang-Feng *et al.*, 1988). These were previously referred to as  $\alpha A$ ,  $\alpha B$ , and un-named respectively, by Shull and Lingrel (1987). Whether or not the two other genes are expressed remains to be established. One of these, designated ATP1AL2 (previously referred to as  $\alpha C$ ; Shull and Lingrel, 1987), has a high nucleotide (76-81%) and aa (80-89%) homology compared to the three well characterised isoforms and is physically linked on the chromosome to the ATP1A2 gene locus. The second, ATP1AL1, has lower nucleotide (68-70%) and aa (66-75%) homology (Orlowski and Lingrel, 1988). Svedlov *et al.* (1987), have also found two extra genomic copies of Na,K-ATPase genes whether these are identical to the ATP1AL1 and ATP1AL2 genes is unknown, although one may represent a H,K-ATPase-like gene (a protein structurally related to Na,K-ATPase; Lingrel *et al.*, 1990). It is not known whether these genes represent pseudo-genes, other unknown ion

translocating ATPase's which are highly homologous to the Na,K-ATPase or, possibly undiscovered Na,K-ATPase isoforms (Orlowski and Lingrel, 1988).

The chromosomal location of various isoforms in humans has also been determined. Of the known isoforms ATP1A1 is found on chromosome 1p, ATP1A2 is found on chromosome 1q, ATP1A3 is located on chromosome 19, the genomic copy of the  $\beta$ 1 isoform (designated ATP1B) is found on chromosome 1 (Yang-Feng *et al.*, 1988). Another  $\beta$ 1 isoform-like gene exists, and has been designated ATP1BL1, this gene is thought to represent a pseudogene, and is located on human chromosome 4 (Yang-Feng *et al.*, 1988). The genomic location of the  $\beta$ 2 gene has been determined to be chromosome 11 in the mouse (Malo *et al.*, 1990). Because of the location of other homologous genes from mouse chromosome 11 in the human genome, the  $\beta$ 2 gene in humans is likely to be located on chromosome 17 (Malo *et al.*, 1990). Other work on the genomic location of Na,K-ATPase genes has been performed on the mouse (*Mus musculus* and *Mus spretus*; Kent *et al.*, 1987) and the American mink (*Mustela vison*; Matveeva *et al.*, 1987; Khlebodarova *et al.*, 1988).

The genomic DNA copies of the horse  $\alpha$ 1 isoform (Kano *et al.*, 1989), the human  $\alpha$ 2 (Shull *et al.*, 1989) and  $\alpha$ 3 (Ovchinnikov *et al.*, 1987; Ovchinnikov *et al.*, 1988) isoforms have been most closely studied and, in common with the 4 human  $\alpha$  subunit-like genes reported by Shull *et al.* (1987), they are approximately 20-30 Kb (kilo bases) in size. They also have a similar structural arrangement in that they have 23 coding regions or exons, and 22 noncoding regions or introns. These introns are located at identical positions within the genes, with two exceptions. These exceptions are:- 1; the position of the 1<sup>st</sup> intron in the  $\alpha$ 3 isoform interrupts between aa's 2 and 3 of the sequence, whereas in the  $\alpha$ 1 and  $\alpha$ 2 isoform genes this intron is located between aa's 4 and 5, and 2; the location of the 10<sup>th</sup> intron in the  $\alpha$ 3 gene interrupts the nucleotide sequence 2 base pairs to the 3 prime side, of the corresponding intron location of the  $\alpha$ 1 and  $\alpha$ 2 genomic sequences. The size of the introns between isoforms has also been shown to be highly variable (Kano *et al.*, 1989; Shull *et al.*, 1989). The genomic sequence of the  $\alpha$  subunit most intensively studied is the human  $\alpha$ 2 isoform or ATP1A2 sequence (Shull *et al.*, 1989; Sverdlov *et al.*, 1989). For this gene nearly 1600 base pairs of DNA sequence upstream (5' side) of the transcription initiation site, have been determined. Sequences sharing homology with the recognition sequences of numerous transcription factors (ie transcription factors ap1, ap2, cp1, cp2, sp1, nf1, CACCC factor) and hormone response elements (glucocorticoid and thyroid hormones), have



been discovered (Shull *et al.*, 1989). The significance of these discoveries has not yet been investigated. The structure and sequence of the ATP1B gene has also recently been reported (Lane *et al.*, 1989). This gene comprises 26.7 Kb of DNA and is encoded by 6 exons. The 5' untranslated nucleotide sequence of this gene contains 2 major transcription initiation sites at positions 510 bp and around 200 bp upstream of the coding region. The 5' untranslated sequence is extremely G+C rich (79%), and sequences upstream of the transcription initiation sites contain 12 potential recognition sites for the sp1 transcription factor. The significance of these sites is as yet undetermined. The genomic structure and sequence of the  $\beta$  pseudogene (called ATP1BL1) has been determined. It shares 88% nucleotide homology with the  $\beta$ 1 isoforms coding sequence, but it contains no introns (Ushkaryov *et al.*, 1989; Lane *et al.*, 1989).

#### 1.IV.iii. The Na,K-ATPase enzyme, as a member of a family of ion translocating ATPase's.

The 5 different isoforms (or pseudo genes) of the  $\alpha$  subunit of Na,K-ATPase are members of a family of related genes. Other proteins which share aa homology with this family also exist. These include proteins from as distantly related species as E.coli (Hesse *et al.*, 1984) and yeast (Serrano *et al.*, 1986). The most closely related of these to the  $\alpha$  subunit of Na,K-ATPase, is the H,K-ATPase, which has been cloned from rat and pig gastric cDNA libraries (Shull and Lingrel, 1986; Maeda *et al.*, 1988). The H,K-ATPase shares large sections of aa homology with the Na,K-ATPase  $\alpha$  subunit with an overall homology of 62% (Shull and Lingrel, 1986). Although regions of the H,K-ATPase are clearly homologous to Na,K-ATPase, it is thought that it does not require a  $\beta$  subunit to function (Sweadner, 1989). A protein of similar molecular weight to the unglycosylated form of the  $\beta$  subunit of Na,K-ATPase has however, been shown to be non-covalently associated with H,K-ATPase (Okamoto *et al.*, 1989; Okamoto *et al.*, 1990). The Ca-ATPase, another protein sharing homology with Na,K-ATPase, also has an associated glycoprotein, although this protein shares no homology with the  $\beta$  subunit of Na,K-ATPase (Leberer *et al.*, 1989). Both H,K-ATPase and Ca-ATPase are also structurally similar to Na,K-ATPase, as they all have similar hydropathy profiles (Jorgenson and Anderson, 1988). Whether or not H,K-ATPase binds cardiac glycosides (as is the case for Na,K-ATPase), is still a matter of debate (Suzuki and Kaneko, 1989; Sweadner, 1989). Other genes sharing aa sequence homology with Na,K-ATPase also exist. These

include various Ca-ATPase's of the endoplasmic and sarcoplasmic reticulum (with approximately 25% aa homology; Lingrel *et al.*, 1990; MacLennan *et al.*, 1985; Brandl *et al.*, 1986; Lytton and MacLennan, 1988; Lytton *et al.*, 1989; Genteski-Hamblin *et al.*, 1988; and Burk *et al.*, 1989) and the plasma membrane (also with approximately 19% aa homology; Lingrel *et al.*, 1990; Shull and Greeb, 1988; Greeb and Shull, 1989; and Strehler *et al.*, 1990), the H-ATPase of yeast (aa homology approximately 17%) (Serrano *et al.*, 1986), and the K-ATPase of E.coli (aa homology approximately 18%) (Hesse *et al.*, 1984). The different isoforms of the Na,K-ATPase are produced by separate genes, whereas, the isoforms of both fast and slow twitch skeletal muscle endoplasmic reticulum Ca-ATPase are produced by alternatively spliced genes (Lytton and MacLennan, 1988; Lytton *et al.*, 1989; Genteski-Hamblin *et al.*, 1988; Brandl *et al.*, 1987; Korczak *et al.*, 1988). The most conserved regions of homology shared between the members of this gene family appears to be primarily in the ATP binding regions and the phosphorylation sites (Jorgenson, 1988).

#### 1.IV.iv. mRNA's coding for the isoforms of Na,K-ATPase.

In the rat, which is the most widely studied species, the  $\alpha 1$  isoform encodes a single sized mRNA of 3.7 Kb, a coding region for a protein of 1023 aa's, a 5' untranslated region of ~240 nucleotides (nt) and, a 3' untranslated sequence of ~330 nt (Shull *et al.*, 1986b). The  $\alpha 2$  (or  $\alpha +$ ) isoform encodes at least two different sized mRNA's one 5.3 Kb, the other 3.4 Kb. These sequences encode a protein of 1020 aa's with ~100 nt 5' and up to ~1850 nt 3' untranslated sequence (Shull *et al.*, 1986b). These different sized RNA's are thought to occur due to the differential use of poly adenylation sites (Young and Lingrel, 1987). The  $\alpha 3$  isoform has a mRNA of 3.7 Kb encoding a protein of 1013 aa's with ~140 nt 5' and ~380 nt 3' untranslated sequence (Shull *et al.*, 1986b). However, a second larger  $\alpha 3$  mRNA species may be produced (Emanuel *et al.*, 1987). No N terminal signal sequence is present in the aa coding sequence of the Na,K-ATPase genes. However, the  $\alpha 1$  and  $\alpha 2$  isoforms have the 5 N terminal aa's removed by post translational processing, the processing of the  $\alpha 3$  isoform was not assessed (Shull *et al.*, 1986b).

The  $\beta 1$  isoform has a coding region of 304 aa's, with up to 460 nt of 5' and 1150 nt 3' untranslated sequence. However, at least five sizes of mRNA are transcribed in the brain and kidney of rat species (Young *et al.*, 1987). The sizes that have been reported

are as follows :- 2.7, 2.35, 1.85-1.75, 1.8-1.7 and 1.4 Kb respectively (Young *et al.*, 1987) or 2.9, 2.6, 2.25, 1.75, and 1.5 Kb respectively (Omori *et al.*, 1988). These are produced by differential use of both five different poly adenylation sites and 2 transcription initiation sites (Young *et al.*, 1987). The  $\beta 2$  isoform encodes a protein of 290 aa's with up to 460 nt 5' and 1510 nt 3' untranslated sequence. A single 3.4 Kb mRNA has been reported for the  $\beta 2$  subunit (Martin-Vasallo *et al.*, 1989). Another feature of both  $\beta$  isoform mRNA's is the homology of the 3' untranslated region. In  $\beta 1$  isoform mRNA's, a 71 nt section of this region is highly conserved (>92%) in all species so far sequenced (Takeyasu *et al.*, 1987). Similarly, in  $\beta 2$  isoform mRNA's, a 104 nt 3' untranslated section exists which is highly homologous between rat and human species (>96%). However, the homologous regions of the  $\beta 1$  and  $\beta 2$  isoform mRNA's, do not share homology with each other. The homologous regions in the  $\beta$  isoforms are as highly conserved between species as the nt sequence of the coding regions of these genes. They are therefore likely to be important for the function or regulation of the  $\beta$  subunit mRNA's, although their precise role has yet to be determined.

#### 1.IV.v. The tissue specific expression of mRNA's coding for the isoforms of Na,K-ATPase.

The expression of mRNA's coding for the isoforms of each subunit has been investigated in different rat tissues during development through foetal to adult life. The results produced so far by a number of independent groups have been highly confusing. Although highly stringent nucleic hybridisations have been carried out with entirely homologous probes, completely contrasting results have been obtained in some cases.

Quantitative analysis of the relative abundance of mRNA's coding for the isoforms of Na,K-ATPase, between different tissues of adult rats has been performed by Young and Lingrel (1987). Other investigators (Sverdlov *et al.*, 1988; Mercer *et al.*, 1988; Herrera *et al.*, 1987; Emanuel *et al.*, 1987; Martin-Vasallo *et al.*, 1989), have produced qualitative results showing the abundance of these mRNA's on Northern blots, although they have made no attempt to quantify these results.

The experimental results from Young and Lingrel (1987) have been quantified by comparing the intensities of autoradiographic signals obtained from slot blot nucleic acid hybridisation analysis. The signal intensity from each tissue was measured, and standardised against the signal obtained from brain tissue. Brain tissue was chosen as it

contained mRNA for each of the  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ , and  $\beta 1$  isoforms of Na,K-ATPase. Figures in parentheses represent the abundance of mRNA (signal per  $\mu\text{g}$  of total RNA) in each tissue relative to brain tissue. The results from Young and Lingrel (1987) showed that  $\alpha 1$  isoform mRNA was present in all the tissues studied. These tissues were as follows; kidney (7.0), brain (1.0), heart (0.7), lung (0.3), stomach (0.3), skeletal muscle (0.1), and liver ( $<0.05$ ). The  $\alpha 2$  isoform mRNA was present in all tissues studied except liver tissue. The relative abundance of mRNA in each tissue compared to brain tissue was as follows; skeletal muscle (1.5), brain (1.0), heart (0.1), stomach ( $<0.05$ ), kidney ( $<0.05$ ), and lung ( $<0.05$ ). Of the tissues studied,  $\alpha 3$  mRNA was only present in brain (1.0), stomach ( $<0.05$ ) and lung ( $<0.05$ ). The  $\beta 1$  isoform of Na,K-ATPase, in a similar fashion to the  $\alpha 1$  isoform was also present in all the tissues studied. The relative abundance of mRNA in each tissue compared to brain tissue was as follows; kidney (2.0), brain (1.0), stomach (0.5), heart (0.2), muscle (0.1), lung ( $<0.05$ ), and liver ( $<0.05$ ). It has been estimated (Young and Lingrel, 1987) that the relative abundance of the isoform mRNA's for Na,K-ATPase, compared to each other in brain tissue is in the ratio 1: 1.2: 0.8: 5.2 ( $\alpha 1$ :  $\alpha 2$ :  $\alpha 3$ :  $\beta 1$ ). The estimates of the abundance of the isoform mRNA's in different tissues, should however be treated with caution. Considerable variation in the values obtained can occur. A startling example of this, is the relative level of mRNA for the  $\alpha 1$  isoform in kidney tissue compared to liver tissue. Gick *et al.* (1988a) have reported that the ratio of the abundance of the  $\alpha 1$  isoform mRNA in kidney tissue compared to liver tissue was 5:1. The comparable figures from Young and Lingrel (1987) gave a ratio of  $> 140:1$ .

A quantitative study of the abundance of mRNA for the  $\beta 2$  isoform of Na,K-ATPase, has not yet been reported. However, from Northern blots published by Martin-Vasallo *et al.* (1989), it is clear that the  $\beta 2$  isoform mRNA was present in the brain, kidney, lung and heart tissues of adult rats.

Investigations have been carried out to determine the abundance of mRNA's for the isoforms of Na,K-ATPase during the development in the rat, through foetal to adult life. The only quantitative experiments, that have investigated this topic, were performed by Orłowski and Lingrel (1988). Other research groups (Emanuel *et al.*, 1987; Herrera *et al.*, 1987; Martin-Vasallo *et al.*, 1989) have performed Northern blot hybridisation analysis on RNA samples extracted from rat tissues during different developmental stages. However, the relative abundance of the mRNA's in these blots was not quantified. In the experiments by Orłowski and Lingrel (1988), the relative abundance of



isoform mRNA's, was quantified by measurement of the intensities of the autoradiographic signals obtained from slot blot nucleic acid hybridisation. The experiments were performed on total RNA samples from rat brain, kidney, heart, lung and skeletal muscle tissues from 10 days before birth to 60 days after birth. The measurements of autoradiographic signals obtained were standardised against the measurement of the abundance of the  $\alpha 1$  isoform in each tissue, at the earliest stage of development. The results of these experiments showed that, the changes in the relative abundance of the mRNA for the isoforms during development, in different tissues, followed a highly complicated pattern. In most cases the abundance of mRNA for all the isoforms increased during the development of rat from foetal to the juvenile (25 days old) stage. A maximum level of abundance was reached when rats were 15-30 days old. However, exceptions to this were, the pattern of abundance of the;  $\beta 1$  isoform in brain,  $\alpha 3$  isoform in heart,  $\alpha 1$  and  $\beta 1$  isoforms in lung, and the  $\alpha 2$  isoform in skeletal muscle tissue. The abundance of the  $\alpha 3$  isoform in heart and the  $\alpha 1$  and  $\beta 1$  isoforms in lung were maximal at birth whereas, the  $\beta 1$  isoform from brain and the  $\alpha 2$  isoform from skeletal muscle continued to increase up to 60 days of life.

Little is known about what difference the isoforms of Na,K-ATPase subunits make to the function of the Na,K-ATPase enzyme or in which tissues these isoforms are expressed (Although the latter has been inferred from mRNA studies; Schneider *et al.*, 1988). The main problem has been that until recently, although Na,K-ATPase enzyme abundance could be assessed, the isoform composition of these enzyme units was unknown. Differences between the isoforms have been detected by SDS polyacrylamide gel electrophoresis. The  $\alpha$  subunits of Na,K-ATPase show different migration rates during electrophoresis, giving two bands called ( $\alpha$ ) and ( $\alpha+$ ) (the  $\alpha+$  band being of an apparently higher molecular weight protein). Translation of  $\alpha$  isoform mRNA's in vitro suggests that the ( $\alpha$ ) band migrates at the same position as the  $\alpha 1$  isoforms translated product and the ( $\alpha+$ ) band, at the same position as the  $\alpha 2$  and  $\alpha 3$  isoforms translated products (Schneider *et al.*, 1988). It has been shown that the Na,K-ATPase enzyme migrating with the ( $\alpha+$ ) band has higher affinity for the Na,K-ATPase inhibitors ouabain and strophanthidin, and has a different sensitivity to trypsin and sulphhydryl cross linking agents (Sweadner, 1989). Recently, specific monoclonal antibodies for  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$  and  $\beta 1$  isoform proteins have been produced (Shyan and Levenson, 1989; Shyan *et al.*, 1990b; Urayama *et al.*, 1989; Arystarkhova *et al.*, 1989). These will facilitate future studies where the abundance of proteins of specific subunit isoforms can be determined

in cells and tissues. Studies have already shown that the tissue distribution of different isoform proteins is similar to that inferred from the mRNA studies cited above (Shyan and Levenson, 1989).

#### 1.IV.vi. Future directions of research on the molecular biology of Na,K-ATPase.

A number of problems and questions on the molecular biology of Na,K-ATPase need to be answered, including :- 1, Do the aa changes in the sequence of the  $\alpha$ 1 isoforms of Na,K-ATPase between distantly related species, significantly alter the structure, function or regulation of the enzyme in these species? How do these aa changes relate to aa changes in other ion translocating ATPase's? 2, What is the structure, function, and genomic organisation of the two unknown Na,K-ATPase " $\alpha$  subunit-like" genes (called ATP1AL1 and ATP1AL2). Are they true isoforms of Na,K-ATPase and if so, in which tissues are they expressed? Are these Na,K-ATPase " $\alpha$  subunit-like" genes other ion translocating ATPase's and if so, what ions do they translocate, or are these genes actually pseudogenes? 3, What is the human chromosomal location and structural organisation of the gene for the  $\beta$ 2 isoform. 4, What transcriptional regulatory elements are present in the genomic copies of the  $\alpha$ 1,  $\alpha$ 3, and  $\beta$ 2 isoform genes, and how do these relate to the elements present in the genomic  $\alpha$ 2 and  $\beta$ 1 genes? What transcription factor/hormone receptor complexes actually regulate the Na,K-ATPase genes, and what is the mechanism of their action? 5, What is the function (if any) of the two sizes of mRNA for the  $\alpha$ 2 isoform and the five or more sizes of mRNA for the  $\beta$ 1 subunit. 6, What function is served by the removal of the five N-terminal aa's from the primary translation product of the  $\alpha$ 1 and  $\alpha$ 2 isoforms? 7, What is the function of the regions which are highly homologous between species and which are located in the 3' untranslated region of  $\beta$  subunit mRNA's? 8, What determines the level of expression of the isoforms of Na,K-ATPase subunits in different tissues and during development?

#### 1.V. The Regulation of Na,K-ATPase.

The regulation of the Na,K-ATPase enzyme can take place at many levels. In general, there are two main types of regulation 1, the adjustment of the activity of the enzyme and 2, modulating the number (or density) of active enzyme units in the plasma membrane. The first category includes regulation of the enzyme by its substrates, ie Na, K, and

ATP, and also by its possible interaction with other unknown intracellular regulatory molecules, either directly or indirectly (via second messenger systems or intermediates). The second category, the modulation of the number of active enzyme units, involves regulation of 1; new transcription or degradation of mRNA 2; translation and the coordinate production of the  $\alpha$  and  $\beta$  subunits proteins and 3; the insertion or removal of the active enzyme units, from the plasma membrane.

### 1.V.i. The acute regulation of Na,K-ATPase enzyme activity.

#### 1.V.i.a. Substrate regulation.

Regulation of the activity of the Na,K-ATPase enzyme by its substrates under physiological conditions is dependent on both the substrate concentrations and the affinities of the enzyme for these substrates. In principle, either the intracellular Na concentration ( $[Na]_i$ ), extracellular K concentration ( $[K]_o$ ) or intracellular ATP concentration ( $[ATP]_i$ ) could be rate limiting for the activity of the enzyme under normal conditions. As previously mentioned, normally  $[Na]_i$  is about 10 mM,  $[K]_o$  is about 4.5 mM and the  $[ATP]_i$  is usually greater than 1 mM. The activity of the purified enzyme has been measured under different Na, K and ATP concentrations (Jorgenson, 1986). The concentrations of substrates giving half maximal activation ( $K_{0.5}$ ) of enzyme activity have been determined to be, at a  $[Na]$  of 37 mM, a  $[K]$  of approximately 0.5 mM and, at  $[ATP]_i$  of approximately 0.5 mM (Katz, 1988) (see figure 4).

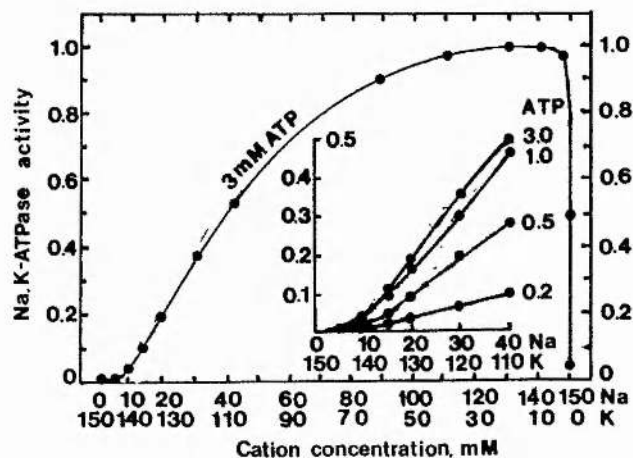


Figure 4. Activity of pure Na,K-ATPase as a function of Na, K, and ATP concentrations. Initial rates of ATP hydrolysis were measured at the concentrations of ATP and cations indicated, Isotonicity was maintained by exchanging NaCl and KCl. Adapted from Jorgenson, (1986).

In the case of K and ATP, the normal physiological concentrations of these substrates are near those that nearly saturate the activity of the enzyme (~1 mM [K] and ~2 mM [ATP]). Small changes in the concentrations of these substrates would therefore have very little effect on enzyme activity, only a large drop in the concentration of K or ATP would alter enzyme activity markedly (Katz 1988). For Na the situation is entirely the opposite, as the normal  $[Na]_i$  is below the  $K_{0.5}$  value, it has been predicted that a 50% increase in  $[Na]_i$  from 20 to 30 mM would double the Na,K-ATPase enzyme activity (Katz, 1988).

In contrast to these findings, it has been suggested (Katz, 1988), that the saturating effect of increasing ATP concentration on the enzyme activity, determined on lysed cell membranes or purified Na,K-ATPase, may not be representative of the situation *in vivo*. In intact cells, it has been shown that uptake of K (ouabain-sensitive  $^{86}Rb$  uptake) has a linear non-saturating dependence on  $[ATP]_i$  (Soltoff and Mandel, 1984; Tessitore *et al.*, 1986). This suggests that *in vivo*, ATP concentrations may be rate limiting on enzyme activity. Factors affecting the synthesis and hydrolysis of ATP as well as its access to Na,K-ATPase may therefore affect the enzymes activity (Katz, 1988). A similarly contrasting situation to that of  $[ATP]_i$ , exists in the case of  $[K]_o$ . In intact kidney tubules, there is a linear dependence of K uptake (ouabain-sensitive  $^{86}Rb$  uptake) on the  $[K]_o$ , up to a concentration of at least 5 mM (Katz, 1988; Doucet *et al.*, 1979). Whereas, in permeabilised kidney tubules, the enzyme activity was shown to be saturated at  $[K]_o$  of 5 mM (Doucet *et al.*, 1979). However, in both the cases of K or ATP, the greatest change in Na,K-ATPase activity is still likely be caused by a large drop in the concentrations of these substrates, as may occur in anoxia or hypokalemia (Rossier *et al.*, 1987).

In contrast to the ambiguity of the effect of  $[K]_o$  and  $[ATP]_i$  on enzyme activity, the action of altering the  $[Na]_i$  is much clearer. The Na affinity ( $K_{0.5}$ ) of purified sheep kidney Na,K-ATPase enzyme preparations is 37 mM (Jorgenson, 1980), and the normal  $[Na]_i$  is 10-19 mM (Beck *et al.*, 1982). On the basis of these facts it has been calculated that an increase in the  $[Na]_i$  from 20-30 mM would double the Na,K-ATPase turnover rate (activity). As with purified enzyme, measurements of intact kidney tubules treated with monensin (a Na ionophore) showed that a change in the  $[Na]_i$  from 10 to 25 mM, resulted in a nearly doubled rate of ouabain-sensitive  $^{86}Rb$  uptake (Katz, 1988). Hence increases in the  $[Na]_i$  (on purified enzyme and Na,K-ATPase in intact cells), result in a large change in enzyme activity.



Little is known about the affinities of different isoforms of the enzyme for Na, K and ATP. Large differences in Na affinity have been shown between the ( $\alpha$ ) ( $K_{0.5}$  for Na = 17 mM) and the ( $\alpha+$ ) ( $K_{0.5}$  for Na = 52 mM;  $\alpha 2$  and/or  $\alpha 3$  isoform) enzyme isoforms in whole cells, which was not present in isolated membranes ( $\alpha = \alpha+$  where  $K_{0.5} = 3.5-12$  mM; Lytton, 1985). The measurement of Na affinity in whole cells and isolated membranes was achieved by taking advantage of the difference in ouabain sensitivity of isoforms of Na,K-ATPase in the rat. Na,K-ATPase enzyme activity was measured in the presence or absence of a ouabain concentration which would selectively inhibit almost all the  $\alpha 2$  and  $\alpha 3$  isoforms, without markedly affecting the activity of the  $\alpha 1$  isoform (Lytton, 1985).

#### 1.V.i.b.1. The inhibition of Na,K-ATPase by cardiac glycosides.

Compounds known to specifically inhibit the activity of Na,K-ATPase include cardiac glycosides such as ouabain (G-strophanthin; Post *et al.*, 1960; Skou, 1960). The binding of ouabain to the enzyme takes place on the outer surface of the membrane (Caldwell and Keynes, 1959), requires ATP and Mg, is stimulated by Na and, is inhibited by K ions (Hansen, 1984). Identical binding capacities of the enzyme for ATP, ADP, and ouabain have been demonstrated (Hansen *et al.*, 1971) and a linear relationship between specific ouabain binding capacity and the maximal hydrolytic activity of Na,K-ATPase has been shown (Baker and Willis, 1972). Experimentally, ouabain binding to cells has been shown to be composed of two components; a saturable specific component, associated with the binding and inhibition of Na,K-ATPase and an unsaturable, non-specific component thought to represent non-specific binding and uptake of ouabain into the cell (Baker and Willis, 1972). The nature of the non-saturable cellular uptake mechanism for ouabain has not yet been characterised. The non-saturable component can be determined when specific glycoside binding to the Na,K-ATPase is specifically inhibited in the presence of extracellular K ions. Specific ouabain binding represents the total ouabain binding determined under K-free conditions, with the linear non-saturable component subtracted (Baker and Willis, 1972). On the basis of these results, specific ouabain binding can be used for quantification of the number of Na,K-ATPase units (Baker and Willis, 1972). Ouabain and potassium have been shown to compete with each other in binding to the  $E_2P$  conformation of the enzyme (Jorgenson,

1986) and hence, under experimental conditions, utilizing high  $[K]_o$ , ouabain is largely excluded from binding to Na,K-ATPase.

#### 1.V.i.b.2. Na,K-ATPase, cardiac glycosides and positive inotropy (in myocardial tissues).

Cardiac glycosides or other putative endogenous inhibitors (endo-ouabains or peptide-like substances released from the hypothalamus; Hauptert, 1988), bind to and inhibit Na,K-ATPase, causing an increase in  $[Na]_i$ . In excitatory tissues such as the myocardium, this stimulates sarcolemmal Na-Ca exchange, increasing the intracellular calcium concentration ( $[Ca]_i$ ). The increase in  $[Ca]_i$ , caused by the inhibition of Na,K-ATPase is thought to be responsible for the increased generation of force during myocardial contraction (positive inotropy; Erdmann *et al.*, 1980; Schwartz *et al.*, 1988). A similar mechanism may be involved in the cardiac glycoside-sensitive release of neurotransmitters from neuromuscular junctions and sympathetic nerve terminals (Torok, 1989). The main link between the inhibition of Na,K-ATPase and neurotransmitter release is thought to be provided by the Na-Ca exchange mechanism.

The isoforms of Na,K-ATPase show a range of sensitivities to cardiac glycosides, in different species. For example, the affinity of ouabain binding to the rat  $\alpha 1$  isoform has a  $K_{0.5}$  of approximately 40  $\mu M$  (Erdmann *et al.*, 1980; Schwartz *et al.*, 1988) whereas, ouabain binding to the human  $\alpha 1$  has a  $K_{0.5}$  of approximately 5 nM (Aiton, 1976; Kawakami *et al.*, 1986a). Despite these large differences in the binding constants between species, there is a good correlation between the % inhibition of Na,K-ATPase and the % maximum increase in myocardial contractile force (Schwartz *et al.*, 1988). In rat ventricle, where both the  $\alpha 1$  and  $\alpha 2$  isoforms of Na,K-ATPase are known to be expressed (Young and Lingrel, 1987), a biphasic relationship has been shown to exist between the increase in the force of myocardial contraction and the concentration of cardiac glycoside present (Adams *et al.*, 1982; Schwartz *et al.*, 1988). The biphasic response of rat ventricle to cardiac glycosides, consisted of; 1, a low concentration effect, with an  $ED_{50}$  (effective dose needed to give a 50% response) of 530 nM, which increased isometric contractile force (in grams) to a maximum of 34% above control values, and 2, a high concentration effect with an  $ED_{50}$  of 19  $\mu M$ , with a maximal increase in contractile force of 84% above control values (Adams *et al.*, 1982). In rat atria, where only a small amount of the  $\alpha 2$  isoform is thought to be present (Young and

Lingrel, 1987), a monophasic relationship existed ( $ED_{50}$  of 45  $\mu$ M) between the increase in the force of contraction and the concentration of cardiac glycosides (Adams *et al.*, 1982). The apparent correlation between isoform presence, their respective cardiac glycoside binding constants, and the increase in the force of myocardial contraction, suggests that this positive inotropic effect was caused by the inhibition of Na,K-ATPase. A good correlation has also been shown between increases in  $[Na]_i$  (caused by inhibition of Na,K-ATPase) and increases in myocardial contractile force (Schwartz *et al.*, 1988).

Cardiac glycosides are not naturally occurring endogenous inhibitors of the Na,K-ATPase in mammalian species. They are normally extracted from certain plant species, although they may be modified synthetically. The possibility of the existence of endogenous circulating inhibitors of Na,K-ATPase with similar properties has been investigated (Hauptert, 1988). Initially, it was shown that a natriuretic substance was present in blood of dogs (DeWardener *et al.*, 1961). This substance appeared to increase Na excretion from the kidney in the absence of; 1, a decrease in the circulating level of mineralocorticoid hormones in the blood, 2, an expansion of blood volume, 3, renal nervous system activity and 4, an increase in glomerular filtration rate or renal blood flow (Bahlman *et al.*, 1967; Lichardus and Nizet, 1971; and Kaloyanides and Azer, 1971). Further experiments then determined that the putative natriuretic substance produced its effect by directly acting upon Na transport in frog urinary bladder, and mammalian renal tubule membranes (Hauptert and Sancho, 1979; Fine *et al.*, 1976). The effects of this putative natriuretic substance on Na transport were later found to be associated with the inhibition of Na,K-ATPase (Hillyard *et al.*, 1976). The action of putative natriuretic substances on the development of hypertension, indicated by volume expansion, was shown to be prevented by lesions in the anterioventral third ventricle of the brain (Brody *et al.*, 1978). A natriuretic substance has since been extracted from the hypothalamus and is thought to act by modulating renal tubule Na reabsorption and vascular smooth muscle tone, via the regulation of Na,K-ATPase activity (Hauptert, 1988). In the short term it is also thought to increase cardiac output by increasing cardiac contractility (Hauptert 1988); presumably by a similar mechanism to that postulated for the action of cardiac glycosides. The role of this putative hypothalamic Na,K-ATPase inhibitor may however be more complicated, as its release from the hypothalamus has been shown to be inhibited by the presence of another natriuretic substance, atrial natriuretic peptide (ANP; Crabos *et al.*, 1988). A number of Na,K-ATPase 'inhibitor-like' substances have been partially purified from blood plasma, urine, the hypothalamus, the heart, and cerebral spinal fluid

(Hauptert, 1988). The proposed chemical properties of these 'inhibitor-like' substances are however diverse and fall roughly into two categories being either peptide-like or non-peptide-like substances (Hauptert, 1988; Hauptert, 1989). The identity of an inhibitor(s) of Na,K-ATPase from hypothalamus so far remains unresolved. Recently, the structure of three related peptide inhibitors of Na,K-ATPase activity, isolated from porcine duodenal extracts were reported (Araki *et al.*, 1989). The physiological and pathological roles of these inhibitors remains to be established.

#### 1.V.i.b.3. Na,K-ATPase, natriuretic substances, and the aetiology of essential hypertension.

The idea of an endogenously circulating inhibitor of Na,K-ATPase has been linked to abnormalities in Na,K-ATPase activity found in animals with volume expanded hypertension (Hauptert, 1988). Na,K-ATPase abnormalities (measured in erythrocytes) are limited to around 10% of human patients with essential hypertension (Diez *et al.*, 1987). Alteration in Na,K-ATPase function is thus probably limited to a discrete section of hypertensive patients and may be a cause or, an effect of hypertension. The patients with essential hypertension caused by Na,K-ATPase abnormalities, exhibited altered enzyme activities (decreased binding affinity for Na and increased  $V_{max}$ ) and, their erythrocytes had a higher than normal  $[Na]_i$  (Diez *et al.*, 1987). This evidence suggested a possible conformational alteration in the structure of Na,K-ATPase, a change of the isoform composition of enzyme units, or a change in some, as yet unknown regulatory component of the enzyme. The Na affinity of the enzyme has previously been demonstrated to be altered, in response to insulin treatment (Lytton, 1985). The change in  $V_{max}$  suggests a change in the number of enzyme units, this may be in response to the increase in the  $[Na]_i$  that was also found (see section 1.V.ii.).

Changes in the isoform composition and the abundance of Na,K-ATPase mRNA in hypertensive rats, have been investigated by Herrera *et al.* (1988). Hypertension was induced by two schemes; 1, uninephrectomised animals were treated with deoxycorticosterone (a mineralocorticoid which maintains or increases Na reabsorption) and were given a high salt diet (DOC-salt model) and; 2, animals were infused with pressor doses of Angiotensin II (which causes vasoconstriction of smooth muscle and release of corticoids (aldosterone) from the adrenal glands; A II model). Na,K-ATPase mRNA abundances were measured in aorta, left ventricular myocardium and skeletal



muscle tissues. In the DOC-salt model 2-3 fold increases in mRNA abundance were detected for the  $\alpha 1$  and  $\beta 1$  isoforms in aorta and skeletal muscle, but these increases were absent in ventricle. A 3-15 fold decrease in  $\alpha 2$  isoform mRNA abundance was found in aorta and ventricle but there was no change in skeletal muscle. The abundance of the  $\alpha 3$  isoform mRNA (which is only present in older rats), was also shown to decrease 3 fold in ventricle. In the A II model, a 3-15 fold decrease was similarly found in the abundance of the  $\alpha 2$  isoform mRNA in ventricle and aorta whereas, no increase in the abundance of the  $\alpha 1$  isoform mRNA was found in these tissues. Likewise no increase  $\beta 1$  isoform mRNA was found in aorta. The parallel changes in the abundance of the  $\alpha 1$  and  $\beta 1$  isoform mRNAs found in each tissue, in both animal models, was suggested to indicate that these mRNA's may be coordinately regulated. The regulation of the  $\alpha 1$  and  $\beta 1$  isoforms did not seem to be related to increases in vascular pressure as, the  $\alpha 1$  and  $\beta 1$  isoform mRNA's were increased in skeletal muscle (which is not directly affected by intravascular pressure) as well as in aorta. The increases in these mRNA's in skeletal muscle and aorta were also suggested to be tissue specific as, no increases in  $\alpha 1$  and  $\beta 1$  isoform mRNA's were found in ventricular tissue. Herrera *et al.*, (1988) have suggested that the increases in  $\alpha 1$  and  $\beta 1$  isoform mRNA's in skeletal muscle and aorta, were secondary changes probably caused by increased Na influx's in these tissues, produced as a consequence of DOC-salt treatment. Increased Na influx's in response to DOC-salt treatment have been demonstrated previously (Jones and Millar, 1978; Nagaoka *et al.*, 1987). The decreases in the abundance of the  $\alpha 2$  and  $\alpha 3$  isoform mRNA's in aorta and ventricle were however, likely to be caused by the increase in intravascular pressure. This was because no decrease in  $\alpha 2$  and  $\alpha 3$  mRNA abundance was found in skeletal muscle whereas, decreases of a similar magnitude were found in aorta and ventricle in both DOC-salt and A II animal models. Furthermore in aorta or ventricle, no changes in these mRNA's were found in animals treated with DOC-low salt or subpressor doses of AII. The mechanism of how changes in intravascular pressure, change the expression of the isoforms of Na,K-ATPase is unknown.

#### 1.V.i.c. Vanadate as an inhibitor of Na,K-ATPase activity.

Another inhibitor of Na,K-ATPase, is the oxide of vanadium, vanadate ( $\text{VO}_4^{3-}$ ). This inhibitor was discovered by accident as a contaminant of commercial ATP preparations (Cantley *et al.*, 1977). Vanadate is thought to act by binding reversibly to the cytoplasmic

surface of Na,K-ATPase, at the phosphorylation site (Cantley *et al.*, 1978). Vanadate inhibition is increased by high extracellular [K] (Beague *et al.*, 1980). Vanadate is a naturally occurring compound which is present in cells at physiological concentrations likely to have a possible inhibitory effect (Grantham, 1980; Jorgenson, 1980). It has been suggested that vanadate may play a role in the intracellular regulation of Na,K,ATPase, as it inhibits the enzyme in dog kidney with a  $K_i$  of 100 nM (Cantley *et al.*, 1977; Westenfelder *et al.*, 1981; and Day *et al.*, 1980). However, the role of vanadate as an inhibitor of Na,K-ATPase is not clear as, the effect of vanadate is not specific, it also inhibits Ca-ATPase, H,K-ATPase, dynein (a contractile protein) and microbial alkaline phosphatase (O'Neal *et al.*, 1979; Grantham, 1980). Vanadates action also requires entry into cells where it can bind to cell proteins (mentioned above) and, intracellular reducing agents such as NADH or glutathione may convert it to the relatively inactive vanadyl form (Cantley and Aison, 1979; Macara *et al.*, 1980; Schuurmans-Stekhoven and Bonting, 1981).

#### 1.V.i.d. Hormonal regulation of Na,K-ATPase activity.

Hormones, neurotransmitters, and growth factors such as insulin, glucagon, dopamine, noradrenaline, angiotensin II, vasopressin, and epidermal growth factor have been postulated to regulate the activity of Na,K-ATPase in an indirect fashion (not binding directly to Na,K-ATPase itself), via secondary messenger systems. The action of insulin on Na,K-ATPase is probably produced via increases in the  $[Na]_i$ , which increases enzyme activity due to increased Na availability. Insulin has been shown to increase Na influx in rat hepatocytes by increasing amiloride sensitive Na-H exchange. However, the increased Na influx found, may be a secondary effect produced by the effect of insulin on cellular growth and proliferation (Fehlmann and Freychet, 1981). The increase in Na,K-ATPase activity produced by insulin in hepatocytes was mimicked by monensin (a Na-H antiporter) (Gick *et al.*, 1988a; Fehlmann and Freychet, 1981), which further indicates that the effect of insulin may be mediated by increases in  $[Na]_i$ . However Lytton (1985), claimed that in adipocytes, stimulation by insulin was achieved by a shift in the  $K_{0.5}$  of Na binding to the ( $\alpha+$ ) ( $\alpha_2$  and/or  $\alpha_3$  isoforms) enzyme units from 52 mM to 33 mM. As the ( $\alpha+$ ) isoform enzyme represented 75% of the total Na,K-ATPase expressed, an increase in Na efflux would be achieved without increasing the  $[Na]_i$  (Lytton, 1985). Indeed it was calculated that the change in the  $K_{0.5}$  of Na binding from 52 to 33 mM

would result in an increase in the fractional activity of the ( $\alpha+$ ) enzyme from 5 to 18% of the maximum (assuming  $[Na]_i = 20$  mM; Lytton, 1985). The action of glucagon on Na,K-ATPase in rat hepatocytes was also stimulatory. The effect of glucagon was of a similar magnitude to that of insulin. The mechanism of its action was not mediated by increases in Na influx, however it was suggested that glucagon may increase Na,K-ATPase activity by enhancing K efflux (Fehlmann and Freychet, 1981).

Dopamine, which is produced in kidney proximal convoluted tubules, has been shown to inhibit Na,K-ATPase activity in this nephron segment (Aperia *et al.*, 1987; Felder *et al.*, 1989). The inhibitory action of dopamine on Na,K-ATPase has been shown to involve a Pertussis toxin sensitive G protein complexed to the dopamine receptor (Bertorello and Aperia, 1988). The secondary messenger involved in dopamine inhibition is unclear, although stimulation of protein kinase C (PKC) in rat proximal tubules inhibits Na,K-ATPase. PKC has been implicated as a secondary messenger because the inhibition of Na,K-ATPase was blocked by the PKC inhibitor, sphingosine (Bertorello and Aperia, 1989). However, Meister *et al.*, (1989) have suggested that the inhibitory action of dopamine on Na,K-ATPase activity in rat and monkey renal tubule cells, is brought about by stimulation of adenylate cyclase activity. The cAMP produced stimulates a cAMP-dependent protein kinase which in turn phosphorylates a phosphoprotein (DARPP-32). DARPP-32 is an inhibitor of protein phosphatase-1 and -2B (Felder *et al.*, 1989). The inhibitory effect of a dopamine agonist on Na,K-ATPase activity was blocked by a specific inhibitor of cAMP-dependent protein kinase (Meister *et al.*, 1989). Furthermore, the action of PKC on Na,K-ATPase activity, also remains to be determined as in different tissues it has been found to be inhibitory (Bertorello and Aperia, 1989) or stimulatory (Lattimer *et al.*, 1989).

The action  $[Ca]_i$  on Na,K-ATPase activity has also been investigated and is thought to be mediated by the action of Ca-sensitive cellular proteins. At least three proteins may modulate Na,K-ATPase activity when free  $[Ca]_i$  is transiently elevated in response to hormones and other stimuli (Yingst, 1988). These three are PKC (as previously mentioned above), calnaktin and calmodulin (Yingst, 1988). Calnaktin is a 35 kDa protein which has been postulated to inhibit Na,K-ATPase enzyme activity in the presence of high free  $[Ca]_i$  (Yingst, 1988). Despite calnaktins effect on Na,K-ATPase activity (50% inhibition in the presence of 1  $\mu$ M  $[Ca]_i$ ; Yingst, 1988), it has no effect on the activity of other membrane ATPases such as Ca- or Mg-ATPase. Calmodulin, a 18 kDa protein, increases the inhibition of Na,K-ATPase in rat brain, in the presence of Ca

(>10  $\mu\text{M}$ ; Cirillo *et al.*, 1984). Calmodulin is thought to interact with Na,K-ATPase via an intermediary, as purified calmodulin itself has no action on purified Na,K-ATPase (Yingst, 1988). However, at low free [Ca] (1  $\mu\text{M}$ ), calmodulin has also been shown to have a stimulatory effect on Na,K-ATPase activity (Powis, 1985).

The actions of noradrenaline, vasopressin and angiotensin II have all been shown to stimulate Na,K-ATPase activity (Beach *et al.*, 1987; Lynch *et al.*, 1986; Brown and Douglas, 1989). The mechanisms of action of these hormones are as yet unknown. However, the stimulatory actions of vasopressin and a phorbol ester analogue (phorbol myristate acetate) in rat hepatocytes were not additive, suggesting that the two agents share a common pathway, probably involving diacylglycerol formation (Lynch *et al.*, 1986).

Epidermal growth factor (EGF) has been shown to stimulate Na,K-ATPase activity in fibroblasts. Little is known about its mechanism of action, but it is thought activation of Na,K-ATPase is mediated by EGF induced phosphorylation. This may be produced by the action of the EGF receptor associated tyrosine kinase (Yarden *et al.*, 1982). The mechanism of stimulation of Na,K-ATPase by phosphorylation either directly or indirectly, remains to be determined.

#### 1.V.ii. Long term regulation of Na,K-ATPase abundance.

##### 1.V.ii.a.1. The effect of $[\text{Na}]_i$ .

Normal mammalian cells have similar  $[\text{Na}]_i$  of around 5-15 mM. They also have similar  $[\text{K}]_i$  of around 140 mM. Despite these similarities, the rate of Na leak (or influx) and, Na,K-ATPase activity (Na efflux), can both vary by over two orders of magnitude (Lechne, 1988). Because of the involvement of the Na gradient provided by the Na,K-ATPase enzyme in the uptake of nutrients (sugars, amino acids and phosphate; see section 1.II.), it has been proposed that more metabolically active cells, transporting more nutrients, would leak more Na and would consequently require a higher level of Na,K-ATPase activity (Lechne, 1988). For a given cell type, Na leak and Na,K-ATPase activity would vary in parallel with the need for the cell to modulate exchanges with the extracellular environment (Lechne, 1988). In view of the reliance of the cell on its Na gradient, a feedback regulatory mechanism, would seem to be an efficient way for the cell to regulate the  $[\text{Na}]_i$ . A feedback regulatory mechanism would operate by responding to



changes in  $[Na]_i$  (caused by changes in Na influx or efflux), by adjusting the level of Na influx or efflux, to bring the  $[Na]_i$  back to its normal value. Changes in  $[Na]_i$  have already been shown to alter the level of Na,K-ATPase activity (Na efflux; See section 1.V.i.a.), achieved by direct modulation of enzyme activity. Longer term regulation of Na,K-ATPase activity caused by a persistent change in  $[Na]_i$  from its normal value, is also possible.

The long term regulation of Na,K-ATPase activity by changes in  $[Na]_i$  has been examined by a number of research groups. This has been investigated by experimentally altering the  $[Na]_i$ . The  $[Na]_i$  can be altered experimentally in cultured cells by a number of different means, these include growth in:- sub-lethal doses of ouabain (Kim and Smith, 1985; Brodie and Sampson, 1989; Pollack *et al.*, 1981a), medium containing a low [K] (causing Na,K-ATPase inhibition; Boardman *et al.*, 1974; Pollack *et al.*, 1981a; Pollack *et al.*, 1981b; Cramb *et al.*, 1989; Kim and Smith, 1985; Werdan *et al.*, 1984; Bowen and McDonough, 1987; Ismail-Beigi *et al.*, 1988; Pressley *et al.*, 1988), medium containing the Na ionophores nystatin or monensin (Cramb *et al.*, 1989; Barlet-Bas and Doucet, 1988) or the Na channel activator veratridine or the blocker tetrodotoxin (Kim and Smith, 1985; Brodie and Sampson, 1989). Most of these treatments have been shown to cause an increase in  $[Na]_i$  with the exception of tetrodotoxin which caused a decrease  $[Na]_i$ . It has been shown that when the  $[Na]_i$  was increased, the level of Na,K-ATPase activity also increased (Jorgenson, 1986). Increases in Na,K-ATPase activity create an extra load on existing Na,K-ATPase units and leave the cell with a lower capacity to respond to any further increases in the  $[Na]_i$  (Lechne, 1988). If increases in the  $[Na]_i$  are sustained, then the cell could respond by increasing its Na and K pumping capacity by increasing Na,K-ATPase unit numbers. Where the  $[Na]_i$  has been increased by the means cited above, this has unanimously been shown to be the case. Alternatively, in response to a sustained decrease in the  $[Na]_i$  as found in the presence of tetrodotoxin, the cell could respond by decreasing its Na and K pumping capacity by decreasing Na,K-ATPase unit numbers, this response has also been demonstrated (Brodie and Sampson, 1989).

The exact means by which the cell brings about a change in Na,K-ATPase enzyme numbers has been to some extent disputed. Pollack *et al.* (1981b) have suggested that in HeLa cells (a human cervical carcinoma cell line), the entire increase in Na,K-ATPase numbers (produced in response to low [K] treatment), could be accounted for by a decrease in the rate of degradation or removal of Na,K-ATPase units from the plasma

membrane. The half time of the degradation of Na,K-ATPase was 5.4 hours in normal growth conditions and 12.8 hours in low [K] growth conditions (Pressley, 1988). The degradation rate of Na,K-ATPase enzyme was determined by monitoring the rate of loss of [<sup>13</sup>C]-radiolabelled Na,K-ATPase enzyme, from the plasma membrane of these cells (Pollack *et al.*, 1981b). Wolitzky and Fambrough (1986) working on chicken skeletal muscle cells, also found that the degradation rate of Na,K-ATPase units was decreased during up-regulation of Na,K-ATPase numbers by low [K] or veratridine treatment (see above). However, the increase in Na,K-ATPase numbers caused by the decrease in the degradation rate was slower acting (degradation half time of 31 hours in normal growth conditions and 64 hours with growth in veratridine) than that found by Pollack *et al.* (1981b). An increase in the rate of biosynthesis was also detected. This was a rapid, but transient increase, with the biosynthesis rate reaching maximum within 15 hours before falling to control values after 36 hours. The increase in the biosynthesis rate was thought largely to be responsible for the initial increase in plasma membrane Na,K-ATPase unit numbers. The work of other investigators on the Madin-Darby canine kidney (MDCK) cell line (Bowen and McDonough, 1987), has shown that the transient increase in the rate of synthesis (caused by growth in low K) is sustained for at least 22 hours. The effect of increased biosynthesis (on the number of Na,K-ATPase units located in the plasma membrane) was delayed, as an estimated time of 3-6 hours was required for the newly synthesised Na,K-ATPase units to be processed and inserted in the plasma membrane (Wolitzky and Fambrough, 1986; Pollack *et al.*, 1981b; Karin and Cook, 1986). The length of the lag time has been disputed however as Caplan *et al.* (1990) have determined that the delay in MDCK cells had a half time of only 75 minutes. The decreased degradation rate of Na,K-ATPase units found with veratridine treatment was thought to be responsible for maintenance of the increased level of Na,K-ATPase units found after 36 hours (a maximum of 70% increase), as by this time the biosynthesis rate had returned to normal (Wolitzky and Fambrough, 1986). Brodie and Sampson (1989) also studied biosynthesis and degradation in rat skeletal muscle cells using veratridine or tetrodotoxin treatments. They also found a similar 70% increase in Na,K-ATPase numbers after 36 hours. However, they found no decrease in the degradation rate of Na,K-ATPase compared to controls, the half time being 21 hours in each case. Other studies have published degradation half times as long as 3-4 days in hypothyroid and T3 treated rat kidney (Tamkun and Fambrough, 1986; Lo and Edelman 1976; Lo and Lo, 1980). The

significance of variations in the degradation rate of Na,K-ATPase enzyme units in different tissues, of different species, is not yet known.

Increases in Na,K-ATPase enzyme units caused by increases in biosynthesis rate and decreases in degradation rate both have, to some extent, a delayed effect. In cells where potentially rapid changes in  $[Na]_i$  are possible (ie epithelial cells engaged in vectorial Na transport), a rapid increase in Na,K-ATPase numbers is required, to prevent large changes in the  $[Na]_i$ . To provide this rapid increase in Na,K-ATPase units, a pool of readily accessible, inactive, cryptic or masked Na,K-ATPase units has been suggested (Barlet-Bas and Doucet, 1988; Bowen and McDonough 1987; Verry *et al.*, 1989; Wolitzky and Fambrough, 1986). These units would be inserted into the plasma membrane rapidly to increase pumping capacity, and save the time required for the synthesis of new enzyme. Barlet-Bas and Doucet (1988), have shown that, the increase in  $[Na]_i$  caused by growth in nystatin (a sodium ionophore), resulted in a large increase in the number Na,K-ATPase units, which was not dependent on protein synthesis. The intracellular pool of Na,K-ATPase units represented some 60% of the total enzyme units in chick myotubes (Wolitzky and Fambrough, 1986) and 31% in cultured chick sensory neurons (Tankum and Fambrough, 1986). Wolitzky and Fambrough (1986) found that this pool in chick myotubes was not depleted during growth in veratridine, but was slightly increased. The increase in Na,K-ATPase units found at the cell surface after veratridine treatment was therefore, not due to the removal of units from an intracellular pool. It has been shown, by the use of a subcellular fractionation techniques that most of the intracellular Na,K-ATPase enzyme is associated with the golgi complex (Mircheff *et al.*, 1989). The evidence for intracellular pools of Na,K-ATPase units is however controversial, as some authors have reported that no significant intracellular pool of enzyme units exists (Caplan *et al.*, 1985).

After the up-regulation (increase) of the number of Na,K-ATPase units on the surface chick myotubes (produced by veratridine treatment), it has been shown that the number of units in myotube plasma membranes could then be down regulated (decreased) by tetrodotoxin treatment (Takeyasu *et al.*, 1989). The down-regulation occurred without any delay and did not decrease the total cellular (located intracellularly and in myotube plasma membranes) level of Na,K-ATPase, or affect the abundance of  $\alpha$  or  $\beta$  subunit mRNA's (Taormino and Fambrough, 1990). This suggests that the Na,K-ATPase units were removed to an intracellular site. Consistent with this idea, Takeyasu *et al.* (1989) have performed experiments where a second round of up-regulation was induced by the

re-initiation of veratridine treatment. The authors have suggested that the intracellularly located Na,K-ATPase units were re-inserted into the membrane without a delay. The reason for the differences in Na,K-ATPase regulation by alteration of the  $[Na]_i$  in different tissues is unknown.

#### 1.V.ii.a.2. Regulation of Na,K-ATPase mRNA's by changes in the $[Na]_i$ .

The increase in the biosynthesis rates of Na,K-ATPase subunits could be mediated by an increase in the efficiency of translation of already existing Na,K-ATPase mRNA. Alternatively, increases in the biosynthesis rates could also result from an increase in the abundance of Na,K-ATPase mRNA's. The latter possibility might be achieved (as previously discussed with the Na,K-ATPase enzyme units) by either decreasing the degradation rate of the mRNA's (increasing mRNA stability; the life span of mRNA) or by increasing the rate of synthesis (transcription). Several laboratories have shown that the abundance of Na,K-ATPase mRNAs are increased during increases in  $[Na]_i$ , either transiently (Cramb *et al.*, 1989; Bowen and McDonough, 1987) or for an extended period (Pressley *et al.*, 1988). Takeyasu *et al.* (1989), found that in response to veratridine treatment the increase in the  $\alpha$  subunit mRNA was not concomitant with that of the  $\beta$  subunit mRNA. The abundance of the  $\beta$  subunit mRNA increased by 3 fold after 10 hours of veratridine treatment, before falling back to near normal levels after 24 hours. The  $\alpha$  subunit mRNA abundance by contrast, remained constant until between 18-24 hours of treatment, the abundance then increased by 80% after 24 hours, where its level remained thereafter (up to at least 48 hours). These studies however, have not shown whether the increases in Na,K-ATPase mRNA abundances were caused by increased rates of transcription or by increased mRNA stability.

#### 1.V.ii.b. Regulation of translation of the subunits of Na,K-ATPase.

Another possibility for the regulation of Na,K-ATPase enzyme is that production of one of the two subunits or their respective mRNA's might be rate limiting. Most of the evidence so far suggests that production of  $\alpha$  and  $\beta$  mRNA's (Bowen and McDonough, 1987) and proteins (Geering, 1988) is coordinated (alterations in the regulation of Na,K-ATPase produce similar changes in both the  $\alpha$  and  $\beta$  subunits). However, Geering *et al.* (1989), showed that in *Xenopus* oocytes, much less  $\beta$  subunit protein was produced than



$\alpha$  subunit protein. The excess  $\alpha$  subunit protein was associated with the membrane, but was highly trypsin sensitive and was consequently inactive (as functional enzyme is relatively resistant to trypsin proteolysis (Geering *et al.*, 1985a; Geering *et al.*, 1987)). Extra  $\beta$  subunit protein produced from microinjected  $\beta$  subunit mRNA, increased the proportion of trypsin resistant  $\alpha$  subunit protein able to perform cation dependent conformational changes, concomitantly with an increase in the number of ouabain binding sites on the plasma membrane. This evidence suggests that the association of the  $\beta$  subunit with the  $\alpha$  subunit leads to a structural rearrangement of the  $\alpha$  subunit, which is part of the process of maturation of enzyme (Geering *et al.*, 1989).

#### 1.V.ii.c. Regulation of Na,K-ATPase by hormones.

A number of hormones have been implicated in the long term regulation of the expression of Na,K-ATPase enzyme units. These include aldosterone, cortisol and deoxy-corticosterone (which have a general mineralocorticoid-like action), corticosterone and the synthetic steroid dexamethasone (which generally have a glucocorticoid-like action), thyroid hormones (triiodothyronine (T3) and possibly thyroxine (T4)) and other hormones such as progesterone.

##### 1.V.ii.c.1. Mechanisms of steroid and thyroid hormone action.

The general mechanism of action of all these hormones on the regulation of their responsive genes is thought to be similar (Evans, 1988). It has been proposed (Rousseau, 1984) that hormonal regulation of gene transcription is a receptor mediated process. The hormone enters the cell by a passive process and binds to its receptor. It is at present unclear whether these receptors are located in the cytoplasm or the nucleus of cells (Burnstein and Cidlowski, 1989) (cytoplasmic forms would have to migrate to the nucleus). However, in the nucleus, the receptor hormone complex binds to accessible specific DNA sequences located within the vicinity of target genes known as hormone responsive elements. Binding of the hormone-receptor complex to these elements produces an alteration in the rate of transcription of the gene concerned (usually an increase). The increase in RNA produced by this action in turn produces an increase in the protein for which the gene codes (Rousseau, 1984; Burnstein and Cidlowski, 1989; Samuels *et al.*, 1989). The receptors for this group of hormones show remarkable

similarity and are thought to comprise a supergene family (Evans, 1988; Green and Chambon, 1986). The receptors have been shown to have at least three homologous domains, one of which is responsible for the recognition and binding of the hormone-receptor complex to specific DNA sequences (Wang *et al.*, 1989). Based on three aa differences in one section of this domain, (which plays a key part in determining the specificity of binding of the complex to DNA; Green and Chambon, 1987; Mader *et al.*, 1989) the hormone receptors have been divided into two groups (Mader *et al.*, 1989). Members within each group are thought to bind the same or similar hormone response elements, whereas, the two groups bind different hormone response elements (Mader *et al.*, 1989). Receptors with the highest binding affinity for, 1, mineralocorticoids (called type I receptors) 2, glucocorticoids (called type II receptors) and 3, progesterone, are in one group, whereas the receptor binding thyroid hormones is classified in the other group, which also contains the oestrogen, retinoic acid and vitamin D3 receptors (Mader *et al.*, 1989). Generally, the responsiveness of tissues to a particular hormone is determined by the expression of a receptor for that particular hormone, in the tissue concerned (Strahle *et al.*, 1989). Besides mineralocorticoid and glucocorticoid hormone-receptor complex's binding the same or similar hormone response elements, these hormones also bind each other receptors. Thus, another mechanism to provide tissue specific regulation by these hormones is necessary. The main physiological problem, associated with the cross reactivity of mineralo- and gluco-corticoids, to each others receptors, involves the action of mineralocorticoids. Mineralocorticoid hormones, circulate in the vascular system at much lower concentrations than glucocorticoid hormones. Type I hormone receptors would therefore mostly be occupied by glucocorticoid hormones. This problem has been shown to be circumvented, by the breakdown of glucocorticoid hormones to inactive forms, within mineralocorticoid target cells (Funder *et al.*, 1988; Gaeggler *et al.*, 1989). Recently, the original idea of the nuclear action of steroid and thyroid hormones has been questioned. Hormones and receptors mediating hormone effects in the absence of gene transcription have been found (Duval *et al.*, 1983; Watson and Muldoon, 1985). These include, oestrogen and oestrogen receptor complex's which bind to microsomes (Watson and Muldoon, 1985), glucocorticoid receptors which bind to tRNA (Ali and Vedeckis, 1987) and a T3 hormone receptor which has been located in the endoplasmic reticulum has now been cloned and sequenced (Cheng *et al.*, 1987).

### 1.V.ii.c.2. Regulation of Na,K-ATPase by mineralocorticoids and glucocorticoids.

The action of mineralocorticoids on Na,K-ATPase, has been most studied in specific segments of kidney nephrons, and in certain epithelial cultured cell lines with similar characteristics. The kidney is the major site of action of mineralocorticoids (although receptors may also exist in regions of the brain; Anderson and Fanestil, 1976). Aldosterone the primary mineralocorticoid, is thought to manifest its actions in these cells in two phases, these phases have been designated as the early and late responses (Gick *et al.*, 1988a). The early response is thought to involve a specific increase in the apical Na conductance by increases in either, the number, or activity of amiloride sensitive Na-H exchangers (Petty *et al.*, 1981; Fukuda and Aperia, 1988). This in turn produces an increase in the  $[Na]_i$  which leads to an acute increase in Na,K-ATPase activity within 3 hours of the onset of aldosterone treatment (Petty *et al.*, 1981; Gick *et al.*, 1988a). The second or the late response of aldosterone, involves a direct increase in the number of Na,K-ATPase units via *de novo* synthesis of Na,K-ATPase subunits. Increases in Na,K-ATPase biosynthesis rates were inhibitable by a competitive antagonist of mineralocorticoid receptor binding, spironolactone, but not by amiloride, a Na-H exchange inhibitor (Geering *et al.*, 1985b; Geering *et al.*, 1982). The early response of aldosterone is however controversial as some experiments have failed to detect an increase in Na,K-ATPase activity after 3 hours treatment (in rat cortical collecting tubules; Mujais *et al.*, 1984a). Recently, experiments on rat cortical collecting tubules have shown that the acute effect (~3 hours) of increased  $[Na]_i$  and the action of aldosterone are independent of each other (Barlet-Bas and Doucet, 1988). In these experiments, aldosterone, was shown to increase Na,K-ATPase unit numbers via protein synthesis, this action was blocked by the protein synthesis inhibitor, cycloheximide. Whereas, treatment with the Na ionophore nystatin, which increases the  $[Na]_i$ , increased Na,K-ATPase unit numbers without requiring protein synthesis, presumably by the activation of previously masked units (Barlet-Bas and Doucet, 1988). Barlet and Doucet (1987) and, Barlet-Bas *et al.*, (1988) have also suggested that the action of aldosterone may be dependent on the presence of the thyroid hormone T<sub>3</sub>.

The site of production of mineralo- and gluco-corticoids is the adrenal gland. Their action has been studied by removing this organ (adrenalectomy) and replacing the hormones, by injection to give known concentrations. Many studies have shown that adrenalectomy lowers nephron Na,K-ATPase activity (Landon *et al.*, 1966; Chignell and

Titus, 1966; Barlet and Doucet, 1988), and this could be restored by the administration of large (pharmacological) doses of mineralocorticoids (aldosterone, deoxycorticosterone and triamcinolone (synthetic); Landon *et al.*, 1966) or glucocorticoids (corticosterone and methylprednisolone (synthetic); Chignell and Titus, 1966; Hendler *et al.*, 1972). Adrenalectomised rats placed on high salt diets, retained Na,K-ATPase activity, (Westenfelder *et al.*, 1977), suggesting that the loss of adrenal hormones may in some way, have been complemented by the action of a high [Na], in the blood. In sections of the kidney nephron where Na,K-ATPase activity was reduced by adrenalectomy (Medullary thick ascending limb, distal convoluted tubule, cortical collecting tubule and medullary collecting tubule), lost activity was replaced by glucocorticoids after 3 hours treatment (Garg *et al.*, 1985; Doucet *et al.*, 1986). Aldosterone replaced activity only in the cortical and medullary collecting tubules (El Mernissi and Doucet, 1983a; El Mernissi and Doucet, 1983b). In support of this, Doucet and Barlet-Bas (1989) have shown that substantial levels of type I, mineralocorticoid receptors exist only in the cortical collecting and medullary collecting tubule segments. However in contrast to the above, restoration of Na,K-ATPase activity in cortical collecting tubules after adrenalectomy, was found to occur after 1-3 days treatment with aldosterone whereas, corticosterone produced no effect on enzyme activity (Mujais *et al.*, 1984b). In the cortical and medullary collecting tubules with short term (3 hours) hormone treatment, enzyme activity was either not restored by aldosterone, corticosterone or the synthetic glucocorticoid dexamethasone (Mujais *et al.*, 1984a) or in another study, was restored by both aldosterone and dexamethasone treatment (El Mernissi and Doucet, 1983b). This apparent contradiction may be explained because, it has been demonstrated that the length of delay of the increase in Na,K-ATPase unit numbers is dependent on the initial number of Na,K-ATPase enzyme units at the onset of hormone treatment (Hayhurst and O'Neil, 1988; O'Neil, 1989).

More recently, the ability of glucocorticoids to increase the abundance of Na,K-ATPase mRNA and the number of ouabain binding sites on the cell surface in neonatal rat myocardial cells has been demonstrated (Orlowski and Lingrel, 1990). Dexamethasone treatment for 4 days was shown to increase the abundance of  $\alpha 2$  isoform mRNA and the number of high affinity ouabain binding sites ( $\alpha 2$  and/or  $\alpha 3$  isoform Na,K-ATPase units) by 2-fold, it also repressed the up-regulation of the  $\alpha 3$  isoform mRNA produced by T3 treatment.



Studies involving the hormonal regulation of Na,K-ATPase mRNA's have been carried out in a frog kidney epithelial cell line (A6 cells) (Verry *et al.*, 1987). Increases in both the rate of *de novo* synthesis and subsequent Na,K-ATPase mRNA abundances, were found after treatment with aldosterone (300 nM) for 6 or 96 hours. The rate of protein synthesis of the  $\alpha$  and  $\beta$  subunits was increased 1.5 to 3 fold, and the abundance of their respective mRNA's, was also increased 2 to 4 fold. The increase in Na,K-ATPase subunit biosynthesis rates and mRNA abundances were both inhibited by spironolactone (a specific mineralocorticoid antagonist; Fenestril, 1968). The increases in mRNA abundances were not, however, blocked by the protein synthesis inhibitor, cycloheximide. This demonstrates that aldosterone acts directly to increase the abundance of Na,K-ATPase mRNA's, without requiring the synthesis of another intermediary protein (Verry *et al.*, 1987). The rate of transcription of Na,K-ATPase genes has also been demonstrated to increase, 45 minutes after the onset of aldosterone treatment (Verry *et al.*, 1988). However, a high dose of aldosterone was used and consequently a response mediated via binding to a glucocorticoid type (II) receptor could not be dismissed. Indeed, experiments by Claire *et al.* (1989) have demonstrated the presence of both type I and type II hormone receptors in this cell line. The steroid hormone regulation of Na,K-ATPase is clearly complicated. The regulation of Na,K-ATPase by mineralo- and gluco- corticoid hormones may be even more complicated in mammalian tissues as separate receptors with highest binding affinity for corticosterone (called type III receptors) and deoxycorticosterone (called type IV receptors) in dog kidney and rat brain tissues have been reported (Ludens *et al.*, 1978; Fanestil and Park, 1981). The function of these putative receptors is unknown. However their existence may explain some of the contrasting results found for the regulation of Na,K-ATPase by mineralo- and gluco-corticoid hormones in the kidney (see the above text). It has however been reported that the type I and type III receptors, previously shown to have separate identities in both rat brain and kidney tissues, are similar if not identical to each other (Krozowski and Funder, 1983; Tashima *et al.*, 1989).

#### 1.V.ii.c.3. Regulation of Na,K-ATPase by thyroid hormones.

The action of the thyroid hormone, triiodothyronine (T3) on Na,K-ATPase has been studied in a number of tissues including rat and guinea pig brain, heart, kidney, liver, skeletal muscle and intestine. In all these tissues except brain, T3 increased Na,K-

ATPase enzyme activity and ouabain binding sites in euthyroid or hypothyroid rats (Ismail-Beigi and Edelman, 1970; Curfman *et al.*, 1977; Lin and Akera, 1978). In rat brain only early in development (up to 3 weeks after birth) was Na,K-ATPase activity and abundance responsive to T3 treatment. During this time, the abundance of both ( $\alpha$ ) ( $\alpha$ 1 isoform) and ( $\alpha$ +) ( $\alpha$ 2 and/or  $\alpha$ 3 isoforms) proteins were increased in hypothyroid rats after T3 treatment (Schmitt and McDonough, 1988). As with the regulation of the number of Na,K-ATPase units by other agents, the regulation of Na,K-ATPase by T3 has been investigated at the various stages of the production of the active Na,K-ATPase enzyme. The action of T3 on Na,K-ATPase has been studied by removal of the thyroid gland (thyroidectomy), followed by T3 replacement by injection. In thyroidectomized rats, T3 replacement caused increases of similar magnitude in the rate of biosynthesis of both the  $\alpha$  and  $\beta$  subunits of Na,K-ATPase after 8 or 22 hours in rat kidney (Lo and Lo, 1980). The degradation rates of Na,K-ATPase subunits were unchanged between hypothyroid and T3 treated rats. (Lo and Lo, 1980; Lo and Edelman, 1976). Changes in the rate of biosynthesis of the Na,K-ATPase  $\alpha$  subunit after T3 treatment have also been investigated *in vitro*, by the estimation of the abundance of protein produced by the translation of extracted guinea pig kidney cortex mRNA. After T3 treatment, the abundance of immunoreactive  $\alpha$  subunit protein, was increased two fold over controls. This suggested that T3 had caused an increase in the abundance of Na,K-ATPase  $\alpha$  subunit mRNA (McDonough, 1985). The effect of T3 on the level of  $\alpha$  ( $\alpha$ 1 isoform) subunit mRNA abundance has been studied in rat heart (ventricle), kidney and brain tissues (Chaudhury *et al.*, 1987). After 24 hours of T3 treatment, the abundance of  $\alpha$  subunit mRNA in heart and kidney increased 2.6- and 2.5-fold respectively, and after 72 hours, 3.9- and 1.9-fold respectively. In brain, no significant increase in  $\alpha$  subunit mRNA was found after either 24 or 72 hours of T3 treatment. This suggests that in responsive tissues such as heart or kidney, T3 may increase mRNA abundance by increasing the rate of transcription of the Na,K-ATPase genes. This has been shown to be the case in "nuclear run-on assays" (which measure the radioactive incorporation of nucleotides *in vitro*, into specific mRNA transcripts initiated in nuclei *in vivo*) in rat kidney cortex and liver (Gick *et al.*, 1988b). With T3 replacement in hypothyroid rats, Na,K-ATPase  $\alpha$  ( $\alpha$ 1 isoform) and  $\beta$  ( $\beta$ 1 isoform) subunit gene transcription rates were shown to be increased in kidney cortex both by 1.8-fold and in liver 1.3- and 1.4-fold respectively. Correspondingly,  $\alpha$  ( $\alpha$ 1 isoform) and  $\beta$  ( $\beta$ 1 isoform) mRNA abundances also increased in kidney cortex 2.3- and 2.6-fold respectively and in liver, 7.3-fold and

no change respectively. Increases in Na,K-ATPase enzyme activity were also measured. Enzyme activity increased by 2.3- and 1.3-fold in kidney cortex and liver respectively (Gick *et al.*, 1988b). The magnitude of the increases in kidney cortex Na,K-ATPase gene transcription rates, mRNA abundances and enzyme activity were similar suggesting a coordinated up regulation due to increased transcription. The situation in liver was clearly different and may have been complicated by the possible existence of significant concentrations of the other isoforms ( $\alpha_2$ ,  $\alpha_3$  or  $\beta_2$ ). However, it is likely that in liver, both transcriptional and post transcriptional regulation of Na,K-ATPase are modulated by T3. Many of the experiments on regulation of Na,K-ATPase by T3 cited above, were carried out using high doses (T3 = 50-130  $\mu\text{g}/100$  g body weight; except Schmitt and McDonough, 1988, where T3 = 10  $\mu\text{g}/100$  g body weight). When hypothyroid rats were treated with a physiological dose (1  $\mu\text{g}/100$  g body weight) of T3 no increase in ouabain-sensitive ATPase (Na,K-ATPase) activity was found in the liver (Haber and Loeb, 1986). Furthermore, no increase in biosynthesis rate of the  $\alpha$  and  $\beta$  subunits of Na,K-ATPase was shown after treatment of toad bladder cells with T3 ([T3] = 60 nM; Geering *et al.*, 1982). These results question the significance of results obtained with high doses of T3. However, Kim and Smith (1984) reported significant increases in ouabain binding with T3 concentrations from physiological ( $10^{-10}$  M) to supraphysiological doses ( $10^{-7}$  M), with a maximal 60% increase in Na,K-ATPase units obtained at  $10^{-8}$  M.

A recent study by Orłowski and Lingrel (1990) has suggested that T3 treatment in primary cultures of neonatal rat myocardial cells increases the abundance of  $\alpha_2$ ,  $\alpha_3$ , and,  $\beta_1$  isoform mRNA's without increasing the abundance of the  $\alpha_1$  isoform mRNA. The magnitude of these increases was 7-, 7- and 4- fold for the  $\alpha_2$ ,  $\alpha_3$ , and,  $\beta_1$  isoform mRNA's respectively. The increase in the  $\alpha_3$  isoform mRNA occurred the most rapidly, reaching near maximum after 2 days. The increase in the  $\alpha_2$  and  $\beta_1$  isoform mRNA's occurred more slowly, and only reached maximum abundance after 8 days treatment. The concentration of T3 (in the range of  $10^{-6}$ - $10^{-10}$  M) found to produce the highest effect on the abundance of all the isoform mRNA's was also at  $10^{-8}$  M, which is in agreement with Kim and Smith (1984). Cell cultures treated with concentrations greater than  $10^{-8}$ M did not elicit as great an increase in mRNA abundance, this effect may be due to the down regulation of T3 receptor concentration following chronic incubation with high concentrations of hormone (Orłowski and Lingrel, 1990). The effect of T3 in similarly derived neonatal rat non-myocardial cells was also studied (Orłowski and Lingrel, 1990). In these cells, although  $\alpha_1$  and  $\alpha_3$  isoform mRNA's were present, T3 had no effect on



their abundance. However, a small effect on the abundance of the  $\beta 1$  isoform mRNA was found. T3 induced a 3-fold increase in the number of high affinity ouabain binding sites ( $\alpha 2$  and/or  $\alpha 3$  isoform Na,K-ATPase units) in myocardial cells, after 4 days treatment (Orlowski and Lingrel, 1990). The reasons for the contrasting responses of Na,K-ATPase isoforms to thyroid hormone treatment in different cell populations are not known.

Previous results have led some authors to suggest that T3 plays a permissive role and is required for the stimulation of Na,K-ATPase activity by other hormones such as aldosterone (Barlet *et al.*, 1988; Barlet and Doucet, 1987). However, the presence of T3 does not seem to be a prerequisite for the action of corticosterone in rat kidney and submandibular gland (Bartolomei *et al.*, 1983; Klein *et al.*, 1984a), and these two hormones have been suggested to work via parallel independent pathways (Klein *et al.*, 1984b). Dexamethasone treatment has however been shown to repress the increase in  $\alpha 3$  isoform mRNA abundance induced by T3 in neonatal rat myocardial cells (Orlowski and Lingrel, 1990).

#### 1.V.ii.c.4. Regulation of Na,K-ATPase by progesterone and oxytocin.

Two other hormones which have been studied to a lesser extent are progesterone and oxytocin. Progesterone has been shown to inhibit Na,K-ATPase activity in *Xenopus* (frog) oocytes during their maturation process (Richter *et al.*, 1984; Richter and Passow, 1985). The mechanism for this inhibition is unknown however, regulation of gene transcription is a distinct possibility. Oxytocin on the other hand, was shown to stimulate Na transport in toad bladder, in a synergistic manner with aldosterone. However, oxytocin had a lower but non-additive effect (compared to aldosterone) on the rate of biosynthesis of Na,K-ATPase  $\alpha$  and  $\beta$  subunits (probably  $\alpha 1$  and  $\beta 1$  isoforms). Oxytocin, aldosterone, and oxytocin plus aldosterone, increased the biosynthesis rates 1.9-, 2.6-, and 2.7-fold for the  $\alpha$  subunit, and by 1.6-, 2.2-, and 2.9-fold for the  $\beta$  subunit, respectively (Girardet *et al.*, 1986).

#### 1.V.iii. Unanswered questions regarding the regulation of Na,K-ATPase.

A considerable amount of information on the regulation of the Na,K-ATPase remains to be elucidated. This includes information on the following items:- 1, What determines

the difference between the substrate binding affinity properties of the purified enzyme compared to the properties of the enzyme in whole cells? 2, What are the identities and physiological role(s) of the putative endogenous natriuretic substances which inhibit Na,K-ATPase? What is their mechanism of action and, how similar is this action to the action of cardiac glycosides? 3, Is there a primary lesion in the regulation of Na,K-ATPase in certain cases of essential hypertension? How do secondary changes in intravascular pressure affect the expression of the isoforms of Na,K-ATPase? 4, Is there a role for the endogenous intracellular inhibitor of Na,K-ATPase, vanadate? 5, What are the mechanisms of action of hormones such as insulin, glucagon, dopamine, angiotensin II, vasopressin, and noradrenalin, on the regulation of Na,K-ATPase activity? What secondary messenger systems link the hormone-receptor complex's of dopamine, angiotensin II, vasopressin, and noradrenaline to the regulation of Na,K-ATPase activity? 6, What is the role of calcium in the regulation of Na,K-ATPase? 7, How do changes in the  $[Na]_i$  regulate the numbers of Na,K-ATPase enzyme units in the plasma membrane? 8, What is the function of the intracellular pool of Na,K-ATPase units, how is it regulated? 9, Is the production of  $\alpha$  and  $\beta$  subunit mRNA's and proteins coordinated, and if so by what mechanism? 10, By what mechanism(s) do the steroid and thyroid hormones act? 11, What determines the basal level of Na,K-ATPase enzyme production in different tissues? and finally 12, How are the numbers of Na,K-ATPase enzyme units regulated concomitantly with the other cellular proteins using the cells Na gradient?

### **1.VI. MDCK cells; an epithelial cell model.**

The cell line MDCK (Madin-Darby Canine Kidney) was derived in 1958 from the kidney of a normal male Cocker spaniel (Madin and Darby, 1958; Leighton *et al.*, 1969). Based on morphology and the expression of cell surface antigenic determinants, this cell line is thought to originate from the late distal tubule or collecting duct regions of the kidney nephron (Bowen and McDonough, 1987). MDCK cells exhibit many of the characteristics of renal epithelial cells, including the formation of tight junctions and the vectoral transport of salts and water (Bowen and McDonough, 1987). This vectoral transport function, reflects the polar organisation of epithelial cells, which is accomplished by the maintenance of different sets of cell surface components in each membrane domain. MDCK cells possess all the morphological characteristics of epithelial

cells in that they have both apical and basolateral (basal and lateral) membrane domains or compartments (Simons and Fuller, 1985). The apical (luminal or mucosal) membrane is invaginated with microvilli and is oriented towards the incubation medium. The basolateral (serosal) membrane has many structures associated with it including :- tight junctions (zona occludens), intermediate junctions (zona adherens), desmosomes, and gap junctions in the lateral membrane and, in the basal membrane, hemidesmosomes which hold the cell to the extracellular matrix (see figure 5.). The two plasma membrane domains are divided by the tight junctions which encircle the apex of the cell and seal neighbouring cells to each other. Intermediate junctions and desmosomes connect the lateral membranes of adjacent cells (Simons and Fuller, 1985).

MDCK cells when grown in culture, form a confluent monolayer of cells. Two strains of MDCK cells (strain I and II) have been derived. Monolayers of these two strains show many different properties, including varying electrical resistance. Strain I MDCK cells form tight monolayers with resistances over 3000 ohm/cm<sup>2</sup>, whereas strain II cells are more leaky to the passage of ions and exhibit resistances of only around 100 ohm/cm<sup>2</sup> (Simons and Fuller, 1985). The electrical resistance of MDCK monolayers is derived from the tight junctions between cells. The tight junctions act as gates to regulate the passage of ions from one side of the monolayer to the other, they are also impermeable to larger molecules (Simons and Fuller, 1985). When grown on impermeable supports such as glass or plastic, monolayers of MDCK strain I cells form blisters (cysts, vesicles or domes), which expand, distend and then collapse. These

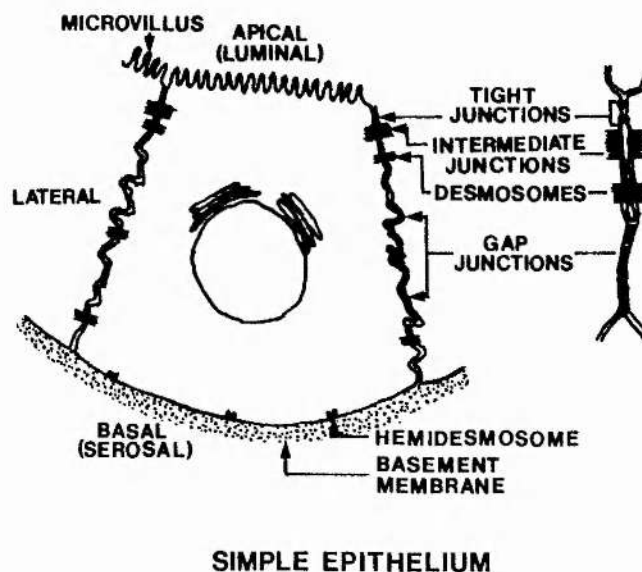


Figure 5. A simplistic diagram of an epithelium showing the structural characteristics of the plasma membrane of epithelial cells such as MDCK. Adapted from Simons and Fuller (1985).

blisters are thought to represent interstitial collections of actively absorbed components from the medium (actively transported solutes accompanied by water ; Leighton *et al.*, 1969; Rabito *et al.*, 1978). The properties of the cell monolayer in blistered areas have not been found to be significantly different from those in non blistered areas and hence, blisters are not thought to represent a special structural component of epithelial monolayers (Rabito *et al.*, 1978). Blisters are not formed when MDCK cells are grown on permeable supports, as this allows free access to both surfaces of the cell monolayer.

#### 1.VI.i. The polarity of monolayers of MDCK cells.

The apical-basolateral polarity of MDCK cells found at the morphological level, also extends to the molecular level. MDCK cells secrete laminin and heparin sulphate proteoglycan only through the basolateral membranes to form the basement membrane (Hubbard *et al.*, 1989). This secretion is stimulated by the initial attachment of MDCK cells to the substrate, via binding of type I collagen receptors to collagen (Rodriguez-Boulan and Salas, 1989). Elements of the cytoskeleton also show cellular polarity. In the apical domain, microvilli are composed of bundles of actin filaments which are linked to the terminal web, which is a dense network of spectrin (also known as foderin) molecules overlying a layer of intermediate filaments. (Alberts *et al.*, 1989). In the basolateral domain, the structural proteins, ankyrin and foderin are attached to major cell membrane proteins such as Na,K-ATPase (Nelson and Veshnock, 1987; Koob *et al.*, 1987; Morrow *et al.*, 1989; and Nelson and Hammerton, 1989) and the Erythrocyte band 3 anion exchange protein (Low, 1986) which is also present in kidney epithelia (Kudrycki and Shull, 1989). Intermediate filaments form a framework which links the nuclear matrix to desmosomes and hemidesmosomes, structurally connecting adjacent epithelial cells (see figure 5.) (Handler, 1989). Plasma membrane lipids are also polarised. The diffusion of lipids in the outer leaflet of the membrane bilayer, between basolateral and apical membrane domains, is restricted. This restriction, is probably mediated by proteins within the tight junctions of the cell. Consequently, lipids not able to flip between the outer and inner leaflets of the membrane bilayer are trapped in their respective membrane domains (Cereijido *et al.*, 1989). It is thought that the apical membranes are enriched in phosphatidylethanolamine, phosphatidylserine and certain glycolipids, and the basolateral membranes are enriched in phosphatidylcholine, phosphatidylinositol and sphingomyelin (Handler, 1989; Rodriguez-Boulan and Salas,



1989). Recent evidence, suggests that the uneven distribution of these lipids reflects both the polarised delivery of these components to their respective domains, and the subsequent inability of lipids to move across tight junctions (Rodriguez-Boulan and Salas, 1989).

The mechanism by which plasma membrane proteins are located in each plasma membrane domain has been investigated extensively. It is thought that all plasma membrane proteins are located together, in the same intracellular compartments during synthesis, until they reach the TGN (trans-golgi network). Here, one or more sorting events occur where it is thought membrane proteins are selected for redirection towards the apical or the basolateral membrane (Rodriguez-Boulan and Salas, 1989). These proteins may have higher affinities for the lipids being directed towards the correct membrane domain (see above; Rodriguez-Boulan and Salas, 1989), and they may thus be incorporated into the same vesicles as lipids destined for the correct membrane domain. It has been suggested that, as apical membrane proteins tend to be specialized and only found in polarized cells. The basolateral membrane would then represent the default pathway for all the other membrane proteins (Hubbard and Steiger, 1989). It has recently been shown that the presence of a glycoso-phosphoinositol (GPI) membrane anchor on membrane proteins caused them to be selected for insertion into the apical membrane (Brown *et al.*, 1989). The GPI membrane anchor is attached to proteins in the endoplasmic reticulum in response to the presence of a hydrophobic signal sequence on the carboxyl terminal end of the protein, which is subsequently cleaved off (Lisanti *et al.*, 1989). However, the presence of a GPI membrane anchor on proteins is probably not the only determinate for the targeting of apical membrane proteins (Brown, *et al.*, 1989). A resorting mechanism may also exist for membrane proteins erroneously inserted into the other membrane domain. Evidence from rat hepatocytes suggests that some of these resorted proteins may be transcytosed to the correct membrane domain (Hubbard and Stieger, 1989; Cereijido *et al.*, 1989).

#### 1.VI.ii. Regulation of Na,K-ATPase in MDCK cells.

The polarity of a number of major plasma membrane proteins such as Na,K-ATPase have also been investigated in MDCK cells. Na,K-ATPase has been shown to be located almost entirely in the basolateral membrane domain (Caplan *et al.*, 1988; Lamb *et al.*, 1981). It has recently been shown that polarisation of a basolateral marker (an un-named

63 kDa protein), but not an apical (an un-named 184 kDa protein) marker, required cell-cell interactions (Vaga-Salas, *et al.*, 1987). Cell-cell interactions also resulted in the formation of the insoluble ankyrin/foderin network (elements of the cytoskeleton). This formed a very high molecular weight, insoluble complex with approximately 50% of the total Na,K-ATPase enzyme in the cell (Nelson and Hammerton, 1989; Jesaitis and Yguerabide, 1986; Rodriguez-Boulan and Salas, 1989; Cereijido *et al.*, 1989). Whether the enzyme units in this complex are active or differentially regulated remains to be determined. Furthermore, the mechanism of the attachment of Na,K-ATPase to ankyrin has not yet been determined. When cells were grown in Ca free medium which prevents tight junction formation (Cereijido *et al.*, 1978) and consequently loss of basolateral polarisation, Na,K-ATPase was distributed evenly over the cell surface. When Ca was added tight junctions and cell-cell contacts recovered rapidly, trapping one third of the Na,K-ATPase molecules in the apical domain (Contreras *et al.*, 1989). This enzyme was removed from the apical domain with a half time of 80 minutes but was not reinserted into the basolateral membrane (Cereijido *et al.*, 1989). When explants of MDCK cells were plated on type I collagen, single cells migrated from the explant (Zuk *et al.*, 1989). These cells were fusiform in shape, and showed loss of cell-cell contact and cell polarity. When this occurred, it is thought that the foderin cytoskeletal network had disassembled, and Na,K-ATPase was distributed symmetrically on the cell surface. Also, a larger amount of the Na,K-ATPase enzyme was located intracellularly rather than in the plasma membrane (as determined by an immunofluorescence/ monoclonal antibody assay; Zuk *et al.*, 1989). This intracellular pool of Na,K-ATPase, was thought to represent newly synthesised enzyme which could not be inserted into the plasma membrane, or enzyme that was endocytosed during the change to the fusiform cell shape. When the fusiform MDCK cells reached confluency, they regained cell-cell contacts and the intracellular pool of Na,K-ATPase decreased. This was presumed to represent the insertion of Na,K-ATPase into the basolateral membrane possibly due to the reformation of the cytoskeletal network (foderin/ankyrin; Zuk *et al.*, 1989). However, Caplan *et al.* (1985) found no evidence of a cytosolic pool of Na,K-ATPase (as a result of new synthesis of the enzyme  $\alpha$  subunit). This indicates that the intracellular pool must be formed by the withdrawal previously synthesised enzyme units already inserted into the membrane. It is not known whether these enzyme units can be re-inserted into the plasma membrane in MDCK cells, however in cultured chick skeletal muscle cells this appears to be the case (Takeyasu *et al.*, 1989).



Na,K-ATPase enzyme in MDCK cells has been shown to bind ouabain half maximally (or K<sub>d</sub>) at a concentration of 60-100 nM (Soderberg *et al.*, 1983; Lamb *et al.*, 1981). The highest number of ouabain binding sites reported on confluent MDCK cells is  $8 \times 10^7$  sites per cell (Kennedy and Lever, 1984). Cells grown chronically in concentrations of up to 4 mM ouabain develop ouabain resistance and show biphasic ouabain-sensitive rubidium (Rb) influx curves. This has been postulated to possibly be due to the expression of a low ouabain affinity Na,K-ATPase enzyme (Soderberg *et al.*, 1983). More than one isoform of Na,K-ATPase has been demonstrated to exist in canine brain and heart tissues (McDonough and Schmitt, 1987). The resistant cells, when grown in culture with wild type cells were found to assist the neighbouring wild type cells, enabling them to survive in medium containing ouabain. The means by which this was achieved was unknown although, the sharing of the ion pumping load through gap junctions was dismissed, due to the apparent lack of diffusion of small molecules from cell to cell, monitored by the movement of microinjected fluorescein, during ouabain treatment (Rossi *et al.*, 1985). However, Potapova *et al.* (1990) have shown that ouabain-resistant BHK hamster cells could maintain the membrane potential of ouabain-sensitive human fibroblasts in the presence of ouabain ( $10^{-6}$  M). They postulate that this was probably due ion fluxes through cell-cell gap junctional connections.

Both, a partial nucleic acid sequence for the  $\alpha$  subunit and the full cDNA for the  $\beta$  ( $\beta$ 1) subunit have now been cloned and sequenced in canine (dog) species (Bowen and McDonough, 1987; Brown *et al.*, 1987). The dog  $\alpha$  subunit partial cDNA sequence is from the central portion of the protein between aa's 242 and 746 and exhibits 90% nucleotide homology with the equivalent sheep region (Bowen and McDonough, 1987). Na,K-ATPase in MDCK cells has been shown to be regulated, as in other cells, by changes in  $[Na]_i$  (Bowen and McDonough, 1987). Both Na,K-ATPase  $\alpha$  and  $\beta$  subunit mRNA's and enzyme subunits have been shown to be coordinately increased in response to increased  $[Na]_i$  (Bowen and McDonough, 1987). Preliminary investigations of the hormonal regulation of Na,K-ATPase in MDCK cells have also been carried out. MDCK cells have been shown to express a low affinity, high capacity steroid binding sites, which have greatest affinity for the major mineralocorticoid in dog, deoxycorticosterone (DOC; Ludens *et al.*, 1978). However, DOC has only been shown to stimulate Na transport in MDCK cells after 60 hours incubation, whereas preincubation with another steroid, aldosterone caused stimulation of Na transport in only 4 hours (Simmons, 1977).

1.VI.iii. Questions and information on Na,K-ATPase in MDCK cells, that remains to be determined.

Clearly, with the wealth of information known about Na,K-ATPase in MDCK cells, this cell line represents a good model for further investigation of the regulation of Na,K-ATPase in epithelial monolayers. Information that remains to be determined includes the following :- 1, What is the exact mechanism(s) which sorts apical and basolateral proteins into their respective membrane domains? How is the polarisation of Na,K-ATPase of the basolateral membrane domain achieved? 2, How does cell-cell contact regulate the formation and disassembly of the ankyrin/foderin cytoskeletal network? 3, What is the location of ankyrin binding site on Na,K-ATPase, and what is ankyrin's role in regulating the sub-cellular location of the enzyme? Likewise, what role (if any) does Na,K-ATPase have in the formation of the cytoskeletal network? 4, What isoforms of Na,K-ATPase are expressed in MDCK cells? 5, Is the regulation of Na,K-ATPase different in MDCK cells grown on different substrates? 6, What hormones regulate Na,K-ATPase activity and enzyme abundance in MDCK cells? How do these properties relate to the tissue of origin, the distal tubular or collecting duct epithelium of the kidney? The purpose of this project was to try to answer some of these questions.

CHAPTER 2. GENERAL METHODS.

## 2.I. Introduction.

### 2.I.i. Cell biology.

The subject area called "tissue culture" is a general term with 3 main divisions. These are 1, Organ culture, where the whole or parts of a structurally intact organ are removed from the live animal and maintained in a viable state in culture. This is done in such a way that allows (where appropriate), growth, differentiation and preservation of its architecture and/or function; 2, Tissue culture, where fragments of tissue, not necessarily possessing the organs original structure, are maintained in culture; and 3, Cell culture, where cells are grown in culture, after having been completely dissociated from the organ or tissue of origin (Freshney, 1987).

The first successful culture of organised tissue was carried out by Roux in 1885, who used this technique to prove that the closure of the neural tube in developing chick embryo, was controlled by the constituent cells and not the structures surrounding them *in vivo* (Parker, 1961). Later, Harrison in 1907 devised tissue culture as a method for studying the behaviour of frog cells free of systemic variations (Harrison, 1907).

This illustrates one of the major advantages of tissue culture in that the experimenter has control over the physiochemical environment (pH, temperature, osmotic pressure, O<sub>2</sub> and CO<sub>2</sub> tension). These may be controlled very precisely and may be kept relatively constant. Another advantage is, that virtually the same cell population may be experimented upon at intervals to produce replicate results. These results may also be compared to other experimental data derived previously, to provide a large amount of information about the same cells (Freshney 1987). There are however some disadvantages to cell culture. One of the major disadvantages of cell culture is that when cells are dissociated from their natural environment *in vivo*, they are usually placed in a two dimensional environment. In this environment, cells have no contact with their original neighbours, which are often morphologically and physiologically different cell types. This change in environment, often leads to alterations in the cells normal morphology and physiology. This can lead *in vitro*, to the loss of many of the normal functional characteristics with which the cells were associated, *in vivo*. Changes in cell morphology and function can also be the result of de-differentiation (sometimes caused by the relatively slow or non-growth of terminally differentiated cells being overgrown by un-differentiated cells) and, de-adaption (caused by lack of physical and chemical

stimuli in culture i.e. lack of appropriate hormones, cell-cell contact or cell matrix-interactions) (Freshney, 1987).

Cell lines are usually derived from primary cultures where tissues or dispersed cells are placed in culture for the first time. Cells which adhere to the culture chambers surface or survive in suspension form the basis of the primary culture. When these cells are sub-cultured or passaged they become a cell line. Most cell cultures can only propagate for a limited number of cell generations before losing viability and dying. However some may give rise to a continuous cell line which has a potentially unlimited life-span and is hence immortal. This alteration in a culture is called transformation and may occur spontaneously or be chemically or virally induced (Freshney, 1987). Paradoxically, many continuous cell lines established from tumours frequently show better retention of specialised functions than those derived from normal tissue (Paul, 1970).

Continuous or established cell lines show similar growth kinetics to bacteria. The growth cycle is conventionally divided into three phases, lag phase, log phase and plateau phase. Lag phase is the time following re-seeding of the culture after passaging. This is a period of re-organisation for the cells. Cells replace surface components damaged during subculturing, attach to the substrate, spread out and resume their normal cytoskeletal organisation, to yield their characteristic cell morphology. When the cells have adapted to their new environment they enter log phase which is a period of rapid cell growth and division leading to an exponential increase in cell numbers. This is the period of greatest uniformity, where the cells are in a similar metabolic condition (ie the great majority of cells are growing rapidly), and where their viability is high (90-100%). The growth rate during this period depends not only on the type of cells involved but also on the nutrient supply and seeding density. This phase is terminated one or two cell divisions after confluence (where the surface of the growth chamber is completely covered) is reached. When this occurs the cells enter plateau (stationary) phase where further proliferation or growth is reduced or ceases. This may be due to many reasons including contact inhibition, reduced cell spreading, build up of the toxic products of metabolism or, depletion of nutrients and growth factors in the medium (Freshney, 1987).

### 2.I.ii. Molecular biology.

The expansion of molecular biology and molecular genetic techniques available today began in the early 1970's with the simultaneous development of techniques to transform

bacterial cultures with DNA plasmids and the ability of these DNA molecules to be altered in a precise manner by the cutting and joining of the DNA by restriction enzymes and ligases. Furthermore this process could be monitored by gel electrophoresis (Old and Primrose, 1985). Today, a plethora of molecular biology techniques are available which revolve around the ability to engineer DNA molecules. Several texts have become available giving protocols of a wide range of the currently available molecular biological techniques. The majority of the methods in this chapter involving molecular biological techniques have utilised the texts available including those of Maniatis *et al.* (1982) and Davis *et al.* (1986). Where necessary modifications to techniques have been made by comparison of similar techniques from other sources such as Walker, (1984) and Stewart and Letham, (1973). Some methods have been derived from research papers such as the modified total RNA extraction procedure from Cathala *et al.* (1983). More recently practical methodology text books (Berger and Kimmel, 1987; Sambrook *et al.*, 1989) have provided much more information on the reasoning behind the use of materials and methods in protocols. This has made solving problems with molecular biological techniques much easier.

#### 2.I.ii.a. nucleic acid hybridisation.

Nucleic acid hybridisation is the formation of double stranded nucleic acid molecules by sequence-specific base pairing of complementary single strands of either DNA or RNA. Hybridisation can therefore form double stranded molecules of either DNA, RNA or hybrids containing one strand of DNA and RNA. Hybridisation experiments can either be performed in solution or with one strand of the complementary nucleic acid bound to an inert support such as a membrane filter (mixed phase hybridisation). Solution hybridisation is usually performed for the the purpose of kinetically analysing the association of nucleic acids (Young and Anderson, 1985). The development, in the 1960's, of methods to immobilise nucleic acids on nitrocellulose filters (Nygaard, 1963) and to detect specific sequences with radioactive probes (Denhardt, 1966; Gillespie and Spiegelman, 1965), formed the basis for present day gene detection methods. Several types of filter materials are now available for the binding of nucleic acids. These materials include nitrocellulose (Nygaard, 1963), activated celluloses such as diazobenzoyloxymethyl-cellulose (Alwine *et al.*, 1977) and charge modified nylon (Reed and Mann, 1985). Several methods, are available for the transfer of nucleic acids to filters



such as nitrocellulose. Nucleic acids can be transferred to filters by Southern or Northern blotting and dot or slot blotting.

#### 2.I.ii.a.1. nucleic acid transfer to filters.

Southern and Northern blotting methods involve the transfer (blotting) of DNA and RNA respectively, from electrophoretic gels to inert membrane supports. Individual RNA's can be separated by agarose gel electrophoresis using denaturants such as glyoxal and dimethyl sulphoxide (Macmaster and Carmichael, 1977), or formaldehyde (Lehrach *et al.*, 1977). The RNA can then be transferred from agarose gels by three techniques; 1, capillary blotting, where the capillary action of 20 x SSC through the gel is used to carry the RNA onto the filter 2, vacuum blotting, where the fluid from the gel is drawn through the filter by negative pressure and 3, electroblotting, where nucleic acids are electrophoresed from the gel onto the filter. Transfer and adsorption to nitrocellulose requires the presence of a high salt concentration ie 20 x SSC (see Appendix 3). Although, the reason why high salt conditions are required for the adsorption of nucleic acids to nitrocellulose, has not been determined.

Dot and slot blotting, involves the vacuum filtration of nucleic acids through the spherical holes (dots) or slots cut in special manifolds, onto nitrocellulose or nylon filters. This results in round dots or band shaped slots of nucleic acids deposited on the filter. Nucleic acids can also be spotted onto filters by hand and then dried (Thomas, 1980). Dot blots also require the presence of 20 x SSC for the adsorption of RNA onto nitrocellulose filters. Methods are available for the transfer and adsorption of RNA to nitrocellulose using either denaturing (ie. in the presence of formaldehyde; Meinkoth and Wahl, 1984 ; or glyoxyl; Thomas, 1980), or non-denaturing conditions (Thomas PNAS; Davis *et al.*, 1986). Thomas (1980), has shown that RNA adsorbs to nitrocellulose most efficiently when it is fully denatured. However, they also suggest that RNA's such as globin mRNA do not need to be fully denatured to bind efficiently. This is suggested to be due to the lower amount of secondary structure in globin mRNA compared to ribosomal and transfer RNA's (Thomas, 1980).

Independent of the methods of transfer and adsorption of nucleic acids to filter supports Thomas (1980) have shown that nucleic acids can be easily washed off nitrocellulose filters. Nucleic acids therefore have to be fixed to the nitrocellulose, otherwise they would wash off filters during hybridisation. The binding of nucleic acids

to nitrocellulose is generally achieved by the baking of nitrocellulose filters at 80°C for 2 hours. The mechanism by which nucleic acids irreversibly bind to nitrocellulose is not understood. The transfer of nucleic acids to nylon filters (unlike binding to nitrocellulose filters), does not require high salt conditions (20 x SSC) (Reed and Mann, 1985). Irreversible binding of nucleic acids to nylon filters is generally achieved by baking for short periods (80°C, 30 minutes; Zeta probe, Biorad ), alkali treatment (Hybond N<sup>+</sup>; Amersham international), or UV crosslinking (Duralon-UV; Stratagene).

After nucleic acids have been fixed to the filters, they are ready for hybridisation. Filter hybridisation experiments are usually performed with a radiolabelled nucleic acid probe which is complementary to specific RNA or DNA molecules bound to the filter support. Methods for producing non-radioactively labelled nucleic acids probes are also available. These include the use of biotinylated or digoxigenin labelled probes which are detected by an enzyme linked colourimetric assay. Which ever probe is used, the filters used for hybridisation are normally pre-incubated in a solution similar to that used for the hybridisation itself, but not containing the labelled probe. This process, known as pre-hybridisation, is done to coat the filter in blocking agents to prevent the non-specific adsorption of radiolabelled probe during hybridisation. Several reagents are used to reduce the level of non-specific background. The main reagent for reducing non-specific background is Denhardt's reagent (see Appendix 3.; Denhardt, 1966). However, both denatured sheared heterologous DNA (salmon sperm) and SDS have been successful used as supplementary blocking agents. The pre-hybridisation or hybridisation solutions are normally sealed inside a plastic bag with the filter and incubated at the appropriate temperature in a water bath. After hybridisation is complete the filter is washed in solutions containing detergents (0.1% SDS) and various levels of salt (0.1-6 x SSC), to selectively remove non-specifically bound probe from the filter.

#### 2.I.ii.a.2. factors affecting hybridisation.

Many factors affect both the rate and extent of hybridisation. Some of the more important factors are discussed below.

- 1, The concentration and nature of the labelled probes:- Increasing the concentration of nucleic acid probes, increases the rate of hybridisation. However, for double stranded probes, which re-anneal during hybridisation, the effect of increasing probe concentration

is more limited than for single stranded probes. This is because the rate of double stranded probe re-annealing is also increased, with increasing probe concentration.

2, The molecular weight of the probes:- For the re-annealing of DNA, the rate of hybridisation is proportional to the square root of the molecular weight of the DNA (Wetmur and Davidson, 1968). However for the hybridisation of probes to nucleic acids on filters, two situations can occur. When the amount of specific nucleic acid sequence on the filter is less than the amount of probe, the rate of hybridisation is limited to the rate of formation of the initial duplex between the probe and its filter bound complementary specific nucleic acid sequence (nucleation). However, when the amount of probe is lower than the amount of specific nucleic acid sequence on the filter, the rate of hybridisation is limited by the rate of diffusion of the probe.

3, The temperature of hybridisation:- The temperature at which experiments are performed affects the rate of hybridisation (Marmur and Doty, 1961). The rate of hybridisation shows a broad bell shaped temperature dependence curve, which for DNA-DNA annealing is at a maximum 20-25°C below  $T_m$  (where  $T_m$  is the temperature at which, at equilibrium half of the specific double stranded nucleic acid molecules have "melted" into their single stranded components). A similar relationship also exists for DNA-RNA annealing except that the rate of hybridisation is at a maximum at 10-15°C below  $T_m$  (Birnstiel *et al.*, 1972). The  $T_m$  of RNA-RNA duplexes is about 10°C higher than that of comparable DNA-DNA duplexes, with DNA-RNA duplexes intermediate in stability (Kimmel and Berger, 1987).

4, The base composition of the nucleic acids:- The formation of guanine-cytidine (G-C) base pairing is more stable than adenine-thymine (A-T) or adenine-uracil (A-U) base pairing. This is due to the presence of three hydrogen bond in the G-C base pairs compared to two between A-T base pairs. This increases the thermal stability of nucleic acids with high G-C content. As a result nucleic acids with high G-C contents exhibit elevated optimum hybridisation temperatures (which is related to the  $T_m$  of the nucleic acid). The base composition of nucleic acids also affects the rate of hybridisation. A change in the mole fraction of G-C base pairs from 0.2 to 0.6 causes an increase in the rate of hybridisation of around 1.5 fold (Kimmel and Berger, 1987).

5, The concentration of formamide:- The presence of formamide, destabilises double stranded nucleic acids and consequently, reduces their  $T_m$  (Young and Anderson, 1985). The reduction in  $T_m$  is greater for poly (A-T) than for poly (G-C) base paired nucleic acids (Casey and Davidson, 1977). The rate of hybridisation of nucleic acids in solution

in the presence of formamide follows a multiphasic bell shaped curved relationship. Little change in the rate of hybridisation is found over 30-50% formamide. However, in the presence of only 20% formamide the rate of hybridisation is reduced by about one third (Howley *et al.*, 1979). Hybridisation in solution in the presence of 80% formamide, reduces the rate of DNA-DNA hybridisation by a factor of three. Similarly for DNA-RNA annealing, the rate of hybridisation in 80% formamide is reduced by a factor of 12 (Casey and Davidson, 1977). Similar results are likely for filter hybridisation experiments.

6, The ionic strength of the hybridisation solution:-The rate of re-annealing of DNA with increasing ionic strength (monovalent cations), increases up to at least 3.2 M [Na] (Wetmur and Davidson, 1968). This is thought to be due to changes in the electrostatic interactions between the two strands of nucleic acid polymers (Wetmur and Davidson, 1968). The effect is however, most dramatic at low salt concentrations (at or below 0.1 M Na). DNA-DNA hybridisation in 1.0M NaCl occurs 7 times more rapidly than at 0.18 M NaCl. Decreasing the salt concentration to 0.09 M reduces the rate an additional 5 fold (Kimmel and Berger, 1987). However, the effect of ionic strength on DNA-RNA hybridisations is somewhat different. At 0.18 M NaCl the rate of DNA-RNA hybridisation is equivalent to that of DNA-DNA hybridisation. However, at 1 M NaCl the rate of DNA-RNA duplex formation is only twice that at 0.18 M NaCl. This contrasts sharply with the equivalent figure for DNA-DNA duplex formation given above. The ionic strength of the hybridisation solution also affects the  $T_m$  of the nucleic acids. The  $T_m$  of double stranded DNA increases with increasing ionic strength up to approximately 1.0 M [Na] (Wetmur and Davidson, 1968).

7, The percentage and distribution of mismatches:- The percentage of base pair mismatches in nucleic acids affects the rate of hybridisation. At the temperature optimum (25°C below the  $T_m$  for DNA) the rate of hybridisation is reduced by a factor of 2 for every 10% of mismatches (Bonner *et al.*, 1973). The  $T_m$  of nucleic acid hybrids is also reduced by mismatching. A collection of experiments utilizing solution hybridisation suggest that for every 1% of mismatching the  $T_m$  is reduced by between 0.5 and 1.4°C, depending on the G-C base pair content of the nucleic acid (Anderson and Young, 1985). The  $T_m$  for a specific nucleic acid hybridisation also depends on the distribution of mismatches. A duplex with all mismatches concentrated at one end of the molecule (with the other end having perfectly matched base pairs), will be relatively stable and will have a high  $T_m$ . A comparable duplex with the same amount of mismatches distributed evenly,



say every fifth base pair, will be relatively unstable and have a lower  $T_m$ . The effect of base pair mismatching on the stability of hybrids is dependent on the ionic strength of the solution. At high salt concentrations mismatched hybrids are much more stable than at low concentrations, although the reason for this is unknown (Anderson and Young, 1985).

8, The viscosity of solution:- Little is known about the effect of viscosity on hybridisation, except that as the viscosity of the solution increases, the rate of hybridisation decreases (Anderson and Young, 1985).

9, The pH of the solution:- The effect of pH of the solution on the rate of hybridisation is small. At high salt concentrations (>0.4 M NaCl), the rate of hybridisation changes less than 1.3 fold in the pH range 5-9 (Wetmur and Davidson, 1968).

The above factors which affect the rate and formation of nucleic acid hybrids are obviously complex, and make the prediction of the optimum conditions of hybridisation difficult to assess. A formula has been derived to enable the approximate estimation of the  $T_m$  of DNA-DNA hybrids (Bolton and McCarthy, 1962).

$$T_m = 81.5^\circ\text{C} - 16.6(\log_{10}[\text{Na}]) + 0.41(\%G+C) - (600/n) - 0.63(\% \text{ formamide})$$

where n = the length of the hybrid in base pairs.

Formulas have also been derived for RNA probes hybridising to immobilized RNA (Bodkin and Knudson, 1985)

$$T_m = 79.8^\circ\text{C} + 18.5(\log_{10}[\text{Na}]) + 0.58(\%G+C) + 11.8 (\%G+C)^2 - 0.35(\% \text{ formamide}) - (820/n).$$

A similar formula has been derived for DNA-RNA hybrids, which is identical to that for RNA-RNA hybrids except that the formamide term in the equation is replaced by -0.50(% formamide) (Casey and Davidson, 1977).



### 2.I.ii.a.3. the stringency of hybridisation.

Because of the effect of base pair mismatching on  $T_m$ , (increased mismatching reducing the  $T_m$  (see above)) theoretically, hybridisation conditions for any nucleic acid can be devised such that hybridisation can only occur between perfectly matched sequences. Such conditions of hybridisation are known as highly stringent. High stringency hybridisation conditions usually involve relatively high temperature, low ionic strength and high concentrations of formamide. As the temperature and concentration of formamide are lowered or relaxed, and the ionic strength is raised, hybridisation of nucleic acids with increasing amounts of base pair mismatches can occur. Hybridisation conditions involving low temperature, high ionic strength and low concentrations of formamide are low stringency conditions. The exact level of stringency of a particular set of hybridisation conditions is dependent on the  $T_m$  of the nucleic acid duplex, and this involves many factors as described above. However, using 20% formamide and a low temperature (35°C), to lower the stringency of hybridisation, effective temperatures of 50°C below the  $T_m$  of perfectly matched hybrids can be reached. This allows the hybridisation and detection of duplexes with as much as 35% mismatching (Howley *et al.*, 1979). Generally, high stringency conditions involve hybridisation at 65-68°C in aqueous solution or 42°C in the presence of 50% formamide. High stringency washing of filters (to remove probe adsorbed non-specifically to the filters or nucleic acids) is usually performed at 50-70°C in low salt buffers (0.1 x SSC). For poorly matched hybrids, hybridisation is usually performed at 35-42°C in 50% formamide, and the washing of filters at 40-60°C in 2-6 x SSC (Anderson and Young, 1985). However, the exact conditions of hybridisation and washing have to be determined empirically, as the amount of immobilized complementary nucleic acid and the extent and distribution of base pair mismatches are often unknown.

### 2.I.ii.a.4. the duration of hybridisation.

The length of time hybridisation should be allowed to proceed, depends on the rate of hybridisation, which as stated above depends on a number of factors. The length of time should be kept to a minimum to reduce the level of non-specific background which increases with time. In practice, for double stranded probes, the hybridisation should be allowed to proceed for  $1-3 \times C_0 t_{1/2}$ , where  $C_0$  = the probe concentration,  $t_{1/2}$  = the time

at which 50% of the probe has re-annealed and, where the value of  $C_0t_{1/2}$  is derived from the following formula (Maniatis *et al.*, 1982)

$$C_0t_{1/2} \text{ (time in hours)} = 1/a \times b/5 \times c/10 \times 2.$$

where a = the weight of the probe in  $\mu\text{g}$ , b = the length or complexity (the total length of different or unique sequences present; shorter than the actual length if repetitive sequences are present) of the probe (Kb), and c = the volume of the reaction in ml.

After hybridisation has been allowed to proceed for  $3 \times C_0t_{1/2}$  the amount of single stranded probe left for hybridisation to the filter is negligible.

#### 2.I.ii.a.5. other considerations for hybridisation.

Agents such as dextran sulphate and polyethylene glycol are available which increase the rate of hybridisation (Wetmur, 1975). In the presence of 10% of either dextran sulphate or polyethylene glycol the rate of hybridisation in solution increased 10 fold. This increase in rate was attributed to the exclusion of the DNA from the volume now occupied by these reagents, thereby effectively increasing the DNA probe concentration. However, the effect of these reagents on the rate of hybridisation is dependent on the nature of the probe used (Wahl *et al.*, 1979). For single stranded probes, the rate of hybridisation is increased by 3-4 fold, whereas for double stranded probes the rate is increased up to 100 fold (Wahl *et al.*, 1979). Most of the increase in the rate of hybridisation caused by these reagents is thought to be due to the formation of networks or concatenates. Networks or concatenates occur due to the formation of duplexes of overlapping regions of the single strands of the probe. This leads to a network of probe stands stuck together which can then hybridise to complementary filter bound nucleic acid, amplifying the hybridisation signal (Anderson and Young, 1985). The formation of concatenates due to the use of dextran sulphate or polyethylene glycol complicates the results, especially for quantitative studies. Caution should therefore be used if these reagents are incorporated in experiments designed for this purpose. These reagents also amplify background levels of non-specific adsorption to filters, to a similar extent as the specific hybridisation of the probe (Anderson and Young, 1985).

## **2.II. Materials.**

### **2.II.i. Cell culture and molecular biology reagents.**

**Amersham International plc** (Aylesbury, Bucks):- [21,22-<sup>3</sup>H] ouabain, [2,8-<sup>3</sup>H] adenosine 5'-triphosphate ([<sup>3</sup>H]-ATP) (~40 Ci/mmol), [ $\alpha$ -<sup>32</sup>P] deoxy-adenosine 5'-triphosphate ([<sup>32</sup>P]-dATP) (~3000 Ci/mmol) multiprime DNA labelling kit, Hybond-N<sup>+</sup> blotting membrane, Hyperfilm-MP autoradiography film.

**BDH chemicals Ltd** (Poole, Dorset):- (Anala R grade except where otherwise stated), acetic acid, isoamyl alcohol, boric acid, bromophenol blue (general purpose reagent), butan-1-ol, caesium chloride, calcium chloride, chloroform (chromatography grade), cocktail T scintillant, d-glucose. di-sodium hydrogen phosphate, ethylenediaminetetraacetic acid disodium salt (EDTA), ethanol (absolute), hydrochloric acid (HCL; 36% solution), 8-hydroxyquinoline, isopropanol, lithium chloride, magnesium chloride, magnesium sulphate, manganese chloride, 2-methoxy ethanol, potassium acetate, potassium chloride, sodium acetate, sodium chloride, sodium hydrogen carbonate, sodium hydroxide, formamide.

**Beckman Instruments Inc** (High Wycombe, Bucks):- JA 10 (rotor) 500 ml polycarbonate centrifuge bottles, "Corex" glass centrifuge tubes, SW 65 (rotor) "Ultraclear" 5 ml ultracentrifuge tubes.

**Becton Dickenson** (Cowley, Oxon):- plastipak syringes, 21 gauge microlance needles.

**Biorad chemical division** (Watford, Herts) :-Zeta-Probe blotting membrane.

**Boots plc** (Nottingham):- sterile gauze.

**J Boulton Ltd** (Barking Essex):- pasteur pipettes.

**British Oxygen Co** (London):- 95% air with 5% CO<sub>2</sub>.

**Coulter Electronics Ltd** (Luton, Beds):- "Isoton II", cuvettes.

**Flow Laboratories** :- MDCK cells (strain I; 60 serial passages) obtained in April 1978, stored in the vapour phase of liquid nitrogen and subcultured at intervals.

**Fluka** (Switzerland):- guanidinium isothiocyanate.

**Formachem** (Strathaven, Scotland):- phenol (crystalline).

**Gelman Sciences Inc.** (Brackmills Northampton) :- Biotrace NT nitrocellulose.

**Gibco BRL Ltd** (Paisley, Scotland):- Earle's balanced salt solution (EBSS; calcium, magnesium and bicarbonate free), glutamine (200 mM), kanamycin (10 mg/ml), trypsin

(2.5%), minimal essential medium (Eagles); (MEME with Earle's salts), minimal essential medium (MEM) non essential amino acids, foetal calf serum, newborn calf serum, Nunc multi-well plates, Nunc tissue culture plates (6 cm), plastic culture flasks (175 cm<sup>2</sup>), [<sup>3</sup>H]-HeLa cell total RNA, nunc 50 ml centrifuge tubes, poly A polymerase enzyme, rabbit globin mRNA, ultra pure agarose,  $\phi$ X174-Hae III DNA size markers,  $\lambda$ -Hind III DNA size markers.

**A.R.Horwell** (London):- stainless steel filler cannulae (blasting needles) 10 cm long, 2 mm diameter.

**ICN Biomedicals Ltd** (High Wycombe, Bucks):- "Cellgen" discs CD-6 (collagen membrane, plate inserts) 31 mm diameter, FMC bioproducts "Nusieve-GTG" agarose.

**Ilford** (Mobberley, Chesire) :- Hypam photographic film fixer.

**Kodak Ltd** (Kirkby, Liverpool) :- LX-24 photographic film developer.

**Lakeland plastics** (Windermere, Cumbria) :- Polythene tubing.

**Medicell International** (London):- dialysis tubing.

**Millipore** (Harrow, Middlesex):- "Millicell HA" culture plate inserts 30 mm diameter.

**Pharmacia LKB Biotechnology** (Milton Keynes):- Sephadex chromatography medium G50 (medium grade), oligo (dT) cellulose affinity chromatography medium type 7.

**Oxoid** (Basingstoke, Hamps):- phosphate buffered saline tablets (PBS).

**Sabre International Products** (Redding, Berks):- syringes (assorted sizes).

**Scotlab** (Bellshill, Scotland):- microcentrifuge tube 1.5 ml.

**Sigma chemicals** (Poole, Dorset):- dimethyl sulphoxide (DMSO), ouabain, ampicillin (antibiotic), ethidium bromide, ficoll, lysozyme, sodium dodecyl sulphate, tetracycline (antibiotic), adenosine 5'-tri-phosphate (ATP), bovine serum albumin (BSA) (molecular biology grade and pentax fraction V), cordycepin 5'-tri-phosphate, diethyl pyrocarbonate (DEP),  $\beta$ -mercaptoethanol, polyvinyl pyrrolidone, proteinase K, salmon sperm DNA, sodium dodecyl sulphate, tris Base and tris-HCl.

**Sterilin** (Teddington, Middx):- plastic tissue culture flasks (75 cm<sup>2</sup>). sterile universal containers (30 ml).

**Whatman Ltd** (Maidstone, Kent) :- 3MM filter paper.

**X-OGRAPH Ltd** (Malmesbury, Wilts) :- MULT-E-ROLE X-ray film cassettes. HI-SPEED-X fluorographic intensifying screens.

## 2.III. Methods.

### 2.III.i. Cell biology methods.

#### 2.III.i.a.1. general cell culture.

For the purposes of tissue culture all of the sterile manipulations cited in this text were performed in a Gelman Sciences Class 100 laminar flow hood/cabinet, and all solutions were prepared using double distilled, 18 MOhm filtered water (Millipore Milli-Q water system). Sterile glassware was prepared by autoclaving at 121°C (15 psi) for 30 minutes. The culture medium used for MDCK cells was MEME. The medium was filter sterilised (0.22 µm filter) and stored, in 500 ml aliquots, in sterile bottles at 4°C, until use. At the time of use, 5 ml l-glutamine (2 mM) , 2.5 ml kanamycin (50 µg/ ml), 5 ml MEM non-essential amino acids, 25 ml foetal calf serum (FCS), and 25 ml new born calf serum (NBCS) were added to each bottle of medium under sterile conditions. This was then known as complete medium. Trypsin was used at one tenth of the stock concentration of 2.5%, diluted in EBSS. A stock 20 mM EDTA was also made up in EBSS and was filter sterilised. This was added to the trypsin also at one tenth concentration to form a stock containing 0.25% trypsin and 2 mM EDTA in EBSS. Stocks of kanamycin, glutamine, trypsin-EDTA, FCS and NBCS were stored frozen at -20°C in aliquots of 10, 20, or 100 mls, in sterile universal containers or 100 ml sterile glass bottles. MEM non-essential amino acids were stored at 4°C.

MDCK strain I cells of 66-69 serial passages were stored frozen in the vapour phase of liquid nitrogen. They were kept in complete MEME medium with the addition of 10% DMSO (to prevent the formation of ice crystals during storage). The cells were thawed, and allowed to settle and adhere to the culture flask for 24 hours. After 24 hours, the medium was exchanged for fresh medium without DMSO. The cells were normally grown in a 5 day cycle, at the end of which they had normally formed a confluent monolayer and had entered the stationary phase of the growth cycle.

At the end of each 5 day growth cycle cells were subcultured or passaged. For the purpose of subculturing, cells were trypsinised to remove them from the surface of the culture flask. Trypsin-EDTA solution was heated to 37°C from frozen. The spent medium from the culture flask was poured off and 5 ml of trypsin-EDTA was taken up into a 10 ml graduated, sterile plugged pipette. The trypsin-EDTA solution was then



dispensed into the flask, and was washed over all the surfaces of the flask to remove any residual traces of medium (including Ca and Mg ions). The trypsin solution was then poured off and replaced by a fresh aliquot of 5 mls of trypsin. The flask cap was then tightly resealed and the flask was then incubated at 37°C on a shaking plate (IKA Vibrax Vxr). When cells were completely liberated from the growth surface of the flask (established by viewing on an Olympus CK inverted binocular microscope), 45 mls of fresh complete MEME medium (warmed to 37°C) was added to the culture flask. A sterile large bore blasting needle was connected to a 10 ml syringe, and was used to draw the cell suspension, in and out of the syringe ~12 times. This process was known as cell blasting and was done to break up clumps and aggregates into single cells for accurate cell counting. After completion of cell blasting, a 1 ml sample was removed by pipette and placed into a coulter counting cuvette. An aliquot of 19 mls of isotonic saline (Isoton II) was measured out and added to the cuvette. The number of cells in a 0.5 ml sample of the solution in the cuvette was counted by a Coulter Electronics model ZM Coulter Counter. Cell size distribution was assessed by a Coulter Channalyser 256 and mean cell volume, mean cell H<sub>2</sub>O, and spherical surface area were calculated following transfer of the data to an IBM PC/XT compatible micro computer (Datalink Computers). After the dilution of the sample was accounted for, the number of cells originally contained within the flask was estimated. A new flask was seeded such that, under normal growth conditions, 0.57 mls of complete medium and  $4.17 \times 10^4$  cells were used per cm<sup>2</sup> of growth area of the culture flask. This was done, regardless of the size of the flask used. A mixture of sterile air containing 5% CO<sub>2</sub>, was blown over the surface of the medium in the new flask for ~1 minute. This produced a buffering system with the NaHCO<sub>3</sub> contained within the MEME medium. The passage number was then incremented by one and the flask was returned to incubate at 37°C. Subculturing or passaging was then complete.

#### 2.III.i.a.2. cell culture on culture plate inserts.

When culture plates or multiwell filter inserts (Millicell HA or Cellagen discs) were required for experiments, the cells left over from subculturing were used for their preparation.

For experiments, cell culture plates were seeded with cells as stated above. However, in experiments where Millicell HA or Cellagen disc inserts were required, a different

regime was used. This was because, Collagen discs, had small plastic stilts on their under surface, to prevent collagen membranes from sticking to multiwell plates. As a consequence of the stilts, the culture membranes were located 1-2 mm above the surface of the multiwell plate. Extra medium was therefore needed to cover the surfaces of the culture membranes and to keep the cells submerged in medium. Thus, for the experiments that used Collagen inserts, 0.83 ml of complete medium was used per cm<sup>2</sup> of growth area.

Culture plates were seeded in the same way as culture flasks. However, instead of the plates being gassed with air containing 5% CO<sub>2</sub>, plates were placed in a box which was equilibrated with air containing 5% CO<sub>2</sub> for ~1 minute. The box was then sealed and incubated at 37°C. Culture plate inserts were used in conjunction with Gibco/BRL nunc 6-well multiwell plates. These were prepared by the addition of medium to each well of the multiwell plate, followed by a culture plate insert. Medium was then added to each insert, in order to wet both surfaces of the culture membrane. The multiwell plate was then placed in a box, the box was equilibrated in air containing 5% CO<sub>2</sub> and incubated 37°C (usually for the length of time taken to trypsinise cells). When the culture plate inserts were properly wet, and the medium had equilibrated with the CO<sub>2</sub>, medium containing the appropriate amount of cells for seeding, was added to each culture plate insert. The multiwell plate was then returned to the box, re-equilibrated in air containing 5% CO<sub>2</sub> and re-incubated at 37°C.

#### 2.III.i.b. Na,K-ATPase enzyme abundance:- ouabain binding assay.

##### 2.III.i.b.1. ouabain binding assay performed on culture plates.

As stated in the introduction, the optimal conditions for determining the specific [<sup>3</sup>H]-ouabain binding (Na,K-ATPase enzyme abundance) in MDCK cells had been demonstrated by Lamb *et al.* (1981). Maximal discrimination between specific and non-specific [<sup>3</sup>H]-ouabain binding was found at a ouabain concentration of 2.5 x 10<sup>-7</sup>M ouabain and an incubation time of 20 minutes. In a study by Cereijido *et al.* (1981a), 100% of the [<sup>3</sup>H]-ouabain binding sites in MDCK cells were also shown to be bound at a ouabain concentration of 3 x 10<sup>-7</sup>M. These conditions were therefore utilised for the experiments involving ouabain binding.

For ouabain binding experiments two types of Krebs solutions were used, potassium free (0K) Krebs (see Appendix 3), and high potassium (15K) Krebs solutions. High potassium (15K) containing Krebs, was identical to 0K Krebs except that it had an additional 15 mM KCl. Ouabain solutions were made from  $2 \times 10^{-7}$ M non-radioactive ouabain plus between 0.5 and 6  $\mu$ Ci/ml of [ $^3$ H]-ouabain (giving a final ouabain concentration of  $2-3 \times 10^{-7}$ M), made up in either 0K or 15K Krebs solutions. The method of measurement of ouabain binding to MDCK cells on plates was as follows:-

- 1, Two groups of plates marked 0K or 15K were placed on a incubation plate at 37°C. The first plate of the 0K group was washed 4 x in 0K Krebs buffer at 37°C and the excess buffer was removed from the plate, by the use of a pasteur pipette attached to a water pump.

- 2, An aliquot of 1 ml of radioactive ouabain solution made up in 0K Krebs was then added to the plate and a stop watch was started.

- 3, A second 0K plate was taken, washed as in step 1 and after 1 minute had elapsed, 1 ml of radioactive ouabain solution (0K) was added

- 4, The process was repeated at 1 minute intervals until all the plates marked 0K had been processed.

- 5, At this point, a plate from the group marked 15K was taken, it was washed 4 x in 15K Krebs solution at 37°C. Then, at the next 1 minute interval, 1 ml of radioactive ouabain solution made up in 15K Krebs was added.

- 6, The process in 5, was repeated for each 15K plate in turn at 1 minute intervals, until all the plates had been processed.

- 7, After 20 minutes had elapsed, the 0K plate which was processed first was taken. The radioactive solution was removed from the plate, it was washed 4 x in 15K Krebs at 0°C, the excess liquid was then removed, and 0.5 ml of trypsin solution was added to the plate.

- 8, Each plate was subsequently taken in the order in which they were first processed and, at 1 minute intervals, the plates were processed as in step 7.

- 9, When the cells had detached from the plates due to trypsinisation, 1.5 ml of Krebs solution (15K) was added to each plate to neutralise the effects of the trypsin.

- 10, The cells on each plate were then blasted (drawn up and ejected from a syringe with an attached cell blasting needle) 15 x to break up cell clumps.

11. A sample of the cell suspension was then taken for radioactive counting (1 ml) and another (0.5 ml) for cell counting and sizing (performed by the Coulter Counter).

12, Standard solutions were produced which contained 100  $\mu$ l of each of the radioactive ouabain solutions (0K and 15K), and 0.9 ml of Krebs. These standards were also radioactively counted. Blank plates were used which were processed identically to cell culture plates. The trypsin/Krebs mixture from these blank plates was also radioactively counted. Blank scintillation vials were also radioactively counted these contained scintillation fluid (10 ml) and Krebs solution (1 ml).

13, The number of ouabain binding sites per cell was calculated from the information obtained as in Appendix 1. The specific ouabain binding represented the 0K ouabain binding (specific plus non-specific ouabain binding) minus the 15K ouabain binding (non-specific ouabain binding) figures.

#### 2.III.i.b.2. Ouabain binding to cells grown on culture plate inserts.

Ouabain binding was also performed on culture plate inserts where, the experimental protocol was essentially the same as that for plates. The culture plate inserts were contained within six-well multiwell plates. Therefore, during the washing steps using Krebs buffer, both the inserts and the wells of the plate had to be washed. Inserts themselves were held by forceps during the washing procedure. When radioactive ouabain solution was added to each insert, it also had to be added to each well. Addition of the ouabain solution to the wells, facilitated the access of the ouabain to the basal surface of the MDCK cell monolayer. As the culture plate inserts were raised up above the base of the wells by plastic stilts, extra fluid had to be added to reach the under side of the inserts. For Millipore HA inserts, 0.75 ml of radioactive solution was added to each insert and 1.25 ml to each well. In the case of ICN "Cellagen" collagen inserts, where the stilts were larger (~2 mm), 1 ml was added to each insert and 2.5 ml to each well. A similar situation occurred with the trypsin solution, where for Millipore HA inserts, 0.75 mls of trypsin was added to each insert and 1.25 mls to each well. To neutralise the effects of the trypsin, 1.75 mls of Krebs buffer was added to each insert. However, in the case of the "Cellagen" inserts, the volume of trypsin required was so large that the cells would have been diluted too much for cell and radioactive counting. This was especially the case in low cell seeding density experiments. It was therefore decided to snap off the plastic stilts from each insert with forceps. This was done, such that the collagen membrane of each insert was in contact with the base of the well. The trypsin (1 ml) could then be added to the insert, as the side wall of the insert formed a tight seal with



the bottom of the well and prevented the trypsin from escaping. The trypsin was then neutralised with 3 ml of Krebs buffer.

A potential problem with the culture plate inserts was that large amounts of radioactive ouabain bound to the filter or membrane and plastic mount of the insert might detach during the trypsinisation of cells. While this effect would have been equal for both OK and 15K plates, the extra radioactivity may well have masked the difference between the two sets of plates. To determine a procedure for washing culture plate inserts, blank culture plate inserts (Millipore HA) were incubated in radioactive ouabain and washed as normal. They were then soaked in a stirring bath of ice cold Krebs buffer. At intervals inserts were removed, the cellulose membrane were removed and soaked in 2-methoxyethanol for 30 minutes (to dissolve the cellulose). The membranes were then radioactively counted in 10 ml of scintillant. The radioactivity bound to the cellulose membranes decreased (washed off) for the first 20 minutes and was then relatively stable. Therefore, for the cold (15K Krebs 0°C) wash at the end of the ouabain binding protocol (step 7), culture plate inserts were washed 4 times in 15K Krebs (0°C) as before. The inserts were then rinsed in 2 baths of ice cold Krebs and they were then washed in a stirring bath of ice cold Krebs for 20 minutes. It has been reported, that the differences in affinity of isoforms Na,K-ATPase for ouabain was due to the dissociation rate of the glycoside from the enzyme (Erdmann *et al.*, 1980). The release of ouabain from HeLa and Girardi cells (Na,K-ATPase), has been shown to occur with a half time of 20 hours at 37°C Boardman *et al.* (1972). The concentration half maximal binding (or K<sub>d</sub>) of ouabain to Na,K-ATPase in MDCK cells (60-100 nM; see section 1.VI.) was higher than that of HeLa and Girardi cells (K<sub>m</sub> = 13-27 nM Boardman *et al.*, 1972). However, it was felt that over the course of 20 minutes, at 0°C, only an insignificant amount of the specifically bound ouabain would detach from the MDCK cells, during the elongated period of washing in 15K Krebs buffer. Indeed, these conditions probably led to a considerably lower loss of specifically bound ouabain, than those of Kennedy and Lever (1984; where incubations for 15 minutes at 37°C in isotonic buffers were used). These conditions were selected, to prevent any appreciable dissociation of ouabain from Na,K-ATPase in MDCK cells.



## 2.III.ii. Molecular biology methods.

### 2.III.ii.a. DNA methods.

#### 2.III.ii.a.1. bacterial growth, plasmid DNA transformation and isolation.

The *Escherichia coli* (*E.coli*) bacterial strains, RRI, NB16, DH1, DH5 and JM101, were grown in Luria-Bertani (LB) medium (Maniatis *et al.*, 1982). Bacterial plasmids, based on either pBR322, or its derivative pAT153 were transformed into the *E.coli* strains mentioned above by the  $\text{CaCl}_2$  procedure of Maniatis *et al.* (1982). The incorporation and maintenance of these plasmids was selected for by bacterial growth in the antibiotics tetracycline (15  $\mu\text{g/ml}$ ) or ampicillin (50  $\mu\text{g/ml}$ ), where appropriate. Bacterial cultures containing plasmids were stored frozen in ~20% glycerol at  $-70^\circ\text{C}$ .

The isolation of plasmids from bacterial cultures was performed by a method similar to that of the large scale alkaline lysis method of Maniatis *et al.* (1982). In this method, a starter culture of bacteria, seeded at low density from the frozen glycerol stock, was grown overnight in 10 ml of LB medium with the appropriate antibiotic concentration, at  $37^\circ\text{C}$ . A 1 or 2 litre culture (LB medium with antibiotic) was seeded from the starter culture and grown over night for ~16 hours in an orbital shaker at  $37^\circ\text{C}$ . The bacteria were collected from suspension in the LB medium, by centrifugation at 10000 rpm (11000  $g_{av}$ ), for 10 minutes, in a JA 10 rotor (Beckman Instruments), at  $0^\circ\text{C}$ . The bacterial pellet was resuspended in 50 ml of 50 mM Tris-HCl pH 8.0 and re-centrifuged as above. For a procedure utilizing 1 litre of bacterial culture, the following protocol was used:- The pellet was resuspended in 50 mls of ice cold bacterial lysis buffer (see Appendix 3) and left on ice for 30 minutes. To the bacterial suspension was added 80 mls of a solution containing 0.2 M NaOH and 1% SDS. This was mixed and left on ice for a further 5 minutes. After this time, 40 mls of 3 M potassium acetate pH 4.8 was added to the suspension, this was mixed, and left on ice for 15 minutes. The lysed bacterial suspension was then centrifuged at 8000 rpm (7000  $g_{av}$ ) for 5 minutes at  $0^\circ\text{C}$  (JA 10 rotor). The supernatant was filtered through 2 layers of sterile gauze into a fresh centrifuge bottle. The volume of the supernatant was measured and 0.6 volumes of cold isopropanol ( $-20^\circ\text{C}$ ) was added. This solution was mixed and centrifuged immediately at 8000 rpm (7000  $g_{av}$ ) for 5 minutes (JA 10 rotor). The supernatant from this centrifugation step was decanted away and the pellet was drained and dried under a

stream of nitrogen gas. The pellet was gently resuspended (using a syringe and 16 gauge needle), in 10.8 ml of TE (10 mM tris pH 8.0, 1 mM EDTA). To the TE solution was added, 11.7 g of caesium chloride (CsCl), this was allowed dissolve, before 1.2 ml of 3 mg/ml ethidium bromide was added. The density of the final solution was adjusted, (if necessary) to 1.58 g/ml. The suspension then centrifuged at 40,000 rpm (116,000  $g_{av}$ ) for >40 hours at 20°C (SW 65 rotor in a Beckman L2-65B ultracentrifuge). The plasmid DNA stained by the ethidium bromide dye collected as two bands located half way up the centrifuge tubes. The upper band contained nicked circular plasmid DNA (or relaxed plasmid DNA plus any chromosomal linear DNA) and the lower band contained closed circular plasmid DNA. The aggregated protein and other material at the top of the tubes was removed with a pasteur pipette. The upper, and then the lower bands were then removed from the tubes, by clean pasteur pipettes. The CsCl solution containing the lower band was then extracted with an equal volume of water saturated butan-1-ol, repeatedly, until all of the red colour associated with the ethidium bromide was removed from the CsCl solution (approximately 5 washes). The CsCl/plasmid DNA solution was then dialysed overnight in 2 changes of 5 litres of TE buffer. The plasmid DNA solution was ethanol precipitated overnight with the addition of 2 volumes of absolute ethanol and 0.1 volume of 3 M Na acetate. This solution was then centrifuged for 30 minutes, at 4°C at 11,000 rpm (12,300  $g_{av}$ ), in a JS 13 rotor (J 21-B centrifuge; Beckman Instruments). The supernatant was discarded and the plasmid DNA pellet was resuspended in a small volume of H<sub>2</sub>O (~1 ml).

#### 2.III.ii.a.2. quantification of the abundance of DNA.

The amount and purity of DNA was assessed by spectrophotometry as suggested by Maniatis *et al.* (1982). The abundance of DNA was measured by reading the absorbance of a solution containing the sample at 260 nm. A solution with a DNA concentration of 50 µg/ml has an absorbance value of 1 O.D. (optical density unit), using a light path of 1cm. The purity of nucleic acids was also measured by spectrophotometry. Purity was assessed by measuring the ratio of the absorbance at 260 nm and 280 nm (the 260: 280 ratio; Maniatis *et al.*, 1982). For DNA, a ratio of around 1.8: 1, indicates that the DNA is free of protein (the major contaminant of nucleic acid preparations). Protein has a strong UV absorbance at 280 nm and consequently lowers the 260: 280 ratio.

### 2.III.ii.a.3. restriction endonuclease digestion of DNA.

The restriction digestion of DNA was performed using a variety of restriction endonucleases (obtained primarily from Gibco/BRL, Northumberland Biologicals and International Biotechnologies Inc.). Restriction endonuclease digestion in all cases was performed in accordance with the instruction and buffers provided. Incubations were normally performed at 37°C in salt buffers containing the following range of concentrations; 0-150 mM NaCl or KCl, 0-15 mM Tris-HCl (pH 7-8.5), 0-15 mM MgCl<sub>2</sub>, 0-200 µg/ml BSA and, 0-10 mM dithiothreitol or β-mercaptoethanol. Digests were usually performed overnight on 250 µg of DNA in a volume of 200 µl. The number of enzyme units required for complete digestion of the DNA was calculated by determining the number of the particular endonucleases cutting sites in the substrate DNA. The number of enzyme units were adjusted according to the number of substrate DNA cutting sites relative to the number in the DNA used for the enzyme's unit definition. The activity of the enzyme in overnight digests was also taken into account. This was done by utilizing the approximate amount of enzyme activity remaining after an overnight digestion (as suggested in the manufacturer's instructions), to calculate the minimum number of units required to completely digest the DNA.

### 2.III.ii.a.4. agarose gel electrophoresis of DNA.

To determine the sizes of fragments obtained during the restriction digestion of DNA, agarose gel electrophoresis was employed. Electrophoresis tanks, gel casting equipment and other accessories were obtained from Gibco/BRL. The type of agarose gel used for the electrophoresis of large fragments of DNA (fragments >500 base pairs) was ultra pure agarose (Gibco/BRL), whereas, the agarose used for smaller fragments (10-500 base pairs) of DNA was Nusieve GTG agarose (ICN Biomedicals).

The gel was made up by the addition to the agarose of, one tenth of the final volume of 10 x TBE electrophoresis buffer (see Appendix 3), and water, up to the final gel volume (usually 100 ml). The concentration of gel used for small DNA fragments was 2%, and for large DNA fragments was 1%. The gel was boiled and shaken until all particles had dissolved and the solution was degassed. The gel was cooled to just above gelling temperature (~45°C), at which point ethidium bromide was added to give a final concentration of 0.5 µg/ml. The gel casting equipment was prepared, and the gel was

poured into the mould containing a gel well forming comb, and allowed to set for ~1 hour. When the gel was set, it was placed in the electrophoresis tank and, the tank was filled to just above the level of the gel with 1 x TBE buffer. Aqueous DNA samples were prepared by the addition of one tenth volume of 10 x TBE buffer, and one tenth volume of 10 x gel loading buffer (see Appendix 3). The samples were then loaded into the wells of the gel (volume was normally 30-50  $\mu$ l per well).  $\lambda$  bacteriophage DNA digested with Hind III restriction endonuclease or,  $\phi$  X 174 bacteriophage DNA digested with Hae III restriction endonuclease (Gibco/BRL) were used as DNA size markers during electrophoresis. The gel was then electrophoresed at 125 volts until samples had entered the gel and then at a voltage of no greater than 5 volts per centimetre (5 V/cm). When the bromophenol blue dye had migrated to the end of the gel, electrophoresis was terminated. Due to the presence of ethidium bromide during electrophoresis the gel could be visualised immediately after electrophoresis. Gels that needed to be destained were soaked in water until the background fluorescence was reduced to a satisfactory level (usually ~ 30 minutes). Visualisation of DNA on gels was enabled by the fluorescence of the ethidium bromide which intercalates with DNA. The fluorescence was achieved by absorption of 302 nm UV light from a transilluminator (UV Products).

#### 2.III.ii.a.5. purification of DNA from agarose gels.

The purification of DNA from agarose gels was attempted by several methods including; 1, the dilution of agarose to 0.1 % with buffer (to prevent re-gelling of the agarose), re-melting of the agarose at 70°C and, purification of the DNA by phenol extraction. 2, the freeze fracture of agarose linkages by immersion in liquid nitrogen, followed by phenol extraction. 3, the physical extrusion of DNA and electrophoresis buffer from the agarose by centrifugation (at 14,900  $g_{max}$ ) of the agarose containing the DNA through a "Costar Spin-X" filter (0.22  $\mu$ m pore size; Nothumberland Biologicals). However all of the above methods proved unsatisfactory, as the isolated DNA subsequently radiolabelled very poorly, which suggested the presence of contaminating agarose. This would absorb UV light at 260 nm and increase the DNA solutions absorbance, causing a variable, and considerable over estimate of the DNA concentration and a under estimate of the specific activity of the radiolabelled DNA. The method that was finally used was 4, electroelution using an IBI electroeluter. In this method, the DNA was electrophoresed out of the agarose into a high salt barrier (3 M Na acetate)



placed in a "V"-shaped channel. The high salt dramatically slowed the migration of the DNA by neutralising its negative charges. The DNA was collected from the V shaped channel with the salt and was directly precipitated with 2 volumes of ethanol. After centrifugation at 13000 rpm. the DNA was dissolved in distilled H<sub>2</sub>O (400 µl) and this solution was extracted sequentially with equal volumes of phenol, phenol/chloroform (1:1) and chloroform. The resulting aqueous solution was then reprecipitated with 2 volumes of ethanol and 0.1 volume of 3 M Na acetate.

#### 2.III.ii.a.6. radiolabelling of DNA.

DNA was radiolabelled by the use of a commercial kit ("Multiprime" DNA-labelling system, Amersham International). The kit employed the method of Feinberg and Vogelstein, (1983), which used random sequence hexanucleotides to prime DNA synthesis, performed by the Klenow fragment of E. coli DNA polymerase I. The enzyme added [<sup>32</sup>P]-radiolabelled nucleotides (normally α[<sup>32</sup>P]-dATP; 3,000 Ci/mmol) to the primer/DNA template resulting in DNA with high specific activity (>2x10<sup>9</sup> dpm/µg DNA).

The radiolabelled DNA was separated from un-incorporated nucleotides by Sephadex column chromatography (G50 Sephadex medium grade). Columns containing G50 Sephadex were produced for this purpose. To stop loss of Sephadex from columns, a small piece of siliconised glass fibre was added to the bottom of a pipette (siliconised 2 ml Volac glass). The glass fibre was then overlaid with siliconised glass beads (Sigma Chemicals). Sephadex was suspended in "Milli-Q" H<sub>2</sub>O (Millipore) and autoclaved at 121°C (15 PSI) for 20 minutes. The sterile Sephadex suspension was added to the pipette until a 2 ml volume of packed G50 Sephadex had formed inside the pipette. The column was equilibrated in 6 x SSC (0.9 M NaCl, and 90 mM tri-sodium citrate), by the passage of 2 column volumes of this solution through the column. The ends of the column were sealed with Nescofilm until use. At the time of use, excess liquid above the Sephadex in the column was drained off. The DNA radiolabelling reaction mixture was added to the top of the column followed by small aliquots of 6 x SSC. The progress of the radiolabelled DNA and nucleotides through the column was monitored using a Gieger counter (Series 900 mini monitor; Mini Instruments Ltd.). When radioactivity reached the bottom of the column, the collection of fractions was initiated. Six fractions of 4 drops followed by six fractions of eight drops were collected from the column. The



radioactivity contained within 2  $\mu$ l samples of the column fractions was then determined by Cerenkov counting. Two peaks of radioactivity were observed. The first representing radiolabelled DNA in the void volume (excluded from entering the Sephadex due to its large size), and the second, the unincorporated nucleotides in the total column volume (whose progress in the column was slowed due to entry into the Sephadex beads). The specific activity of the radiolabelled DNA was calculated as in Appendix 2. The fractions of radiolabelled DNA (first peak) were then pooled and used in subsequent nucleic acid hybridisation experiments.

### 2.III.ii.b. RNA methods.

#### 2.III.ii.b.1. total RNA extraction.

A modified version of the total RNA extraction procedure of Cathala *et al.* (1983) was used to isolate total RNA from cell suspensions. All the solutions used for total RNA extraction were as detailed in Appendix 3. All aqueous solutions used DEP-H<sub>2</sub>O (see Appendix 3). Latex gloves (Micro-Touch; Surgikos) were worn at all times.

The outline of the total RNA extraction procedure was as follows:-

- 1, The cell suspension (normally  $\sim 4 \times 10^7$  cells in  $\sim 50$  ml of MEME medium), obtained from trypsinisation of cultured cells, was transferred to a 50 ml Nunc polypropylene centrifuge tube. The tube was centrifuged at 1500 rpm ( $\sim 500 g_{av}$ ) in a MSE Coolspin centrifuge for 5 minutes at 4°C. The supernatant was decanted and both tube and cell pellet were washed in 50 ml ice cold phosphate buffered saline (PBS) and then recentrifuged.

- 2, For every  $10^7$  packed cells, 720  $\mu$ l of cell lysis buffer and 80  $\mu$ l of  $\beta$ -mercaptoethanol were added. A 21 gauge needle and syringe were then used to disrupt the cell pellet, by drawing the solution and the cell pellet into and out of the syringe as forcefully as possible (until the pellet was dispersed).

- 3, When the disruption and homogenisation of the cell pellet was complete, 6.25 volumes of 4 M LiCl was added and the solution was rehomogenised using the syringe and needle (3-6 times). This mixture was then transferred to sterile siliconised glass Corex centrifuge tubes (15 or 30 ml). The mixture was left at room temperature for 20 minutes and then incubated at 4°C overnight, to allow precipitation of the RNA.

4, The next day, the Corex tubes were centrifuged in a JS-13 rotor (J-21B centrifuge Beckman Instruments) at 6500 rpm (4300  $g_{av}$ ) for 90 minutes at 4°C, and the supernatant was discarded.

5, The pellet was rehomogenised using a fresh syringe and needle, in 0.6 volumes (compared to the volume of 4 M LiCl added) of 3M LiCl, recentrifuged at 6500 rpm (4300  $g_{av}$ ) for 30 minutes at 4°C and, the supernatant was again discarded.

6, The pellet was resuspended in 0.1 volume (compared to that of the 4 M LiCl added) of TNESDS. Proteinase K was added to a final concentration of 200-400  $\mu\text{g/ml}$  and the solution was incubated for 1 hour at 37°C. Proteinase K digestion reduced the quantity of interface material, producing a discrete interface during the following phenol/chloroform extractions (step 7). After proteinase K digestion the suspension was transferred to sterile 1.5 ml microfuge tubes for phenol/chloroform extraction.

7, To each microfuge tube was added an equal volume (compared to that of the TNESDS solution added) of phenol/chloroform (1:1). With the lids closed, the tubes were shaken until an emulsion of the phenol and aqueous phases had formed. The tubes were then centrifuged in a Hereaus Biofuge A at 13000 rpm (14900  $g_{max}$ ) for 2 minutes to separate the organic and aqueous phases. Denatured protein collected as a white aggregate at the interface between the two phases. The aqueous (upper) phase was removed using a siliconised pasteur pipette, taking care not to remove any of the interface material. This solution was then transferred to a fresh microfuge tube. The phenol/chloroform extraction procedure was complete.

8, To the original microfuge tube was added a further 0.4 volumes (compared to the original volume of TNESDS added) of TNESDS and the phenol was back extracted and centrifuged as before. The second aqueous phase was aspirated and added to the microfuge tube containing the first aqueous phase. The combined aqueous phases were re-extracted with an equal volume of phenol/chloroform. The aqueous phase from the second phenol chloroform extraction, was finally extracted once more using an equal volume of chloroform.

9, The aqueous phase from the chloroform extraction was then ethanol precipitated. This was achieved by the addition of 0.1 volumes (of the aqueous phase remaining) of 3 M Na Acetate and 2.5 volumes of absolute ethanol. The solution was left overnight at -20°C to precipitate the purified total RNA. The RNA/ethanol precipitation mixture was stored if necessary at -20°C for short terms of up to 1 week or in the vapour phase of liquid nitrogen for longer terms of over 1 week.

10, When required, the microfuge tubes containing the precipitated RNA, were centrifuged at 13000 rpm (14900  $g_{max}$ ) in a microfuge for 20 minutes. The supernatant was discarded and the pellet was washed in 500  $\mu$ l of 70% ethanol (this step was used, to dispose of any excess salt in the pellet, the presence of which can prevent dissolution). The microfuge tubes were then recentrifuged at 13000 rpm (14900  $g_{max}$ ) for 5 minutes, to re-pellet the RNA. The supernatant was discarded and, the pellet was dried off under vacuum and resuspended in DEP-H<sub>2</sub>O.

When total RNA was extracted from intact tissues, the same total RNA extraction procedure as described above was used with the following exceptions 1, 5 ml of lysis buffer was used per gram wet weight of tissue. The quantities of all other solutions were increased in proportion to the increased volume of the homogenate, and 2, the tissue was disrupted and homogenised using a Polytron tissue homogeniser (PT-10/35; Kinematica).

The final method used for the extraction of total RNA was developed from a series of earlier experiments which employed a slightly different extraction protocol. The experimental differences included; 1, in step 1 of the procedure, the cells were centrifuged for 10 minutes, and were washed in only 10 ml of PBS. 2, NaCl was omitted from the TNESDS, and in step 6, the proteinase K procedure was omitted. 3, In step 7, only 0.5 volume of phenol/chloroform was used in the extraction. 4, in step 9, only 2 volumes of ethanol were used for precipitation of RNA. 5, The 70% ethanol wash in step 10 of the protocol was omitted. These differences were amended as described above to improve both the yield and purity of the extracted total RNA.

#### 2.III.ii.b.2. mRNA isolation.

The procedure used to isolate poly A<sup>+</sup> mRNA was similar to that of Berger and Kimmel (1987). For details of the solutions used for the isolation of poly A<sup>+</sup> mRNA see Appendix 3.

An oligo (dT) cellulose column was constructed, using the barrel of a 2 ml syringe, plugged with a piece of boiled ashless cotton wool. A piece of silicon tubing was fixed onto the luer port of the syringe and 250 mg of oligo (dT) cellulose, suspended in 5 mls of DEP-H<sub>2</sub>O was added to the syringe. The tubing was connected to a flow cell within a spectrophotometer, which monitored the light absorbance at 260 nm wavelength. The absorbance reading was recorded directly using a chart recorder.

Total RNA from the extraction procedure was dissolved in DEP-H<sub>2</sub>O at a concentration of <10 mg/ml by heating at 65°C for 15 minutes. The RNA solution was diluted with an equal volume of double concentration loading buffer. The column was pre-equilibrated in loading buffer (when the absorbance reading gave a steady value; ~3 ml), and the RNA solution was added. The RNA which passed through the column (Poly A<sup>-</sup> RNA) was monitored via the UV absorbance of the spectrophotometer and chart recorder. This RNA was collected, reheated to 65°C and re-loaded onto the column. This was done to optimise the absorption of the poly A<sup>+</sup> RNA to the oligo (dT) cellulose. When all of the poly A<sup>-</sup> RNA had been collected for the second time, clearance buffer was applied to the column (to remove SDS and poorly bound material from the column), until the absorbance reading again returned to a steady reading. Elution buffer was then added to the column to collect the poly A<sup>+</sup> mRNA. After the mRNA had been collected (monitored by UV absorbance), the column was washed with DEP-H<sub>2</sub>O (optional) and finally with wash buffer, to remove any residual material still bound to the column. The poly A<sup>+</sup> mRNA was transferred to a Corex tube and precipitated with the addition of 0.1 volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol (ethanol precipitation) and left overnight at -20°C. The mRNA was recovered by centrifugation at 11000 rpm (12300 g<sub>av</sub>) for 30 minutes at 0°C in a Beckman JS 13 rotor.

The poly A<sup>+</sup> mRNA extraction procedure used in some of the experiments had an altered protocol compared to the that detailed above. These alterations included; 1, The loading buffer used 20 mM (instead of 10 mM) tris pH 7.5 and only 0.1% SDS. 2, No clearance buffer was used. 3, The elution buffer used 20 mM (instead of 10 mM) tris pH 7.5 and also contained 0.1% SDS. The elution buffer was also heated to 65°C before its addition to the oligo (dT) cellulose column. The alterations to the procedure were made because very little poly A<sup>+</sup> mRNA eluted from the oligo (dT) column unless the elution buffer was heated to 65°C. This was probably due to too great an ionic strength in the elution buffer, which even when heated to 65°C gave inconsistent and often poor yields of mRNA.

### 2.III.ii.b.3. quantification of RNA.

The amount and purity of RNA was assessed (as with DNA) by spectrophotometry as suggested by Maniatis et al. The abundance of RNA was measured by reading the absorbance of a solution containing the RNA sample at 260 nm. A solution containing



RNA at a concentration of 40 µg/ml, using a light path of 1cm, has an absorbance value of 1 O.D. (optical density unit). The purity of the RNA was also estimated by spectrophotometry. The purity was assessed by measuring the ratio of the absorbance at 260 nm and 280 nm (the 260: 280 ratio Maniatis *et al.*, 1982). For RNA, if this ratio was around 2: 1, then the RNA was free of protein (the major contaminant of nucleic acid preparations). Protein has a strong UV absorbance at 280 nm and consequently lowers the 260: 280 ratio. RNA extractions in general were notorious for yielding RNA in a degraded form due to the actions of ribonuclease enzymes, which were difficult to inhibit. The extent of the degradation of the extracted RNA was checked visually by denaturing gel electrophoresis (see next section). Total RNA from animal cells showed two main bands upon electrophoresis which were 28S and 18S in size (S refers to the equivalent size measurements determined by ultracentrifugation; Svedberg coefficient) The equivalent sizes in nucleotides of the rRNA's from MDCK cells were determined to be 4.4 and 2.0 Kb respectively. These two bands were the major ribosomal RNA's of the cell (rRNA) and comprised 95-99% of total cellular RNA. Any degradation of the bands led to extensive smearing of the bands, due to the appearance of smaller sized RNA's.

#### 2.III.ii.b.4. denaturing agarose gel electrophoresis of RNA.

When RNA was to be Northern blotted, the denaturing agarose electrophoresis of RNA was performed essentially as described in Maniatis *et al.* (1982) using formaldehyde as the denaturant. However, when electrophoresis was performed purely for a visual assessment of the RNA's viability, the similar, but more convenient, method of Davis *et al.* (1986) was used.

In the method of Maniatis *et al.* (1982), the agarose ( 1-1.5% final concentration, Gibco/BRL ultra pure) was boiled and degassed in DEP-H<sub>2</sub>O with 0.1 volume (final) of 10 x MOPS buffer added (see Appendix 3). The agarose gel was cooled to ~45°C and, formaldehyde was added to a final concentration of 2.2 M. The solution was mixed and the gel was poured into a gel electrophoresis mould and allowed to set. The RNA samples were dissolved in DEP-H<sub>2</sub>O (volume depending on gel well size), followed by the addition of, 0.44 volumes of 10 x MOPS buffer, 0.78 volumes of formaldehyde, and 2.22 volumes of de-ionised formamide (see Appendix 3). The RNA was then denatured by heating at 65°C for 15 minutes. The denatured RNA was allowed to cool and 0.1 volume of gel loading buffer (see section 2.III.ii.a.4.) was added, the the solution was



then mixed. The agarose gel was placed in the electrophoresis tank and the tank was filled to just above the top of the gel with 1 x MOPS buffer. The RNA samples were loaded into the wells and the gel was electrophoresed initially at 100 volts to run samples out of the wells and then, at 3 V/cm for ~5 hours, or at ~1 V/cm overnight, until the bromophenol blue of the loading buffer had migrated to near the end of the gel. If required the gel was stained and destained as in Maniatis *et al.* (1982). The gel was soaked for 2 hours in several changes of distilled H<sub>2</sub>O, followed by staining for 1 hour in 0.1 M ammonium acetate, 1 hour in 0.5 µg/ml ethidium bromide, 0.1 M ammonium acetate, and 0.01 M β-mercaptoethanol and, destaining for 45 minutes in 0.1 M ammonium acetate, and 0.01 M β-mercaptoethanol.

The method of Davis *et al.* (1986) had several alterations compared to the above method, and these were as follows; 1, the formaldehyde concentration of the gel was only 0.66 M (the formaldehyde concentration was reduced to prevent interference with ethidium bromide fluorescence). 2, ethidium bromide was added and mixed into the gel before gel casting, at a concentration of 1µg/ml. 3, gels were visualised immediately upon termination of electrophoresis by use of a UV transilluminator. Some destaining (1 hour in H<sub>2</sub>O) was sometimes required for the production of a good photographic record. A denaturing gel showing the composition and non degraded nature of RNA routinely obtained using this method of total RNA extraction is shown in figure 1.

#### 2.III.ii.b.5. RNA radiolabelling.

The radiolabelling of rabbit globin mRNA was performed by the use of the enzyme poly A polymerase. This enzyme catalysed the addition of ATP to the 3' end of RNA molecules, and extended the length of existing poly A tails (Sippel, 1973).

The labelling reaction mixture contained 5 µg rabbit globin mRNA, 100 µCi [2,8-<sup>3</sup>H]-ATP (~10 µM), 5 µM cordycepin tri-phosphate, 235 µM ATP, 50 mM tris HCl pH 7.9, 10 mM MgCl<sub>2</sub>, 2.5 mM MnCl<sub>2</sub>, 250 mM NaCl, 0.5 mg/ml BSA, and 6 units of poly A polymerase. The [<sup>3</sup>H]-ATP (dissolved in 50% ethanol) was dried down under vacuum (as suggested in Maniatis *et al.*, 1982) in a microfuge tube, in order to remove its ethanol solvent. The tris, MgCl<sub>2</sub>, and NaCl were dissolved and the pH was adjusted to 7.9 with HCl, before the addition of the MnCl<sub>2</sub> (as the Mn<sup>2+</sup> ions formed a brown precipitate at higher pH values). The ATP, cordycepin tri-phosphate and BSA were made up at 10 x the required concentration and were added as 0.1 x the volume of the final

Figure 1. A RNA agarose denaturing gel (formaldehyde) showing an example of total RNA routinely obtained from total RNA extraction using the procedure outlined in the text. The 28S and 18S bands illustrated are the major ribosomal RNA (rRNA) species.



solution. The dried [ $^3\text{H}$ ]-ATP was resuspended in the final reaction solution (in the absence of the enzyme), and the reaction was started by the addition and mixing of the enzyme. The total reaction volume was 250  $\mu\text{l}$ . After 3 hours, the labelling reaction was terminated, the [ $^3\text{H}$ ]-globin mRNA was ethanol precipitated (2.5 volumes of ethanol and 0.1 volume Na acetate; and centrifuged) three times to dispose of most of the unincorporated [ $^3\text{H}$ ]-ATP. The pellet after the final ethanol precipitation in 70% ethanol was stored at  $-20^\circ\text{C}$  until further purification was performed.

Poly A polymerase has been reported to incorporate most of its substrate (ATP) into poly A tails of between 300 bases and several thousand bases in length (Sippel, 1973). In order to produce a homogeneous population of radiolabelled mRNA with short poly A tail extensions, cordycepin tri-phosphate was added to the reaction mixture. The presence of cordycepin tri-phosphate prevents the extension of poly A tails due its lack of a 3 prime hydroxyl group (Suhadolink, 1970) and therefore attenuates the length of poly A tails added to mRNA.

Both the radiolabelled globin mRNA prepared as described above and [ $^3\text{H}$ ]-HeLa cell total RNA (Gibco/BRL) were then used to determine the efficiency of the extraction procedures for the extraction of total cellular RNA and poly A<sup>+</sup> mRNA (see Chapter 3). This was done by adding a known amount of radioactivity of [ $^3\text{H}$ ]-RNA to the cell pellet immediately after addition of the guanidinium lysis buffer, or for poly A<sup>+</sup> mRNA isolation, by the addition of [ $^3\text{H}$ ]-RNA to previously extracted samples of total RNA. Aliquots of the discarded 4 M LiCl and 3 M LiCl supernatants, the organic phases from the phenol/chloroform extractions and, the ethanol/Na acetate solution discarded from the precipitation step were then sampled and radioactivity determined, to estimate the loss of RNA. Correction factors compensating for the quenching effects of solvents during scintillation counting, were calculated. This was done by counting a known amount of radioactivity in the presence or absence of the solvents involved. For the poly A<sup>+</sup> mRNA extraction procedure, aliquots of all solutions recovered from oligo (dT) cellulose column elutions, were scintillation counted to estimate the loss of radioactivity, and therefore the recovery of [ $^3\text{H}$ ]-RNA, at each stage of the procedure.

### 2.III.ii.c. hybridisation methods.

#### 2.III.ii.c.1. transfer of nucleic acids to filters.

As described in the introduction, two types of analysis of nucleic acids by hybridisation can be performed. These are Southern (DNA) or Northern (RNA) blot hybridisation and, dot or slot blot hybridisation (DNA or RNA).

#### 2.III.ii.c.1.I. Northern blotting of RNA.

For Southern and Northern blots, nucleic acid samples were electrophoresed prior to transfer to filters for hybridisation. The RNA for Northern blots, was electrophoresed as described in section 2.III.ii.b.4.. Gels subsequently used for Northern blotting normally had a volume of 100 mls (1.2% agarose), and were cast in a model H5 (11 x 14 cm) gel former (Gibco/BRL) The transfer or blotting of RNA from gels onto filters was performed by an electroblotting procedure (see section 2.I.ii.a.1.). The transfer of nucleic acids to nitrocellulose filters requires the presence of a high salt concentration (ie 20 x SSC is normally used) in gels and transfer buffers. Buffers of this ionic strength cause unacceptably high electrical resistance and consequently overheat during electroblotting. Nylon filters do not require high salt concentrations in buffers for the adsorption of nucleic acids. This is presumably because these filters adsorb nucleic acids by a different mechanism due to their positively charged surfaces. Nylon filter were consequently ideal for electroblotting. As a result the experiments involving Northern blotting used Zeta-probe (Biorad laboratories Ltd.) or Hybond N<sup>+</sup> (Amersham International plc.) charge modified nylon membranes.

The transfer of RNA from formaldehyde denaturing gels was performed using a Trans-Blot Electrophoretic Transfer Cell (BioRad laboratories Ltd.). After the electrophoretic separation of nucleic acids, the gel was soaked for 15 minutes in 1 x transfer buffer (see Appendix 3.) in order to remove the formaldehyde and equilibrate the gel in transfer buffer. The gel holder of the apparatus was placed in a tray half full of transfer buffer. A Scotch-Brite pad and a piece of Whatman 3MM filter paper, pre-soaked in transfer buffer were then placed in the gel holder. The gel was then placed in the holder, such that there were no air bubbles between it and the filter paper. A piece of nylon filter membrane presoaked in transfer buffer, was placed on top of the gel and all



air bubbles were removed from between the gel and the nylon membrane. This was done by rubbing the membrane from the centre to the edges in order to push the air bubbles out from underneath the membrane. A piece of Whatman filter paper presoaked in transfer buffer, was placed on top of the nylon membrane, and the air bubbles were again squeezed out. Finally another presoaked (in transfer buffer) Scotch-Brite fibre pad was placed on top of the filter paper. The gel holder was closed and fastened. The Trans-Blot electroblotting tank was half filled with precooled (0°C) transfer buffer. The gel holder was inserted into the tank such that the side of gel in contact with the nylon filter faced the positive pole of the tank. The tank was cooled by a cooling coil connected to a chilled bath containing anti freeze (Shell UK Ltd). The water bath was cooled (to -4°C) by two Grant Instrument cooling coil compressors. The tank was filled with transfer buffer, which was then stirred by a magnetic stirring bar. This was done to redistribute the buffer, to prevent localised changes in pH and consequent overheating. The gel was electroblotted for >4 hours at 100 volts. The RNA in the gel migrated towards the positive pole of the apparatus and was absorbed onto the nylon filter. After electroblotting, the gel holder was inverted and opened such that the gel was on top of the nylon membrane. The position of the gels wells were marked on the filter with a soft pencil, and the filter was removed from the gel holder. Zeta-probe nylon membranes were then rinsed in transfer buffer and air dried. If Zeta-probe membranes were not to be hybridised within 2 days, the filters were baked in a vacuum oven for 30 minutes at 80°C. Hybond N<sup>+</sup> nylon membranes were placed on a piece of Whatman 3MM filter paper soaked in 50 mM NaOH, for 5 minutes, then they were rinsed briefly in 2 x SSC and dried. Baking was not required for the fixation of nucleic acids to Hybond N<sup>+</sup> membranes.

#### 2.III.ii.c.1.II. dot blotting of RNA.

The dot blotting of RNA samples was performed by a method modified from Davis *et al.* (1986). In this method, RNA samples are blotted onto nitrocellulose without any form of denaturing but, in the presence of a high salt concentration (20 x SSC). Thomas (1980) found that the efficiency of transfer of RNA was proportional to the extent of denaturation. RNA denatured by heating (50°C, for 1 hour) adsorbed to nitrocellulose with much reduced efficiency compared to RNA denatured by heating in 1 M glyoxal or dimethyl sulphoxide. They (Thomas, 1980) suggested that the reduced efficiency of

RNA denatured by heating was caused by partial renaturation of the RNA during the 12-15 hours of transfer process. This, suggested that the successful transfer of RNA by the method of Davis *et al.* (1986) would require some form of denaturing step. Methods with denaturing steps utilizing formaldehyde, glyoxal (Maniatis *et al.*, 1982) were tested but were found to give high background or irregular hybridisation signals. A method using heat treatment was found to yield more consistent results. Subsequently the method used for denaturing RNA was that of heat denaturing, where RNA samples were heated to 65°C for 15 minutes and then cooled on ice. The samples were serially diluted (4-12 times) in 20 x SSC and then dot blotted as quickly as possible (0-10 µg in 0-200 µl).

The size of dots of RNA blotted onto nitrocellulose filters was regulated by the use of a dot blotting manifold (Gibco BRL; see figure 2). This manifold consisted of three blocks of perspex. Two of the perspex blocks had a grid of 96 (8 x 12) 4 mm diameter holes drilled through each block. Nitrocellulose and Whatman 3MM filters were soaked in DEP-H<sub>2</sub>O, and then equilibrated in 20 x SSC, before use. The manifold was assembled such that the nitrocellulose filter and a piece of Whatman 3MM filter paper were sandwiched between the two blocks of perspex with holes. The blocks were bolted

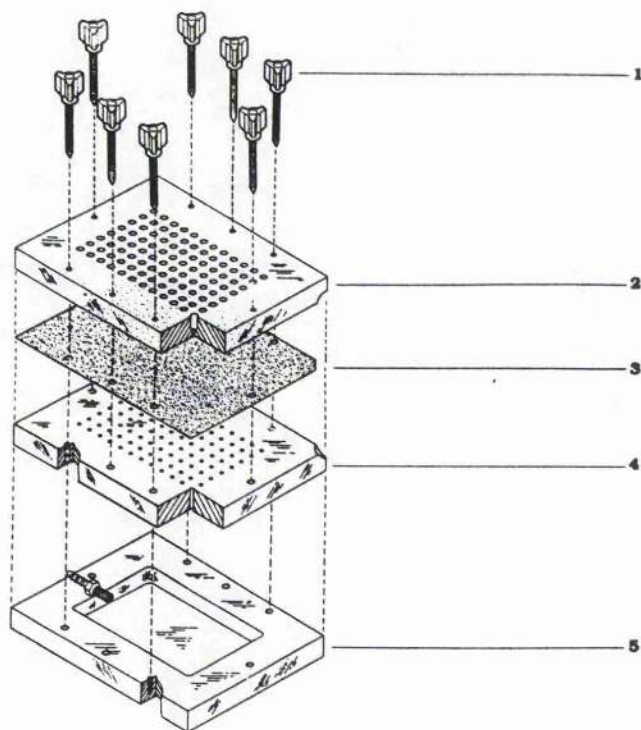


Figure 2. Shows the dot blot manifold apparatus from Gibco/BRL. The apparatus consisted of 1) 8 locating bolts. 2) the sample well template (8 x 12, 4 mm diameter wells). 3) The nitrocellulose or nylon filter on top of a piece of Whatman 3MM filter paper. 4) the filter support block. 5) The vacuum suction chamber.

together with six locating screws. RNA samples (100  $\mu$ l) were applied to the 96 holes (wells). The RNA solution was drawn onto the nitrocellulose or nylon filter into a reservoir, by a low vacuum (5 in of Hg). RNA was retained by the nitrocellulose filter and formed uniform dots. After the RNA samples were filtered through the nitrocellulose, the wells of the manifold were rinsed with 200  $\mu$ l of 20 x SSC to wash the filter and collect any RNA deposited on the side of the wells. The nitrocellulose filter was removed from the manifold and allowed to dry (30 minutes at room temperature). The filters were placed between two pieces of Whatman 3MM filter paper, and baked for 2 hours in a vacuum oven (80°C). The nitrocellulose filter was then ready for hybridisation.

#### 2.III.ii.c.2. nucleic acid hybridisation.

Initial experiments employed hybridisation protocols essentially as described in Anderson and Young (1985). However, later blots were processed using a Hybaid blot processing system. Hybridisation consisted of four stages:- 1, Prehybridisation; where the hybridisation membrane was incubated in a solution with high concentrations of blocking agents in order to block non-specific binding sites. 2, Hybridisation; the membrane was transferred to a solution containing the radiolabelled, complementary nucleic acid probe. The probe was allowed to anneal or re-associate with its complementary nucleic acid sequence immobilized on the membrane. 3, Washing; The membrane was washed in buffers at temperatures and ionic strengths which resulted in the removal of any non-specifically bound probe without removing the probe bound to complementary sequences. 4, Autoradiography; A piece of X-ray film was exposed by the radioactivity bound to the membrane. The image on the X-ray film gave an estimate of the quantity and location of radioactive probe bound to the hybridisation membrane.

#### 2.III.ii.c.2.I. prehybridisation of filters.

For the initial hybridisation experiments, the prehybridisation solution consisted of 50% deionised formamide, 5 x SSC, 5 x Denhardt's solution (see Appendix 3), 50 mM Na phosphate buffer, pH 6.8, and 250  $\mu$ g/ml sonicated salmon sperm DNA (SS DNA) (added just before use).



The Hybaid blot processing system essentially consisted of a mesh and a hybridisation bag (see figure 3). The hybridisation membrane was inserted between the two sides of a folded over plastic mesh. The mesh was then inserted inside the

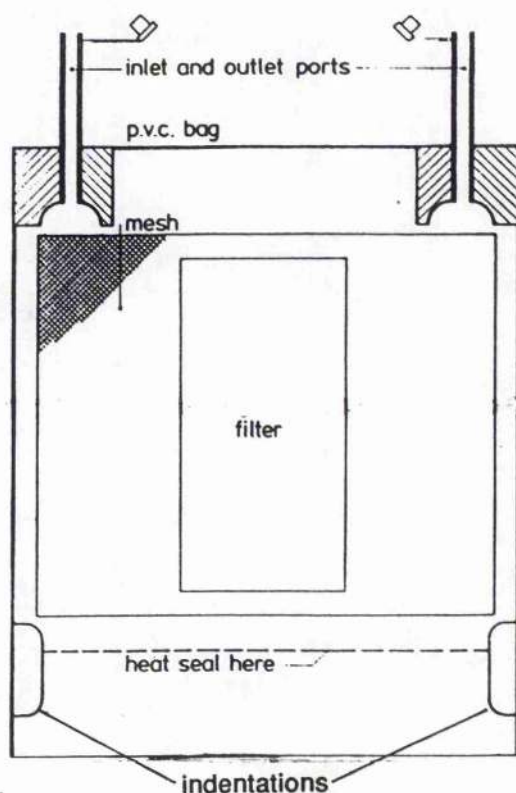


Figure 3. Shows the hybridisation bag and mesh of the Hybaid blot processing system. The hybridisation membrane was enclosed within a mesh, which was inserted inside a PVC bag. The PVC bag has two ports for the addition or withdrawal of hybridisation solutions or air.

hybridisation bag. The hybridisation bag was a thick PVC bag with two stoppered luer ports at the end of two pieces of plastic tubing. The tubes enter the bag at the top corners. The bag was molded with indentations near the base, in order to locate the mesh in an identical position. The bottom of the bag was left open to allow insertion of the membrane. The mesh was inserted into the bag (from the bottom), and forced past the indentations. The bag was then sealed below the indentations using a heated bag sealer (Calor). The prehybridisation solution was heated to the desired temperature (usually 42°C) in a water bath. The SS DNA was then heat denatured at 100°C for 5 minutes, cooled (on ice), added to the prehybridisation solution and thoroughly mixed. The solution (15 ml volume) was taken up into a syringe and added to the hybridisation bag

via the luer port. The port was then stoppered. The syringe was then used to withdraw all the air from the bag via the other luer port. When the prehybridisation solution had covered the mesh and filter, and the air was removed from the bag, the tube was sealed off with a clip, while the syringe was withdrawn from the port. The port was stoppered and the tube unclipped. The bag was then prehybridised overnight (16-24 hours) in a shaking water bath at 42°C.

#### 2.III.ii.c.2.II. hybridisation of filters.

The hybridisation solution consisted of 50% formamide, 5 x SSC, 1 x Denhardt's reagent, 20 mM Na phosphate buffer pH 6.8, 100 µg/ml SS DNA. The radiolabelled probe was added separately before use.

The hybridisation solution was heated to 42°C in a water bath. The radiolabelled nucleic acid probe (see section 2.III.ii.a.6.) and the SS DNA were denatured by heating (100°C for 5 minutes) and, cooled on ice, before their addition to the hybridisation solution. The prehybridisation solution was poured out of the hybridisation bag, and the bag was filled with air using a syringe. The hybridisation solution was split into two halves, these halves were added to each of the bags two ports, and the ports were then stoppered. This was done to ensure an equal distribution of the radioactive probe over the surface of the filter. The bag was rotated 10 times to mix the hybridisation solution with any residual prehybridisation solution. The air was withdrawn from the bag with a syringe. When the hybridisation solution covered the mesh/filter, and the air was completely removed, the tube was sealed off with a clip. The syringe was removed from the port, the port was stoppered and the clip removed. The bag was returned to the shaking water bath, and was incubated overnight (16-24 hours) at 42°C.

#### 2.III.ii.c.2.III. washing of filters.

The washing solutions consisted of 2 x SSC containing 0.1% SDS and 0.1 x SSC containing 0.1% SDS.

After the completion of hybridisation, one port of the hybridisation bag was unstoppered, and air was blown into the bag with a syringe. The hybridisation solution collected at the bottom of the bag and was poured off through the open port. One of the bags ports was then connected to a bottle of wash buffer via a piece of tubing. The other



port was connected by tubing to a vacuum pump, via a trap for collecting waste wash solution. The bag and the bottle of wash buffer were transferred to a water bath at 55°C. A low vacuum was applied to the bag and wash buffer was drawn from the wash bottle into the bag and across the surfaces of the mesh and filter, out of the bag, and was collected in the vacuum trap. The filter was washed for 10 minutes in the 2 x SSC wash buffer and for 10 minutes in the 0.1 x SSC wash buffer.

#### 2.III.ii.c.2.IV. autoradiography of filters.

After washing, the filters were removed from the hybridisation bag and mesh. Without allowing the filter to dry, the filters were sealed into a plastic bag (120 gauge plastic, Lakeland Plastics). The plastic bag was fixed in place in a Mult-e-role X-ray cassette with Hi-Speed-X intensifying screens. All procedures using X-ray film were performed in a dark room. A piece of X-ray film (Hyperfilm-MP, Amersham International) was preflashed, using a Sunpak auto zoom 5000 flash with a Wratten No. 22 gelatin filter. This was done to improve the linearity of the films response to low levels of radioactivity. The intensity of the flash was modulated to increase the absorbance of the film after development by approximately 0.15 absorbance units at 540 nm. The film was secured into the X-ray cassette on top of the plastic bag containing the blot, the film was exposed at -60°C. Incubation at -70°C improves the sensitivity of the film, by 2-fold over that at 20°C (Laskey, 1984). The length of exposure of film depended on the level of radioactivity present on the filter. Filters with hot spots of 2-5, 5-10, or >10 cpm, (assessed using a geiger counter; Series 900 mini monitor; Mini Instruments Ltd) were exposed for approximately 2-3 days, 24-36 hours, or 16-24 hours respectively. After exposure, the film was removed from the cassette for developing. The film was developed for 4 minutes at room temperature in LX-24 X-ray developer (Kodak Chemicals Ltd) and fixed for 3 minutes in Hypam fixer (Ilford). The film was washed in water for >5 minutes and allowed to dry.

#### 2.III.ii.c.2.V. quantification of autoradiographs.

The radioactivity contained within dots or gel bands on autoradiographs was determined by the use of a Shimadzu flying spot scanning densitometer (CS-9000). The scanner moved the X-ray film across a light beam and measures the absorption of the

light. For Hyperfilm-MP the optimal wavelength for measurement was 500 nm. The scanner produced a trace of the level of absorbance as it moved across the surface of the film. The area under peaks of absorbance were calculated by the scanners computer software. The arbitrary units of area were calculated such that 1 unit of area = an absorbance of 0.001 OD units every 0.02 mm. The autoradiographic signals were quantified by comparison to a set of standards of known amounts of radioactivity, or by comparison to a standard curve of double diluted RNA samples (dot blots). Standards of known amounts of radioactivity were produced by counting small aliquots of radiolabelled DNA probe, and dot blotting a series of decreasing amounts of radioactive probe.

CHAPTER 3. ISOLATION AND QUANTIFICATION OF  
TOTAL RNA AND mRNA.

### 3.I. Introduction.

For the majority of this project the cell line MDCK (strain I; Richardson *et al.*, 1981) was chosen as a model system to investigate factors affecting the cell surface expression of Na,K-ATPase. A further aim, was to attempt to estimate whether any changes in the expression of the enzyme could be correlated to changes in the abundance of specific Na,K-ATPase mRNA's. The background information and characteristics of this cell line have been highlighted in section 1.IV..

Optimal procedures to estimate the number of sodium pump units on the surface of MDCK cells were already available and routinely performed in this laboratory (Lamb *et al.*, 1981). Many procedures for the extraction of RNA exist, of which the most popular is probably the method of Chirgwin *et al.* (1979), employing extraction in guanidinium isothiocyanate and the separation of RNA from other cellular material by CsCl density gradient ultracentrifugation. Other methods include the use of guanidinium isothiocyanate and hot phenol (Maniatis *et al.*, 1982), guanidinium isothiocyanate and lithium chloride (Cathala *et al.*, 1983), guanidine hydrochloride (Berger and Kimmel, 1987), SDS and hot phenol (Davies *et al.*, 1986) and, lithium chloride and urea (Auffray and Rougeon, 1980). The method of choice for this project was a modification of the guanidinium isothiocyanate and lithium chloride procedure of Cathala *et al.* (1983), which is outlined in section 2.III.ii.b.. This protocol was compatible with the laboratory equipment available although its main advantage was that it was possible to process relatively large numbers of samples simultaneously (Cathala *et al.*, 1983). Furthermore, the method of Cathala *et al.* (1983) produced higher yields of both total and poly A<sup>+</sup> RNA than the CsCl and ethanol based method of Chirgwin *et al.* (1979). The CsCl based method of Chirgwin *et al.* (1979) was also impracticable, as only a small number of cell samples could be processed at any one time.

The most commonly used method for quantifying the abundance of specific mRNA's, involves assessing the amount of radioactive signal produced from the hybridisation of a radiolabelled DNA or RNA probe (which is complementary to the mRNA of interest). However, the absolute abundance of specific mRNA's cannot be determined, because the efficiencies of total RNA extraction, poly A<sup>+</sup> mRNA isolation, and nucleic acid hybridisation are unknown. In order to compare the abundance of specific mRNA's between different RNA samples the comparisons have to be standardised against the abundance of a parameter which is likely to be constant between

samples. The abundance of specific mRNA's are usually standardised against the amount of total RNA used for each sample. An example of this type of standardisation is found in Young and Lingrel (1987), who used equal amounts of total RNA per sample for the analysis of the abundance of specific Na,K-ATPase mRNA's. When experiments are performed where the level of a specific mRNA species is likely to be modulated compared to controls, the assumption is made that, the amount of total RNA per cell remains unaltered by experimental treatments. This assumption may not always be correct, as the amount of total RNA per cell can be variable (Gick *et al.*, 1988b). In cell culture where the number of cells are known, the amount of total RNA per cell can be estimated. However if the efficiency of total RNA extraction is low, small changes in the efficiency would result in large percentage changes in the estimate of the amount of total RNA per cell. To minimise the effect of variations in the efficiency of total RNA extraction, procedures giving consistently high percentage yields of total RNA or poly A<sup>+</sup> mRNA are required. The premise for the experiments undertaken in this chapter was therefore to determine the percentage yield and range of variation of the total RNA extraction and poly A<sup>+</sup> mRNA isolation procedures.

### **3.II. Methods.**

For the general methods used in this Chapter see Chapter 2.

#### **3.II.i. Rabbit globin mRNA radiolabelling.**

Rabbit globin mRNA was radiolabelled by the enzyme poly A polymerase (as outlined in section 2.III.ii.b.5.). For the [<sup>3</sup>H]-mRNA produced by poly A polymerase to be useful for the estimation of the efficiency of RNA extraction, the radiolabelled mRNA had to be separated from unincorporated nucleotides. Several separation methods were tried, including separation by Sephadex column chromatography, and denaturing agarose gel electrophoresis. Both of these techniques proved unsatisfactory, as very little [<sup>3</sup>H]-mRNA was recovered from the column chromatography and the gel electrophoresis gave a smeared band possibly due to the differing extent of radiolabelling. However, [<sup>3</sup>H]-mRNA was efficiently separated from unincorporated nucleotides by oligo (dT) column chromatography. Unincorporated [<sup>3</sup>H]-ATP does not bind to the column, but elutes with the poly A<sup>-</sup> RNA (in the loading buffer; see figure 1).



The radiolabelling reaction was carried out as in section 2.III.ii.b.5.. The impure [ $^3\text{H}$ ]-mRNA extract from the radiolabelling reaction, which was stored in ethanol, was

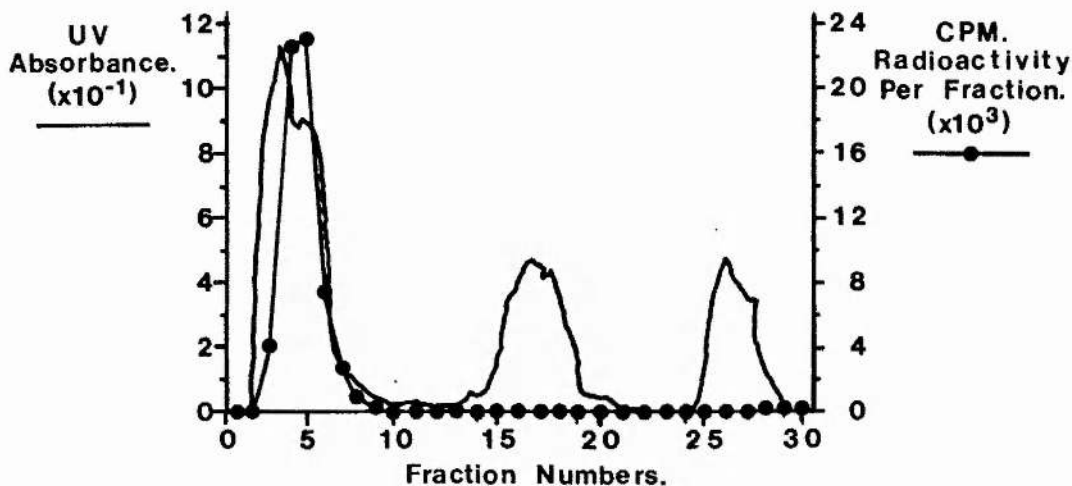


Figure 1. Absorbance (254 nm) and [ $^3\text{H}$ ]-radioactivity of the eluate from an oligo (dT) column. A sample of total RNA (to act as a UV marker) and [ $^3\text{H}$ ]-ATP were added to the column dissolved in loading buffer. This was followed, by the addition of loading buffer, collected in fractions 1-11, elution buffer (at 65°C) in fractions 12-22, DEP- $\text{H}_2\text{O}$  in fraction 23 and, wash buffer in fractions 24-30 (see section 2.III.ii.b.2.). Fractions collected from the column were scintillation counted to determine the level of [ $^3\text{H}$ ]-radioactivity.

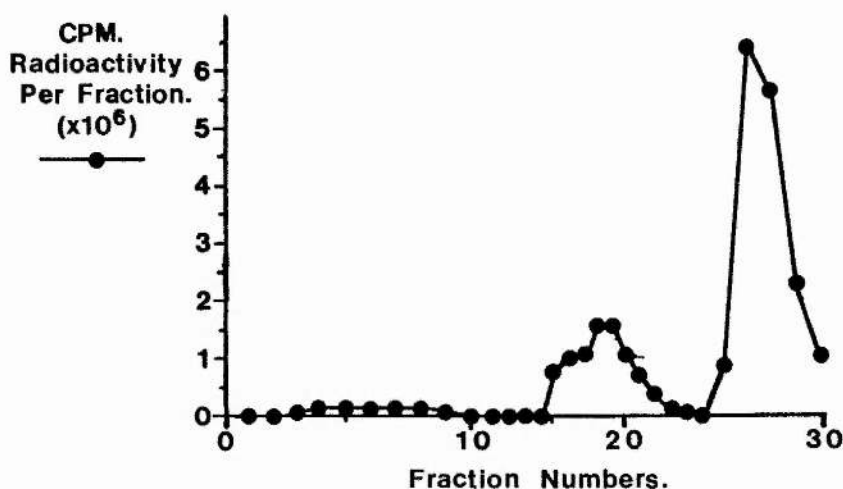


Figure 2. Shows the radioactivity in eluted fractions from an oligo (dT) column. Loading buffer was added to the column in fractions 1-11, elution buffer (65°C) in fractions 12-24, and wash buffer in fractions 25-30.

recovered by centrifugation (13000 rpm for 20 minutes). The pellet was dissolved in DEP-H<sub>2</sub>O, and after the addition of an equal volume of 2 x loading buffer the [<sup>3</sup>H]-mRNA solution was added to an oligo (dT) column. The progress of the [<sup>3</sup>H]-mRNA during chromatography was monitored by assessing the radioactivity in each fraction of the eluate collected from the column (see figure 2).

The point at which the loading, elution and wash solutions were added to the oligo (dT) column was determined by the return of the UV absorbance to a basal level. Although the UV trace was difficult to follow, as only 5µg of mRNA was applied to the column. The purified [<sup>3</sup>H]-mRNA was presumed to be the RNA eluted in the elution buffer (poly A<sup>+</sup> mRNA). The fractions containing the poly A<sup>+</sup> mRNA were ethanol precipitated (2.5 volumes of ethanol and 0.1 volume of 3 M Na acetate). The pellet of [<sup>3</sup>H]-mRNA obtained after centrifugation was then dissolved in 1 ml of DEP-H<sub>2</sub>O, aliquoted (40µl) and stored frozen in the vapour phase of liquid nitrogen. The aliquots of [<sup>3</sup>H]-mRNA were then used to determine the efficiency of both the total RNA extraction procedure and the poly A<sup>+</sup> mRNA isolation procedure.

### **3.III. Results.**

#### **3.III.i. Determination of the efficiency of total RNA extraction using radiolabelled HeLa cell total RNA.**

Two experiments were undertaken to investigate the efficiency of the RNA extraction procedure. These experiments were also used to determine whether the use of the protein denaturant urea, in combination with LiCl offered any improvement over extraction using 4 M LiCl alone in step 3, of the RNA extraction protocol (see section 2.III.ii.b.1.). A combination of 3 M LiCl and 6 M urea was used as a replacement for the 4 M LiCl precipitation step. A known amount of [<sup>3</sup>H]-HeLa cell total RNA (Gibco BRL) was added to each sample of cells lysed in guanidinium isothiocyanate buffer at the beginning of the extraction procedure (step 2). The RNA extraction procedure was continued as in the procedure outlined in section 2.III.ii.b.1., except that samples of solutions discarded during extraction were assessed for radioactivity. This was done to determine the loss of RNA from each stage of the extraction procedure. The amount of radioactive quenching, caused by the presence of extraction solutions during scintillation counting, was determined by counting known amounts of radioactivity in the presence of each solution.

solution. The estimates of quenching were then used to correct the data. The results from the two repeat experiments are given in table 1.

As can be seen from tables 1, the overall extraction procedure utilizing 4 M LiCl (in step 2 of the protocol; see section 2.III.ii.b.1.) was more efficient at recovering RNA, than the equivalent protocol using 6 M urea/3 M LiCl (in step 2 of the protocol). Also it was noticeable that most of the RNA lost from the extractions was lost during the initial precipitation with either 6 M urea/3 M LiCl or 4 M LiCl.

Stage of total RNA extraction.	RNA losses (%).	
	3 M LiCl/6 M urea extractions.	4 M LiCl extractions.
3 M LiCl/6 M urea or 4 M LiCl precipitation.	45.51 ± 9.14	31.64 ± 13.26
3 M LiCl precipitation.	0.74 ± 0.14	1.47 ± 0.06
First Phenol extraction.	0.38 ± 0.21	0.44 ± 0.02
Second Phenol extraction.	1.47 ± 0.61	1.93 ± 0.19
RNA unrecovered.	22.16 ± 0.08	14.83 ± 12.07
Total RNA recovered.	29.75 ± 8.69	49.71 ± 0.93

Table 1. Recovery of [<sup>3</sup>H]-HeLa cell RNA from two different total RNA extraction procedures. Data are corrected for the quenching effect of the various solvents. Figures represent the % of the [<sup>3</sup>H]-RNA lost (or recovered), at each stage of the extraction procedure. Figures indicated represent ± % values from the data of 2 experiments.

Experiments were also under-taken to determine the best method of precipitation of the RNA at the end of the protocol (step 9; see section 2.III.ii.b.1.). Precipitation using 2 volumes of ethanol and 0.1 volumes of Na acetate, 2 M LiCl, 3 M LiCl, or 2.5 volumes of ethanol and 0.1 volumes of Na acetate were performed. As can be seen in table 2, precipitation using 2.5 volumes of ethanol and 0.1 volume of Na acetate produced the best yield of RNA.

Precipitation Method.	% Recovery of [ <sup>3</sup> H]-RNA from RNA extraction.	
	3 M LiCl/6 M urea extraction.	4 M LiCl extraction.
2.5 volumes of ethanol + 0.1 volumes of Na Acetate	20.79	48.78
2.0 volumes of ethanol + 0.1 volumes of Na Acetate	8.28	43.86
2 M LiCl.	3.51	11.03
3 M LiCl.	7.88	11.66

Table 2. Percentage recovery of [<sup>3</sup>H]-HeLa cell RNA from total RNA extraction. Extraction was performed using either 4 M LiCl or 3 M LiCl/6 M urea in step 2 of the protocol (see section 2.III.ii.b.1.) and then finally precipitated (in step 9) with the solutions indicated. The recoveries indicated represent the percentage recovery of the radioactivity added initially to the extractions.

HeLa cell [ $^3\text{H}$ ]-RNA, was produced by the growth of cells in [ $^3\text{H}$ ]-uridine. The RNA extracted from these cells is reported to contain RNA's smaller than 300 nucleotides in length (ie tRNAs; Gibco BRL). RNA's smaller than this size (300 nucleotides) were poorly extracted by the LiCl RNA extraction procedure (Cathala *et al.*, 1983). Thus these tRNA's (and probably 5S rRNA's) and their associated radioactivity would almost certainly be lost during RNA extraction. As the mRNAs of interest in this project are much greater than 300 nucleotides in length, the resulting estimate of the efficiency of the extraction of these large mRNA's would be under estimated. For this reason, it was decided to attempt to radiolabel a mRNA of size greater than 300 nucleotides and investigate its efficiency of extraction. The radiolabelling of globin mRNA was chosen as this was commercially available (Gibco BRL) and, it was approximately 600-650 bases in size (see Methods section 3.II.).

#### 3.III.ii. Determination of the efficiency of the total RNA extraction procedure, using radiolabelled rabbit globin mRNA.

The samples of [ $^3\text{H}$ ]-globin mRNA were used to assess RNA recovery in the same way as the [ $^3\text{H}$ ]-HeLa cell RNA. The [ $^3\text{H}$ ]-mRNA was added to the cell samples after the addition of lysis buffer (step 2, see section 2.III.ii.b.1). The loss of RNA during the extraction protocol was calculated from the loss of radioactivity in discarded solutions. Three separate experiments were performed with different numbers of independent samples (2, 4 and 8 samples respectively). Multiple numbers of cell samples were processed in order to estimate the variation in the efficiency of total RNA extraction both within and between experiments. Samples both within and between experiments were treated identically. However, between experiments more variation in the extraction of cell samples was likely to exist. The mean results of these three experiments are shown in table 3 and the range of values found in each experiment are given in table 4.

#### 3.III.iii. Determination of the efficiency of oligo (dT) column chromatography using radiolabelled globin mRNA.

As the [ $^3\text{H}$ ]-rabbit globin mRNA was purified on an oligo (dT) column, this RNA was ideal for the measurement of the efficiency of poly A<sup>+</sup> mRNA isolation by oligo (dT) column chromatography. A known amount of radiolabelled globin mRNA was added to



Stage of RNA extraction.	RNA losses (%).
4 M LiCl precipitation.	20.84 ± 3.23
3 M LiCl precipitation.	0.39 ± 0.10
First Phenol extraction.	0.76 ± 0.29
Second Phenol extraction.	1.39 ± 0.41
Ethanol/Na acetate precipitation.	0.12 ± 0.05
RNA un-recovered.	7.87 ± 3.45
Total RNA recovered.	68.62 ± 5.11

Table 3. Shows the percentage loss or recovery (where appropriate) of [<sup>3</sup>H]-RNA from each stage of the total RNA extraction procedure. The data was corrected for the quenching effect of the various solvents during scintillation counting. Figures are ± % standard deviations, where n = 14 samples from 3 separate experiments.

samples of total RNA before their application to the oligo (dT) column. This RNA was then used for the isolation of poly A<sup>+</sup> mRNA as stated in section 2.III.ii.b.2.. The eluate from the oligo (dT) column was fractionated and collected during each stage of the purification procedure. The radioactivity contained within each fraction was determined by scintillation counting. The relative recovery of [<sup>3</sup>H]-mRNA in the poly A<sup>-</sup> RNA (loading buffer), poly A<sup>+</sup> mRNA (elution buffer) and washing (wash buffer) stages of

the mRNA isolation procedure was determined (see table 5). The quenching associated with the presence of loading, elution and wash buffers during scintillation counting was also determined. These buffers were found to have no or very little effect on the radioactive counts obtained. Therefore, no quench correction of radioactive counting in these buffer was necessary.

Stage of RNA extraction.	RNA losses (%).		
	Experiment 1.	Experiment 2.	Experiment 3.
4 M LiCl precipitation.	16.16 - 16.71	24.43 - 26.35	18.35 - 21.56
3 M LiCl precipitation.	0.24 - 0.31	0.29 - 0.48	0.35 - 0.53
First phenol extraction.	0.24 - 0.43	0.79 - 1.04	0.60 - 1.51
Second phenol extraction.	1.07 - 1.17	1.62 - 1.95	0.72 - 2.02
Ethanol/Na acetate precipitation.	0.01 - 0.02	0.08 - 0.12	0.13 - 0.17
RNA un-recovered.	1.42 - 7.26	3.70 - 12.13	3.73 - 13.75
Total RNA recovered.	74.26 - 80.69	60.13 - 68.19	65.33 - 73.21

Table 4. Shows the percentage range of losses or recovery (where appropriate) of [<sup>3</sup>H]-RNA from each stage of total RNA extraction in three separate experiments. The data was corrected for the quenching effect of the various solvents during scintillation counting. The number of cell samples extracted in each experiment were 2, 4, and 8, in experiments 1, 2, and 3 respectively.

Stage of poly A <sup>+</sup> mRNA isolation.	RNA in eluted solutions (%).	
	Experiment 1.	Experiment 2.
Loading buffer poly A <sup>-</sup> RNA.	0.78 ± 0.19	2.10 ± 0.34
Elution buffer poly A <sup>+</sup> mRNA.	91.69 ± 0.40	77.88 ± 2.52
Wash buffer.	2.88 ± 1.18	6.46 ± 1.42
RNA un-recovered.	4.65	13.56

Table 5. Shows the percentage loss or recovery (where appropriate) of [<sup>3</sup>H]-globin mRNA from each stage of the poly A<sup>+</sup> mRNA isolation procedure for two identically processed cell samples (see section 2.III.ii.b.2.). Poly A<sup>+</sup> mRNA is recovered from the elution buffer. Figures represent ± standard deviations ( $\sigma$  n-1) of radioactive counts from the solutions sampled, where n = 3 samples.

### **3.IV. Discussion.**

The results obtained using [<sup>3</sup>H]-globin mRNA in total RNA extractions, showed that this procedure was highly efficient, giving average yields of 77.5%, 64.0%, and 68.7% in the experiments shown in table 4. The overall mean recovery of [<sup>3</sup>H]-globin mRNA from the 3 experiments (14 samples) was 68.62% ± 1.37% (SEM). This mean was considerably higher than the comparable 49.71% mean recovery of [<sup>3</sup>H]-HeLa cell RNA (from the extraction protocol using 4 M LiCl). The difference between these mean recoveries probably reflected the loss of small [<sup>3</sup>H]-RNA's from the [<sup>3</sup>H]-HeLa cell RNA. This showed that the [<sup>3</sup>H]-globin mRNA was a better marker for the recovery of large mRNA's than was [<sup>3</sup>H]-HeLa cell RNA.

The vast majority of the [ $^3\text{H}$ ]-RNA lost during the RNA extraction procedure using either [ $^3\text{H}$ ]-globin mRNA or, [ $^3\text{H}$ ]-HeLa cell RNA, occurred during the initial precipitation in lysis buffer and 4 M LiCl. The replacement of the 4 M LiCl used in the initial precipitation step with 6 M urea/3 M LiCl did not improve either the eventual recovery of [ $^3\text{H}$ ]-RNA or the losses encountered using 4 M LiCl in this step. If anything the addition of urea at this stage increased the loss of [ $^3\text{H}$ ]-RNA from the extraction. A variable amount of [ $^3\text{H}$ ]-RNA remained un-recovered, probably due to losses on the surfaces of tubes and pasteur pipettes, or due to the variable quenching effect of biological material contained within solutions scintillation counted. Because, only the quenching effect of the solutions themselves were accounted for in the results.

To assess the variation in the efficiency of total RNA extraction, the recovery of [ $^3\text{H}$ ]-RNA data in table 4 was analysed statistically. When an analysis of variance test was performed on the data for RNA recovery, a significant difference (at the 1% level) was found between the results of each experiment. Cell samples extracted within each experiment showed a smaller variation than that found between experiments. The data within each experiment showed that the variation in any one experiment was only  $\pm 4\%$  (of the total [ $^3\text{H}$ ]-mRNA added) of the mean [ $^3\text{H}$ ]-RNA recovery. This shows that, in experiments where the total abundance of mRNA is unknown (assuming the recovery of total RNA is about 68.6%), the highest recovery of total RNA should be only 12.5% higher than that of the lowest. This represented the difference produced by the variation in the efficiency of total RNA extraction (from 72.7-64.6%). Any changes in total RNA abundance greater than 12.5%, would therefore be expected to reflect real differences in the amount of total RNA present in the cells used for the extraction.

The efficiency of the isolation of poly A<sup>+</sup> mRNA was also investigated briefly, in the likelihood of this procedure being required in some future experiments. The mean percentage recovery of [ $^3\text{H}$ ]-mRNA isolated from the two poly A<sup>+</sup> mRNA isolation experiments was 84.8%. This showed that this procedure, if required, would also give a high percentage recovery. However, variations with oligo (dT) cellulose column chromatography may be found because, other cellular RNA's (mainly ribosomal RNA's) are thought to be isolated along with the mRNA (this contamination may be reduced by a second passage of the RNA through the column; Berger and Kimmel, 1987). The use of oligo (dT) cellulose chromatography in the quantitative analysis of the abundance of mRNA, adds greater variation between samples and should therefore only be used when necessary that is, when the abundance of the specific mRNA of interest or the efficiency

of hybridisation are low. This is because, the amount of total RNA required to obtain a reasonable level of radioactive signal from nucleic acid hybridisation, becomes too great. Both dot blot and Northern blotting techniques (see section 2.III.ii.b.) limit the amount of RNA that can be used for the detection of low abundance mRNA's, as the capacity of filters (nitrocellulose = 80  $\mu\text{g}/\text{cm}^2$  or 10  $\mu\text{g}$  per dot) and the volume of gel wells (50  $\mu\text{l}$  or ~100  $\mu\text{g}$  maximum) can be restrictive.

The data presented in this chapter were previously reported in a preliminary form in Cutler *et al.* (1988).



CHAPTER 4. NUCLEIC ACID HYBRIDISATION (Na,K-ATPase cDNA'S AND mRNA'S).

## 4.I. Introduction.

### 4.I.i. Na,K-ATPase and control DNA probes.

The gene probes available for hybridisation experiments involving Na,K-ATPase were:- 1, The full length cDNAs for the rat  $\alpha 1$ ,  $\alpha 2$  and  $\alpha 3$  isoform genes (Shull *et al.*, 1986b), obtained from Dr. J Lingrel, Dept. of Microbiology and Molecular Genetics, University of Cincinnati College of Medicine. 2, A partial human  $\alpha 1$  cDNA probe (2.4 Kb) corresponding to nucleotides ~600-3000 of the cDNA sequence (Emanuel *et al.*, 1986), provided by Dr. R Levenson, Dept. of Cell Biology, Yale University School of Medicine. 3, A partial rat  $\beta 1$  cDNA probe (0.9 Kb) corresponding to nucleotides ~250-1150 of the cDNA sequence (Mercer *et al.*, 1986) also provided by Dr. R Levenson, Yale University School of Medicine. 4, A partial mouse skeletal muscle  $\alpha$  actin cDNA (1.36 Kb) corresponding to most of the genes translated sequence and the 3 prime untranslated sequence (Minty *et al.*, 1981; Minty *et al.*, 1982).

### 4.I.ii. Conditions of hybridisation of nucleic acid probes.

The optimal conditions for the hybridisation of the partial cDNA for the human  $\alpha 1$  isoform (pHANK) were previously determined in this laboratory by an empirical method and were as those used in the work reported in Unkles *et al.*, (1988). The prehybridisation, hybridisation and washing solutions and conditions were as in section 4.III.i., but all washing was previously performed at 60°C. Under these conditions, a Northern blot of HeLa cell (Human cervical carcinoma cell line) total RNA was shown to hybridise to a single RNA species without any non-specific background (Unkles *et al.*, 1988).

### 4.I.iii. Na,K-ATPase $\alpha$ isoform specific probes for MDCK cells.

As described in section 1.IV.i., the three known isoforms of Na,K-ATPase show 85-86% homology in their amino acid (aa) sequences and 76-79% homology in their nucleotide (nt) sequences (Shull *et al.*, 1986b). The high level of homology of aa and nt sequences is however not uniformly distributed along the length of the  $\alpha$  subunit sequence. Large segments of the aa sequence such as that surrounding phosphorylation

site (the asparagine located at position 369 of the aa sequence) show 100% aa homology and approximately 86% nt homology (Shull *et al.*, 1986b). Other regions such as the N-terminal of the subunit have aa sequences which are relatively diverged and show only 40-50% homology (Shull *et al.*, 1988; Jorgenson, 1988). The homology of a partial cDNA for the  $\alpha$  subunit cloned from MDCK cells, including the phosphorylation site has a nt homology of 90% compared to the sheep  $\alpha$  sequence (Bowen and McDonough, 1987). This information suggests that the nucleic acid homology of the central portions of the  $\alpha$  subunit were almost as homologous between isoforms (within one species) as cDNA's of a single isoform between species. Therefore if probes for the rat isoforms were used to cross hybridise to MDCK RNA, at a level of stringency which would detect each probe's complementary dog isoform, RNA for the other isoforms could also hybridise. To overcome the possible cross hybridisation of different isoforms between species, probes for the rat isoforms would have to be found which exhibited much lower nt homology with other isoforms. This should ensure that when the level of stringency was relaxed for cross hybridisation to MDCK (dog) cell RNA, the isoforms would not cross hybridise with each other. The obvious region within the cDNA sequences, to look for probes having low levels of nt homology between isoforms, was the N-terminal of the  $\alpha$  polypeptide or the 5 prime region of the coding sequence. To determine the restriction maps (the positions at which restriction enzymes cut) of the nucleotide sequence of the three  $\alpha$  isoform cDNA's, the sequences were typed into a data base. The data base was analysed for restriction endonuclease cutting sites by the use of a Staden Analyseq software package run on a VAX/VMS mainframe computer. The main region of divergence of the  $\alpha$  isoforms is contained within the initial 30 aa's of the N-terminal, or the first 90 nt of the coding sequence. Restriction sites were chosen that included this region of the sequence with as little of the extra, more homologous down stream sequence as possible. Restriction enzymes (cutting sites) were also chosen that were, inexpensive to purchase, had few other cutting sites in the cDNA and vector sequences concerned, and had no other fragments of an identical size to the desired probe fragment. The fragments chosen were:-

- 1, rat  $\alpha$ 1, a 186bp NCOI-STUI fragment located at positions 237-422 of the cDNA nt sequence.

- 2, rat  $\alpha$ 2, a 142bp BAMHI-SSTI fragment located at positions 44-185 of the cDNA nt sequence.

3, rat  $\alpha$ 3, a 202bp SSTII-SMAI fragment located at positions 130-331 of the cDNA nt sequence (Shull *et al.*, 1986b; see figure 1).

These restriction fragments were then tested for their homology to the other two isoforms, using the Staden Analyseq computer package. The rat  $\alpha$ 1 fragment had a maximum nt homology of 53.8% and 48.4% with the  $\alpha$ 2 and  $\alpha$ 3 isoforms respectively. The rat  $\alpha$ 2 fragment had a maximum nt homology of 43.7% and 38.7% with the  $\alpha$ 1 and  $\alpha$ 3 isoforms respectively. The rat  $\alpha$ 3 fragment had a maximum nt homology of 54.0% and 47.5% with the  $\alpha$ 1 and  $\alpha$ 2 isoforms respectively. The fragments were then compared to their counterparts in another mammalian species (human) to determine their likely homology to the isoforms in dog species (MDCK cells). The rat  $\alpha$ 1,  $\alpha$ 2 and  $\alpha$ 3 fragments were 85.5%, 89.4% and 92.1% homologous to the equivalent nt sequences in human  $\alpha$  isoforms respectively (Kawakami *et al.*, 1986; Shull *et al.*, 1989; Ovchinnikov *et al.*, 1988). Lathe (1985) showed that theoretically 64% was the minimum nt homology necessary, for the hybridisation signal of a sequence to be greater than the background signal from a cDNA library (equivalent in complexity to a population of mRNAs). This showed that, even if the conditions of hybridisation were relaxed enough to allow the hybridisation of > 85% homologous sequences (the  $\alpha$ 1 isoform fragment), cross hybridisation to the other isoforms should not occur.

#### 4.I.iv. The $\alpha$ actin control probe.

The available nucleotide sequence of the mouse  $\alpha$  actin cDNA (Minty *et al.*, 1982) shares approximately 96% and 86% nt homology with the comparable sequences from rat skeletal muscle  $\alpha$  actin and non-muscle  $\beta$  actin respectively (Zakut *et al.*, 1982; Nudel *et al.*, 1983). This suggests that the mouse  $\alpha$  actin cDNA should also share considerable homology with actin mRNA's expressed in MDCK cells.

#### 4.II. Methods.

The hybridisation experiments were performed essentially as described in section 2.III.ii.c.

Figure 1. A gel showing restriction endonuclease digests of plasmids (vector pBR322) containing Na,K-ATPase  $\alpha$  isoform cDNA's. Lane S represents standard markers ( $\phi$ X-174 DNA digested with Hae III), with sizes in base pairs (bp). Lanes 1-4 represent  $\alpha$ 1 cDNA plasmid digested with Nco I and Stu I; lanes 5-8 represent  $\alpha$ 2 cDNA plasmid digested with BamH I and Sst I; lanes 9-12 represent  $\alpha$ 3 cDNA plasmid digested with Sst II and Sma I restriction enzymes. The arrows indicate the positions of the gel wells and the fragments subsequently used as isoform-specific probes.



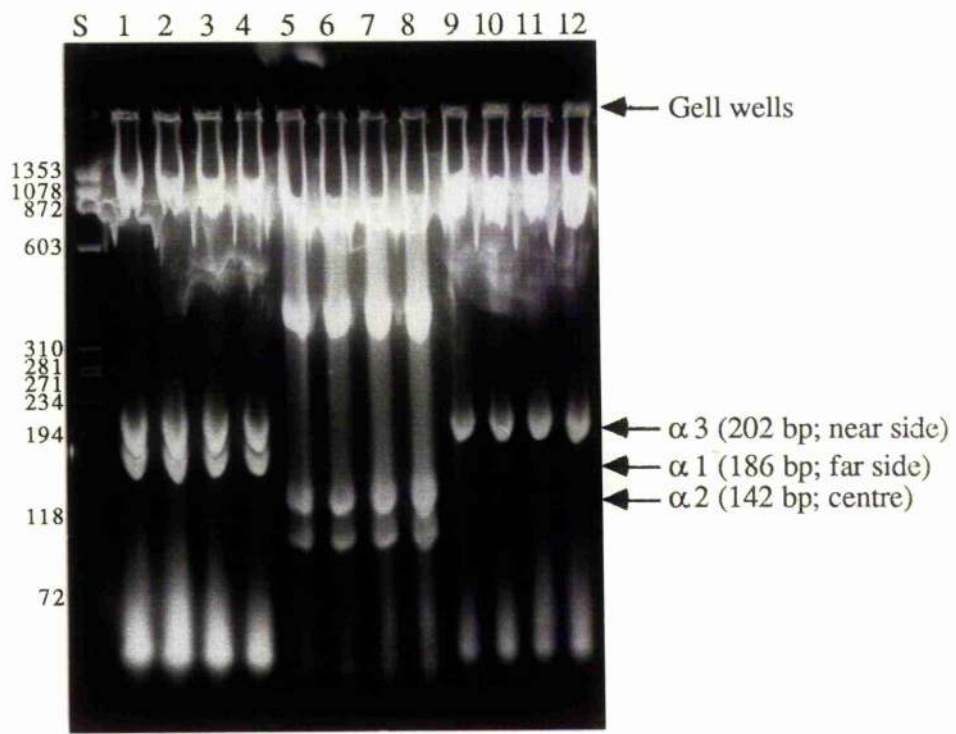
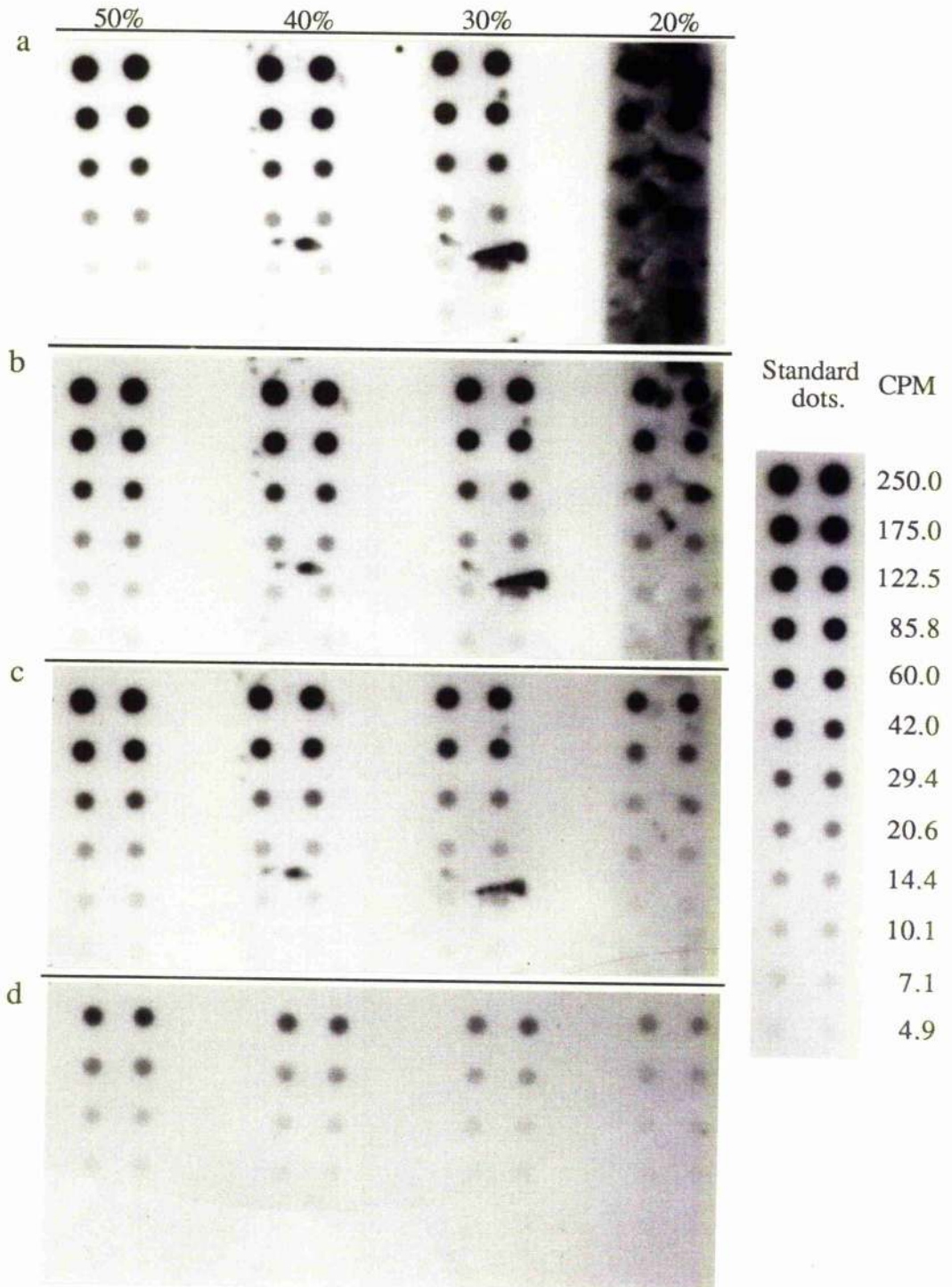


Figure 2 a-d. A series of RNA dot blots, hybridised with radiolabelled pHANK DNA probe in hybridisation solutions containing concentrations of formamide from 20%-50%. The RNA dots are double diluted MDCK total RNA with ~10  $\mu$ g of RNA in the highest concentration dots (top of each column). The four filters containing pairs of double diluted RNA dots were hybridised, and then washed serially in a) 2 x SSC, 0.1% SDS, followed by b) 1 x SSC, 0.1% SDS, c) 0.5 x SSC, 0.1% SDS and d) 0.1 x SSC, 0.1% SDS. All washes were performed at 55° C for 30 minutes. Standard dots containing known amounts of radioactivity are also illustrated.

Percentage formamide.



#### 4.III. Results and Discussion.

##### 4.III.i. Determination of the optimal concentration of formamide for hybridisation.

Howley *et al.* (1979) have suggested that alteration of the concentration of formamide between 30-50% during hybridisation had no effect on the rate of hybridisation. Furthermore, it has been shown that under certain conditions, high concentrations of formamide (70%) do not permit DNA reannealing, but allow almost complete DNA-RNA reannealing (Vogelstein and Gillespie, 1977; Casey and Davidson, 1977). The effect of formamide on the hybridisation of the Na,K-ATPase  $\alpha$  subunit DNA probes was investigated.

A dot blot experiment was performed which used varying concentrations of formamide for the hybridisation of identical amounts of MDCK total RNA. The nitrocellulose filter produced was probed with the pHANK cDNA probe at 42°C, and then washed serially with various ionic strength buffers, at 55°C (See figure 2a-d). As can be seen from figure 2a, formamide appeared to act as a blocking agent, as increasing concentrations of formamide show decreasing levels of background hybridisation to the filter. At the lowest stringency wash (2 x SSC) the hybridisation signal on the highest

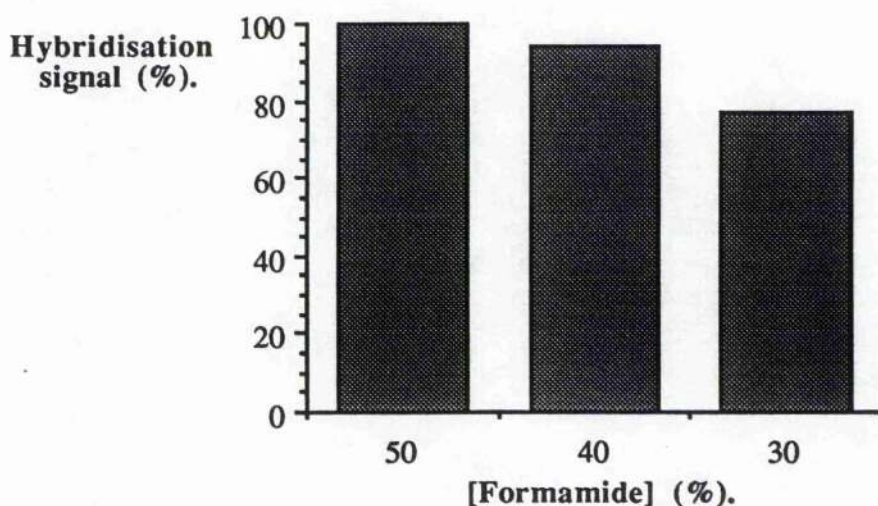


Figure 3. Shows the % amount of signal present on dot blots of MDCK total RNA hybridised with the radiolabelled pHANK DNA probe. The quantification of the signal was determined for hybridisations performed in the presence of 30, 40, and 50% formamide (see figure 2a). Blots were washed in 2 x SSC, 0.1% SDS at 55 °C. The signals were determined as a percentage of the signal present in the 50% formamide hybridisation.



concentration spots of RNA was approximately equal; the relative signal levels are given in figure 3. The 20% formamide dot blot was unquantifiable due to high levels of background. As the level of stringency was increased to the wash in 0.1 x SSC (see figure 2d), the difference between the levels of hybridisation signals in 20-50% formamide became greater (see figure 4). This suggested that some non-specific hybridisation to RNA might be associated with the lower [formamide] hybridisations (especially 20% formamide) under low stringency washing conditions (2 x SSC). The results in figure 4 suggested that the extent of hybridisation in 50% formamide must be greater than that at lower concentrations, producing higher specific signals. At this washing stringency, the hybridisation signal increased by approximately 20% for every 10% increment in the formamide concentration (see figure 4). These results posed the question, would this increase be sustained in formamide concentrations higher than 50%? The data from the blot hybridised in 50% formamide also yielded information about the melting temperature of the pHANK-RNA hybrid during the washing of filters ( $T_w$ ), and this is illustrated in figure 5. The data reported in figure 5, suggested that 50% dissociation of the pHANK-RNA hybrid (50% loss of radioactive signal) at a washing temperature of 55°C ( $T_w$ ) would occur in a wash buffer containing a  $\log_{10} [Na]$  of -1.32 or ~50 mM

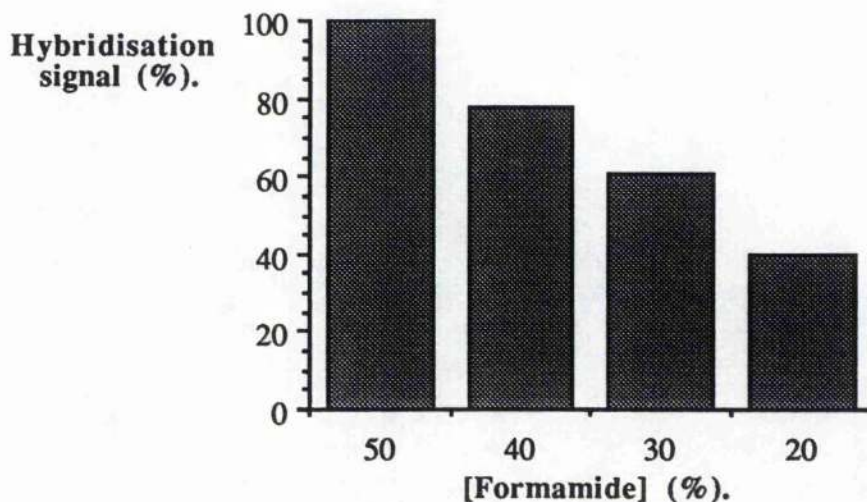


Figure 4. Shows the % amount of signal present on dot blots of MDCK total RNA hybridised with the radiolabelled pHANK DNA probe. The quantification of the signal was determined for hybridisations performed in the presence of 20, 30, 40, and 50% formamide (see figure 2). Blots were washed in 2 x SSC, 0.1% SDS followed serially by washes in 1 x, 0.5 x, and 0.1 x SSC with 0.1% SDS at 55°C. The signals were determined as a percentage of the signal present in the 50% formamide hybridisation.



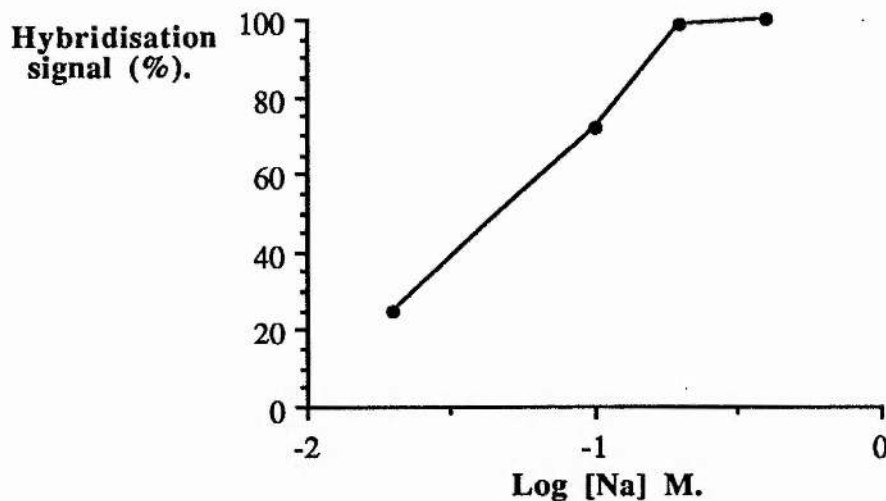


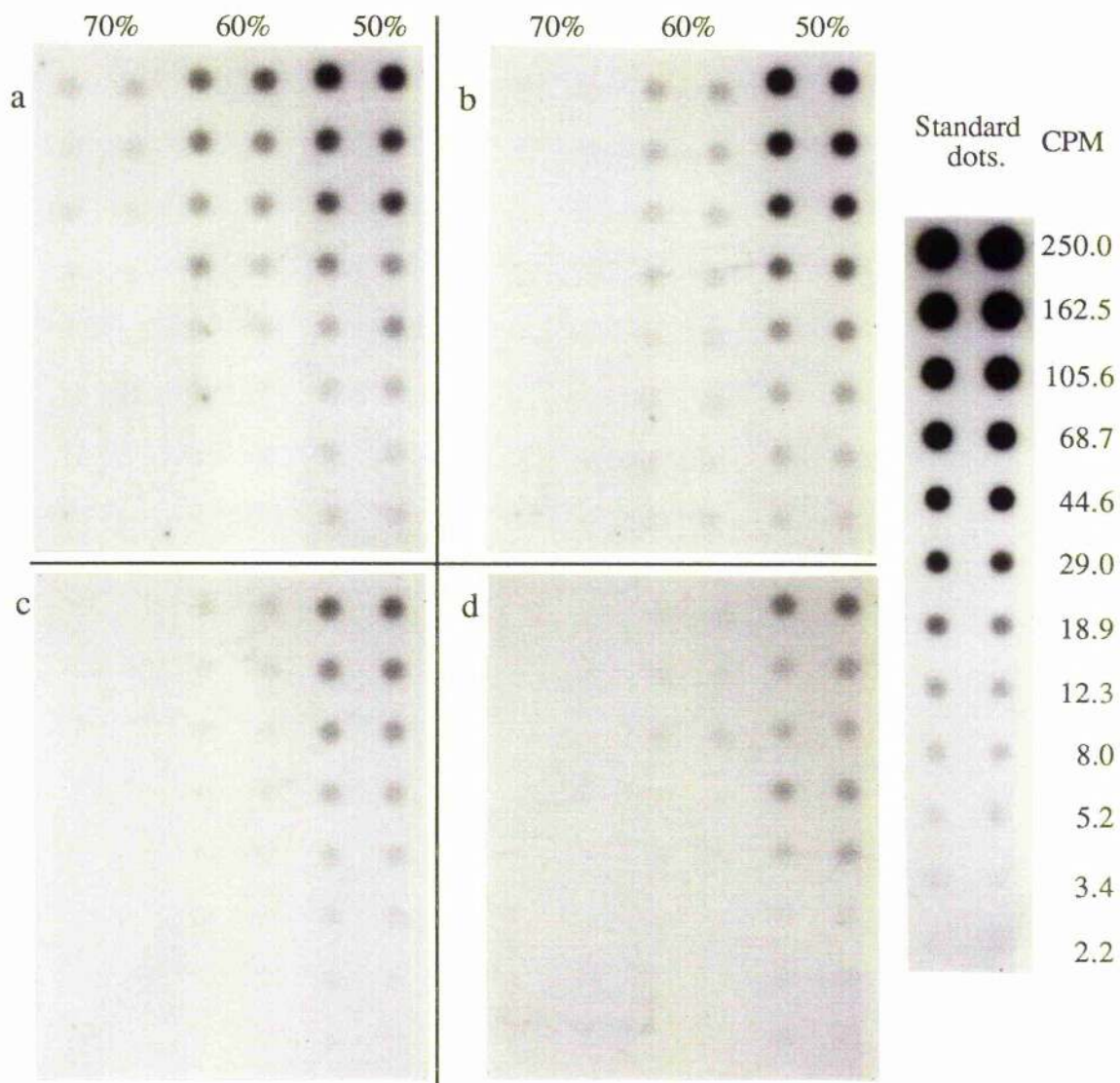
Figure 5. Shows a plot of the amounts of signal obtained from hybridisation of MDCK total RNA with a radiolabelled pHANK DNA probe in 50% formamide (see figure 2). The figures are expressed relatively to the signal obtained after washing in 2 x SSC ( $\log_{10} [\text{Na}] = -0.4$ ). The  $\log_{10} [\text{Na}]$  of each of the washing buffers is plotted against relative signal strength.

[Na] (0.25 x SSC). Inputting this data into the formula for the melting temperature ( $T_m$ ) of DNA-RNA hybrids reported by Casey and Davidson (1977; see section 2.I.ii.a.2.), suggested that the  $T_m$  under the conditions of hybridisation ( $T_{\text{hyb}}$ ; in ~1M [Na] and 50% formamide) was 54.5°C. Hence hybridisation in 50% formamide (42°C) was occurring at 12.5°C below the  $T_{\text{hyb}}$ . This figure was within the optimum range of 10-15°C below  $T_{\text{hyb}}$ , for DNA-RNA hybridisation (Birnstiel *et al.*, 1972).

To examine whether higher concentrations of formamide (>50%) would produce an increased level of signal, another experiment was performed, where the concentrations of formamide were varied between 50-70%. The NCOI-STUI restriction fragment of the rat  $\alpha 1$  cDNA was used as a probe, so that the  $T_m$  of the hybrid formed with the MDCK Na,K-ATPase  $\alpha 1$  isoform mRNA, might also be determined. The hybridisation was performed at 38°C, because of the likely low homology of this probe to the MDCK mRNA. The resulting dot blot was washed serially at increasing stringencies (see figure 6). The results from this experiment (see figure 6 and 7) showed that there was a dramatic decrease in hybridisation signal when the concentration of formamide was increased above 50%. Any possible increase in the amount of hybridisation signal

Figure 6 a-d. A series of RNA dot blots hybridised with the rat  $\alpha 1$  isosorm-specific probe (Nco I- Stu I DNA fragment) in hybridisation solutions containing concentrations of formamide from 50%-70%. The RNA dots are double diluted MDCK total RNA with 20  $\mu\text{g}$  of RNA in the highest concentration dots (top of each column). The three filters containing pairs of double diluted RNA dots were hybridised, and then washed serially in a) 2 x SSC, 0.1% SDS at 40°C, followed by b) 0.1 x SSC, 0.1% SDS at 40°C, c) 0.1 x SSC, 0.1% SDS at 45 °C and d) 0.1 x SSC, 0.1% SDS at 50°C. All washes were performed for 10 minutes. Standard dots containing known amounts of radioactivity are also illustrated.

Percent formamide.



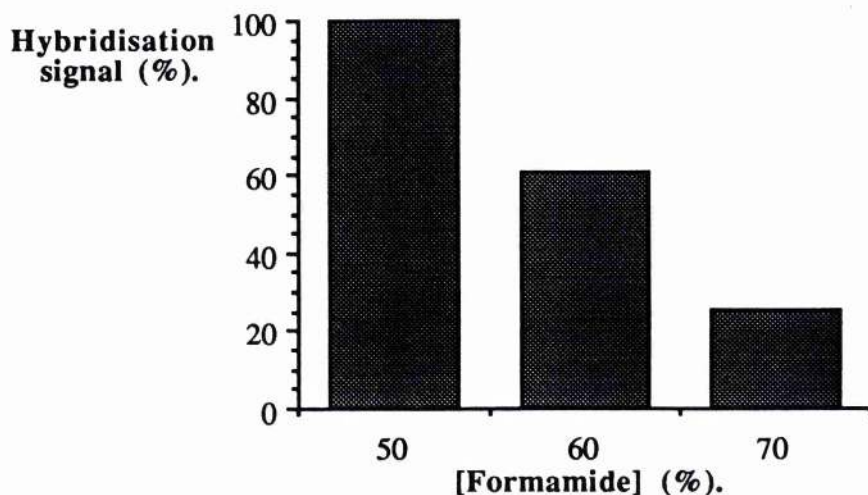


Figure 7. Shows the % amount of signal present on dot blots of MDCK total RNA hybridised with the radiolabelled rat  $\alpha 1$  NCOI-STUI DNA probe (see figure 6a). The quantification of the signal was determined for hybridisations performed in 50, 60, and 70% formamide. Blots were washed in 2 x SSC, 0.1% SDS at 40°C. The signals were determined as a percentage of the signal present in the 50% formamide hybridisation.

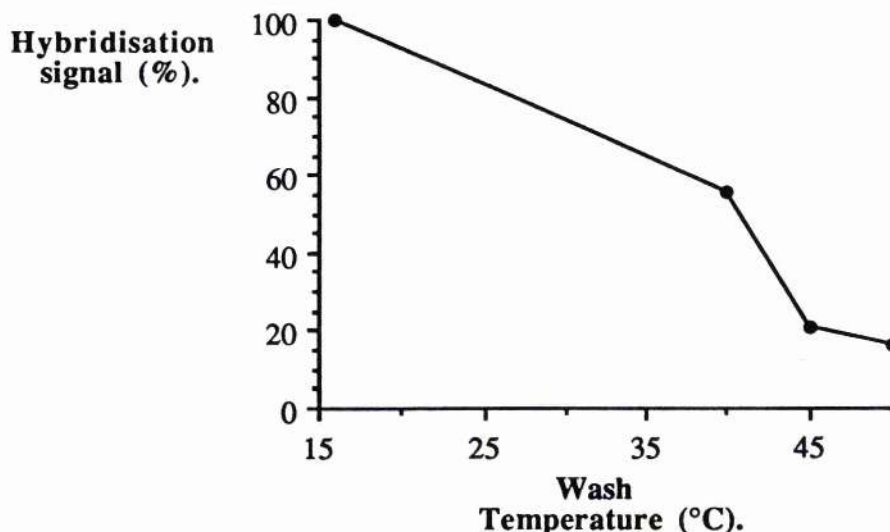


Figure 8. Shows a plot of the relative signals obtained from hybridisation of MDCK total RNA with the radiolabelled rat  $\alpha 1$  NCOI-STUI DNA probe in 50% formamide (see figure 6). The figures are expressed as the relative (% of maximum) radioactive signal remaining bound to the filter, after sequential washing in buffers with increasing temperature. The washing temperature at 16°C was a figure theoretically derived from a wash in 2 x SSC, 0.1% SDS at 40°C (see text).

resulting from a lowered rate of probe (DNA-DNA) reannealing, must have been more than offset by a reduction in the rate of DNA-RNA annealing. The washing temperature in 0.1 x SSC, 0.1% SDS which was equivalent in stringency to the wash in 2 x SSC, 0.1% SDS at 40°C, was calculated from the formula of Casey and Davidson (1977; see section 2.I.ii.a.2.). This temperature, and those of the other washes in 0.1 x SSC, 0.1% SDS, were then plotted against the relative level of signal (see figure 8.). The  $T_w$  of the Rat  $\alpha 1$  NCOI-STUI - MDCK  $\alpha 1$  mRNA hybrid was assessed from the dot blots hybridised in 50% formamide (see figure 6). The  $T_w$  of the hybrid was calculated to be between 41-42.5°C, depending on whether the hybridisation signal at the washing temperature of 16 or 40°C was used as the maximum level of signal (or 100%). Using the formula of Casey and Davidson (1977) the  $T_{hyb}$  was calculated to be 47.5-49°C, and therefore, the optimum temperature range for hybridisation was 32.5-39°C (Birnstiel *et al.*, 1972).

#### 4.III.ii. Determination of the optimum hybridisation and washing conditions of the rat $\alpha 1$ isoform-specific probes.

Experiments were performed to determine the best hybridisation and washing conditions for the use of the rat  $\alpha 1$  NCOI-STUI DNA probe, in several species. The probe was hybridised to dot blots of total RNA from rat kidney, MDCK cells, HeLa cells, and E.coli bacteria (Boeringer Mannheim). The latter serving as a control, as Na,K-ATPase is not thought to have an homologous counterpart in prokaryotes. Hybridisation of the probe was performed in 50% formamide at six different temperatures (28, 33, 38, 43, 48, 58°C respectively). The washing of filters was performed in parallel, in 0.1 x SSC, 0.1% SDS, at ten different temperatures (25-70°C inclusive at 5°C increments). Due to the scale of the experiment, only a single dot of RNA per species was used for each independent data point (an individual washing temperature at any single temperature of hybridisation). The resulting autoradiographs of the dot blots from this experiment are illustrated in figure 9. Hybridisation at 28°C resulted in the precipitation of SDS, this consequently increased the viscosity of the solution and impaired the hybridisation reaction.

Quantification of the radioactive hybridisation signals determined for rat kidney total RNA, MDCK cell total RNA, HeLa cell total RNA, and E.coli RNA, are shown in figures 10 a, b, c, and d, respectively. As can be seen in figure 10 a) hybridisation



Figure 9 a-j. A series of RNA dot blots hybridised with the rat  $\alpha 1$  isosorm-specific probe (Nco I- Stu I DNA fragment) at temperatures between 28-58°C (in hybridisation solutions containing 50% formamide). Columns of four single RNA dots consisting of R) 1.2  $\mu$ g of rat kidney total RNA, M) 12  $\mu$ g of MDCK total RNA, H) 12  $\mu$ g of HeLa cell total RNA, and E) 12  $\mu$ g of E. coli RNA were hybridised at temperatures from 28-58 °C. The columns of RNA dots were then washed independently, in parallel in 0.1 x SSC, 0.1% SDS at 5°C increments from 25°C to 70°C, illustrated in a-j respectively. Standard dots containing known amounts of radioactivity are also illustrated.



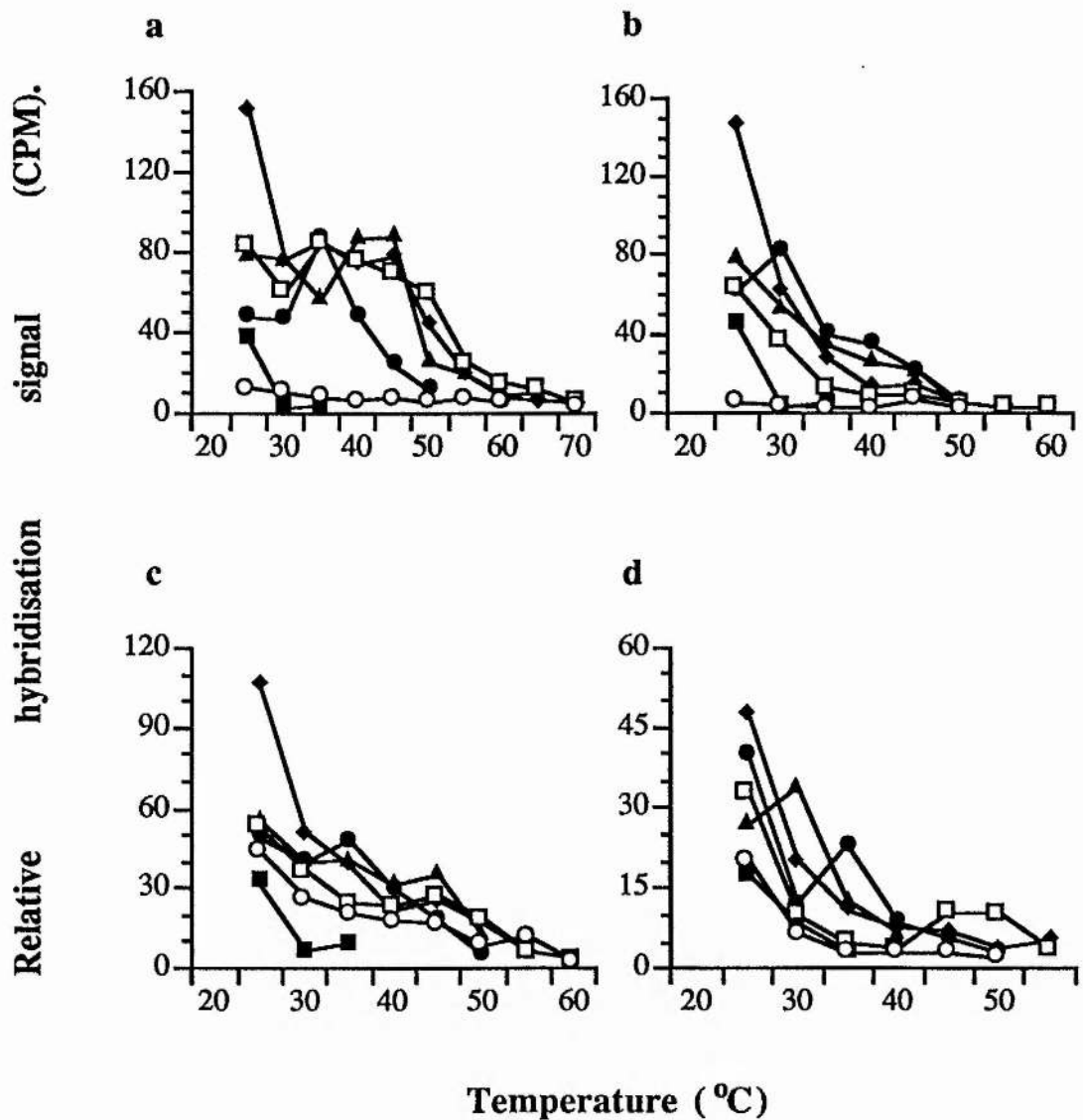


Figure 10. Shows the hybridisation signals (converted to CPM of radioactivity via the standard curve) of radiolabelled rat  $\alpha 1$  NCOI-STUI DNA probe, hybridised to dots of a) rat kidney total RNA b) MDCK cell total RNA c) HeLa cell total RNA and d) E.coli RNA, at different hybridisation and washing temperatures. The RNA dots in each hybridisation and washing condition were processed in parallel. Six hybridisations were performed at 28  $^{\circ}\text{C}$  (■), 33  $^{\circ}\text{C}$  (●), 38  $^{\circ}\text{C}$  (▲), 43  $^{\circ}\text{C}$  (◆), 48  $^{\circ}\text{C}$  (□), and 58  $^{\circ}\text{C}$  (○) respectively. The abundance of signals was determined by comparison to a set of known radioactive standard dots.

signals with rat RNA, were not consistent until washing temperatures increased to around 45 $^{\circ}\text{C}$ . The hybridisation temperature which yielded the highest levels of signal was 48 $^{\circ}\text{C}$ . The  $T_w$  of the DNA-RNA hybrid was calculated as the temperature at which 50%

of the signal was washed off the RNA dots on the filter. Using the average of the signals produced by hybridisation at 38, 43 and 48°C (which were relatively similar), the  $T_w$  in 0.1 x SSC (washing buffer) was calculated to be approximately 51°C. Inputting the  $T_w$  into the formula reported by Casey and Davidson (1977) and adjusting the figures for the [formamide] and [Na] present during hybridisation, the  $T_{hyb}$  was calculated to be 57.5°C. This suggested that the optimum hybridisation conditions were in the range of 42.5 to 47.5°C (10-15°C below  $T_{hyb}$ ; Birnsteil *et al.*, 1972). This theoretical range of values was in reasonable agreement with the value of 48°C determined empirically. Graphical representations of the radioactive signals obtained from hybridisation to MDCK cell, HeLa cell, and E.coli total RNA are presented in figures 10 b-d respectively. For MDCK RNA, the highest signal levels were achieved at a hybridisation temperature of 33°C, and the  $T_w$  (using the average of data for hybridisation at 33 and 38°C) in 0.1 x SSC (washing buffer) was approximately 37.5°C. From this value the optimal temperature range for hybridisation was calculated to be 29 to 34°C (Casey and Davidson, 1977; Birnsteil *et al.*, 1972). For HeLa cell RNA, the radioactive signals for a range of hybridisation temperatures, were very similar, with possibly 38°C being the best on average. Using averages of the data for hybridisation at 33-48°C, the  $T_w$  was calculated to be approximately 42.5°C, and this value was used to calculate the optimum temperature range for hybridisation of 34 to 39°C (Casey and Davidson, 1977; Birnsteil *et al.*, 1972). Hybridisation with E.coli RNA, exhibited substantial background signals at wash temperatures below 40°C, which suggested that when using this DNA probe, wash temperatures (in 0.1 x SSC) should always be kept above 40°C, if high levels of non-specific hybridisation are to be prevented.

The data from hybridisations to rat RNA (kidney) and human RNA (HeLa cells), showed that the difference between  $T_w$  (and hence the  $T_m$ ) of the two DNA-RNA hybrids (rat-rat and rat-human) was approximately 8.5°C. The percentage homology shared by the rat  $\alpha 1$  probe and the human  $\alpha 1$  nt sequence was 85.5%, or in other words, 14.5% of the bases were mismatched between the two sequences. This showed that the effect of mismatches on the reduction of the  $T_m$  of DNA-RNA hybrids was approximately 0.6°C per for every 1% of mismatch, under these conditions of hybridisation and washing. This was within the range of values reported, for the effect of mismatches on the  $T_m$  of hybrids (0.5-1.4°C; Anderson and Young, 1985). The  $T_w$  of MDCK RNA was 5°C below that of human  $\alpha 1$  (HeLa cells) RNA, which suggested that the homology of the MDCK  $\alpha 1$  mRNA to the rat  $\alpha 1$  probe was approximately 8.5% lower than that of the

human  $\alpha 1$  mRNA. This implied that the equivalent MDCK  $\alpha 1$  mRNA effectively shared approximately 77% homology with the rat  $\alpha 1$  probe. The percentage homology of MDCK  $\alpha 1$  mRNA sequence to the rat  $\alpha 1$  probe was obviously approximate, as the location as well as the total number of mismatches, would also affect the  $T_w$  or  $T_m$ . Sheep and pig  $\alpha 1$  cDNA sequences (Shull *et al.*, 1985; Ovchinnikov *et al.*, 1986) have a 2 aa or 6 nt deletion from within the region complementary to the rat  $\alpha 1$  NCOI-STUI DNA probe (at nt positions 65-70 of the NCOI-STUI DNA fragments sequence). If the Na,K-ATPase  $\alpha 1$  isoform sequence from dog species also had this deletion, it would reduce the stability of rat DNA-MDCK RNA hybrids. This could explain the apparently high level of mismatches (and low hybridisation and washing temperatures) found between the rat  $\alpha 1$  NCOI-STUI probe and MDCK  $\alpha 1$  mRNA.

The conditions of hybridisation and washing determined for the detection of MDCK  $\alpha 1$  isoform mRNA, using the  $\alpha 1$  isoform specific rat probe, are low stringency conditions. These low stringency conditions, capable of detecting hybrids with as low an homology as approximately 77%, should almost certainly allow the hybridisation of the  $\alpha 2$  and  $\alpha 3$  isoform specific probes to their dog mRNA counterparts. This is because, the rat  $\alpha 1$  specific probe has the lowest level of homology with its counterparts in other species compared to the other isoform specific probes (see earlier in this Chapter).



CHAPTER 5. ISOFORMS OF Na,K-ATPase IN RAT  
TISSUES AND MDCK CELLS.

## 5.1 Introduction.

The optimum conditions for the hybridisation of the Na,K-ATPase  $\alpha$ 1 isoform-specific probe (a restriction endonuclease fragment isolated from the 5 prime end of the rat  $\alpha$ 1 isoform cDNA) to RNA isolated from rat tissues and MDCK cells, was determined in section 4.III.ii. The hybridisation conditions for the other rat  $\alpha$  isoform-specific DNA probes ( $\alpha$ 2 and  $\alpha$ 3) should in theory, be similar to those of the  $\alpha$ 1 isoform, because all the isoform probes have similar  $T_m$ 's (as calculated from the formula reported by Casey and Davidson, 1977). However, it was necessary to show that the other probes (as well as the  $\alpha$ 1 probe) actually hybridised to their corresponding mRNA's. This could only be determined by the use of a Northern blotting technique. The best approach to ensure the detection of isoform mRNA's was to hybridise under optimum conditions. That was, to hybridise to mRNA's which were completely homologous to the probes concerned (ie mRNA's from rat tissues). Rather than perform test experiments yielding data on the hybridisation of the isoform-specific probes alone, a more informative approach was taken, which would also provide information on the regulation of Na,K-ATPase in rat tissues. Alterations in Na,K-ATPase mRNA abundance have been shown to occur during the development of hypertension in certain rat strains (Herrera *et al.*, 1988). Therefore, while investigating the conditions of hybridisation of the isoform-specific probes, an investigation into possible alterations in the abundance of the  $\alpha$  and  $\beta$  isoform mRNA's in the Milan strain of hypertensive rats was undertaken.

### 5.1.i. Milan hypertensive rats.

The Milan hypertensive rat strain is a selectively inbred strain of Wistar rat, which shows a mild variant of primary hypertension (Bianchi *et al.*, 1973a; Bianchi *et al.*, 1973b). The genetic lesion associated with Milan hypertensive rats is manifested in a renal malfunction and consequently, the lesion in this strain is different from those of other hypertensive rat strains. The systolic blood pressure of these hypertensive rats rises up to 160-170 mmHg between 4 to 7 weeks of life, which is 30-40% above normal. The mean arterial blood pressure however, is generally only raised by about 20-25%. These rats also show a mild degree of left ventricular hypertrophy (of around 15-20%), and a reduced kidney size (10% smaller than normal). Evidence suggests that hypertension in

this rat strain may be due to a decreased number of glomeruli (25% lower than normal) in adult animals (Genest *et al.*, 1977). This leads to a reduced amount of ultrafiltration, and increased circulating blood volume. The amount of ultrafiltration is eventually increased to normal levels by a compensatory rise in blood pressure, which consequently results in the circulating blood volume returning to normal (Bianchi *et al.*, 1979a; Bianchi *et al.*, 1979b).

As outlined in the general introduction (section 1.V.i.b.3.), in a limited number of cases (~10% in man, Diez *et al.*, 1987) changes in the regulation of Na,K-ATPase may be the cause or an effect of the developing hypertension. As stated previously, changes in Na,K-ATPase mRNA abundance in certain animal models of hypertension, have been reported (Herrera *et al.*, 1988). In response to hypertension (induced by nephrectomy and deoxycorticosterone (DOC)/high salt or angiotensin II (A II) treatment), Herrera *et al.* (1988), showed a 2-3 fold increase in the abundance of both  $\alpha 1$  and  $\beta 1$  isoform mRNA's (in aorta and skeletal muscle tissues) and a 3-15 fold decrease in the abundance of  $\alpha 2$  and  $\alpha 3$  isoform mRNA's (in aorta and left ventricle). They suggested that the increase in  $\alpha 1$  and  $\beta 1$  isoforms was due to increased Na influx into the cells of the tissues concerned, whereas the decrease in the  $\alpha 2$  and  $\alpha 3$  isoforms was directly in response to increases in vascular pressure. This conclusion was reached because only in cardiovascular tissues were the  $\alpha 2$  and  $\alpha 3$  isoforms down regulated, and this response was not found in treated but non-hypertensive animals. Measurements of Na,K-ATPase isoform mRNA abundance in the myocardial tissues of a different hypertensive model, such as the Milan rat strain, should help to reveal if these results and conclusions also apply more generally in other animal models of hypertension.

#### 5.I.ii. Isoforms of Na,K-ATPase in MDCK cells.

The background information on the presence (or absence) of isoforms of Na,K-ATPase has already been outlined in section 1.VI.i.. As the MDCK cell line was derived from dog kidney, it would be expected to express the same isoforms of Na,K-ATPase as the tissue of origin. The exact nature of the expression of isoforms in kidney tissues has been to some extent controversial. The blots so far reported, showing the expression of the isoforms in whole kidney RNA samples have all agreed on the presence of substantial amounts of  $\alpha 1$  and  $\beta 1$  isoform mRNA's. The presence and levels of expression of  $\alpha 2$  and  $\alpha 3$  isoform mRNA's however, are still the subject of debate. Both Emanuel *et al.*

(1987) and Svedlov *et al.* (1988), have reported signals for  $\alpha 2$  and  $\alpha 3$  mRNA's on Northern blots using whole kidney RNA extracts. Therefore, the presence of all of the isoforms of Na,K-ATPase in MDCK cells could not be ruled out, although the  $\alpha 1$  and  $\beta 1$  isoforms were likely to be the major isoforms present.

## **5.II. Methods.**

The methods in this Chapter were as described in Chapter 2.

## **5.III. Results and Discussion.**

### **5.III.i. Na,K-ATPase isoform mRNA's in rat tissues.**

The relative abundance of the mRNA's for each of the isoforms ( $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ , and  $\beta 1$ ) of Na,K-ATPase was determined in various tissues from normal Wistar rats. Total RNA was extracted from the brain, kidney, lung, ventricular myocardium, atrial myocardium, and aorta. The probes specific for the isoforms of Na,K-ATPase were as described in section 4.I.iii.. The amounts of total RNA isolated per gram wet weight of tissue are illustrated in figure 1. The yield of total RNA indicated for rat aorta in figure 1,

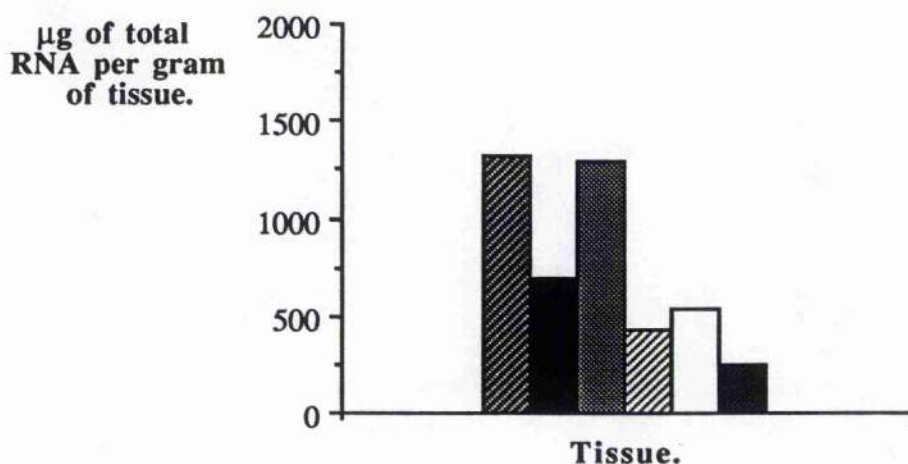
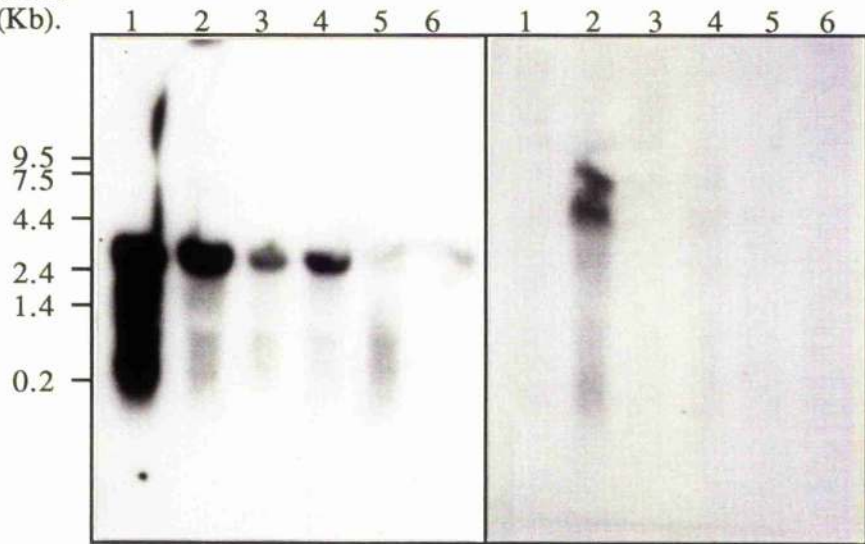


Figure 1. Yield of total RNA ( $\mu\text{g}$  RNA/gram wet weight of tissues) isolated from various rat tissues. The tissues extracted included kidney (▨), brain (■), lung (▩), ventricle (▧), atria (□), and aorta (■).

Figure 2. Northern blots of rat total RNA from 1) kidney (30  $\mu$ g), 2) brain (30  $\mu$ g), 3) lung (30  $\mu$ g), 4) ventricular myocardium (30  $\mu$ g), 5) atrial myocardium (8.7  $\mu$ g), and 6) aorta (4  $\mu$ g). Filters were hybridised at 48°C (in 50% formamide) with the Na,K-ATPase isoform-specific probes ( $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 3, and  $\beta$ 1) as indicated. Filters were finally washed at 65 °C in 0.1 xSSC, 0.1% SDS for 10 minutes.

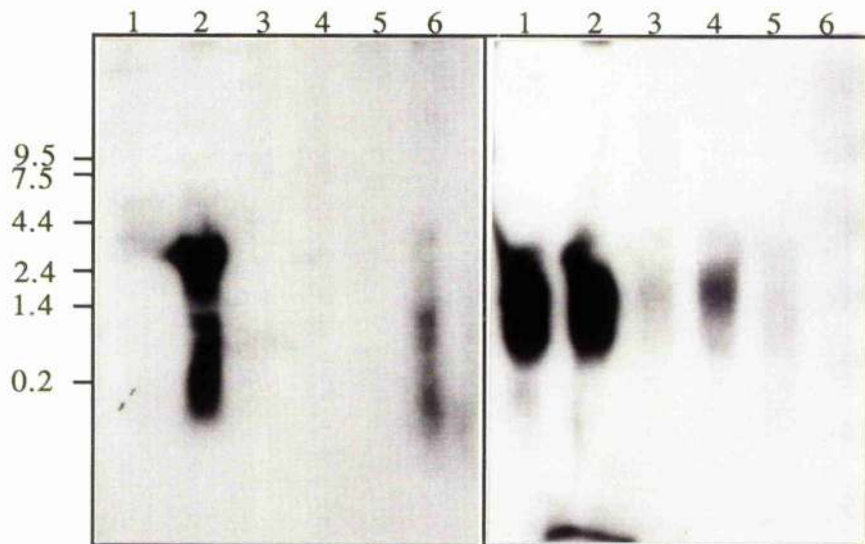


Size  
Markers  
(Kb).



$\alpha 1$

$\alpha 2$



$\alpha 3$

$\beta 1$

was probably an underestimate, due to difficulties encountered in homogenising this tissue during RNA isolation.

Samples from the rat tissues were then subjected to Northern blot analysis, to determine the abundance of mRNA for the Na,K-ATPase isoforms. The autoradiographs of these Northern blots are illustrated in figure 2. The percentage incorporation of radioactivity into the  $\alpha$  isoform-specific probes using the Klenow fragment of DNA polymerase was low. The  $\alpha 2$  isoform-specific probe only incorporated approximately 4.7% of the radioactivity in the labelling reaction. The low incorporation of radioactivity into the  $\alpha 2$  specific probe may have been the reason for the low intensity of the band on the  $\alpha 2$  specific Northern blot. The relative abundance of the mRNA's for the Na,K-ATPase isoforms as shown in figure 2, was similar to that found by Young and Lingrel (1987). Of the tissues studied, the brain exhibited a much greater abundance of  $\alpha 2$  and  $\alpha 3$  isoform mRNA's; any low levels of  $\alpha 2$  or  $\alpha 3$  mRNA present in the other tissues were not detectable under the conditions used.

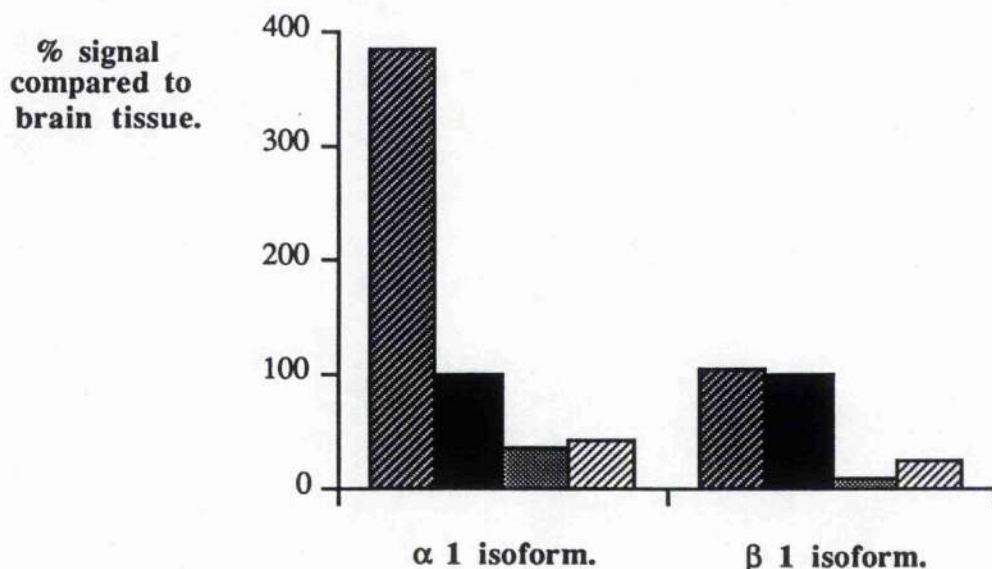


Figure 3. The relative abundance, compared to brain tissue, of the  $\alpha 1$  and  $\beta 1$  isoform mRNA's of Na,K-ATPase. The tissues illustrated are kidney (▨), brain (■), lung (▩), and ventricle (▧). The blots of atrial myocardium and aorta were not quantified because of the presence of background signal and due to the low signal intensity.

The relative tissue abundances of the  $\alpha 1$  and  $\beta 1$  isoforms compared to levels in the brain were assessed and are illustrated in figure 3. The relative abundance of the isoforms shown in figure 3 was assessed by scanning densitometry. Both the bands and lower

molecular weight signals on the autoradiographs were scanned (see figure 2). The bands were of a size corresponding to the reported values for the isoforms of Na,K-ATPase (Young and Lingrel, 1987; Young *et al.*, 1987). Northern blots of kidney RNA using the  $\alpha 1$  isoform-specific probe, also showed two other bands, which were smaller in size (ie  $\sim 2.1$  and  $\sim 0.4$  Kb) than the reported value of the  $\alpha 1$  isoform mRNA (3.7 Kb). These signals may have resulted from the hybridisation of mRNA's transcribed from another gene(s), whose sequence(s) was very similar to that of the  $\alpha 1$  probe. Similarly, the  $\alpha 3$  specific probe also hybridised to lower molecular weight mRNA species, which were present in the Northern blots of brain total RNA (see figure 2). These autoradiographic signals indicated that the  $\alpha 3$  probe, showed high levels of specificity for some low molecular weight mRNA's, as no similar signals were found in RNA samples from other tissues. It is therefore probable that some of the lower molecular weight signals present on blots were the result of degradation of the RNA by RNase during extraction, rather than non-specific adsorption of the probe to the filter or to non-homologous RNA species. Degradation may have occurred because RNase enzymes are notoriously difficult to inhibit. Lower molecular weight mRNA's were also present on the Northern blots reported by others (Young and Lingrel, 1987; Emanuel *et al.*, 1987; Martin-Vasallo *et al.*, 1989; Orlowski and Lingrel, 1988; and Schneider *et al.*, 1988) although, no reference was made as to their origin. The relative abundances (to brain tissue) of the  $\alpha 1$  and  $\beta 1$  isoform mRNA's determined were of a similar magnitude to those reported by Young and Lingrel (1987). The abundance of  $\alpha 1$  mRNA in kidney and ventricle and the  $\beta 1$  in kidney were however less abundant in this study (3.8 x, 0.4 x, and 1.0 x respectively) than the previously reported values (Young and Lingrel, 1987; 7.0 x, 0.7 x, and 2.0 x, respectively). These differences may to some extent reflect the lower level of accuracy produced when quantifying Northern blots compared to slot (or dot) blots used by Young and Lingrel (1987). The lower accuracy of measurements from Northern blots is produced because the non-linearity of the autoradiographic film's response to radioactivity cannot be accounted for. In dot or slot blotting, a standard curve of dots or slots, comprised of known levels of radioactivity, can be used to adjust hybridisation signals obtained for the non-linear response of the film.

### 5.III.ii Na,K-ATPase isoform mRNA's in Milan hypertensive rats.

The initial hybridisations of Northern blots, using the Na,K-ATPase isoform specific probes, produced blots without an appreciable amount of non-specific background (see figure 2). This suggested therefore, that under similar hybridisation and washing conditions, the dot blotting technique would produce accurate estimates of the total abundance of specific mRNA's, even if some degradation of RNA samples occurred during extraction. The RNA dot blotting technique was therefore employed to determine the abundance of the mRNA's of the isoforms of Na,K-ATPase in normotensive and hypertensive rat tissue samples. Twelve to fourteen week old male hypertensive rats of

Tissues weighed (significance).	Weight of normotensives (grams).	Weight of hypertensives (grams)
Whole body. (**)	391.5 ± 10.8	442.0 ± 28.4
Tissues weighed (significance).	Wet weight : body weight ratio of normotensives.	Wet weight : body weight ratio of hypertensives
Kidney. (***)	3.48 ± 0.17	2.51 ± 0.18
Left ventricle.	1.30 ± 0.05	1.37 ± 0.16
Right ventricle.	1.16 ± 0.03	1.31 ± 0.14
Atria.	0.28 ± 0.03	0.32 ± 0.02

Table 1. The total body weight, and the wet weight: body weight ratio ( $\times 10^{-3}$ ) of various tissues from hypertensive and normotensive Milan rats. The figures represent  $\pm$  standard deviations, where  $n = 6$ , except for normotensive myocardial tissues where  $n = 4$  and kidney where  $n = 12$ . The statistical significance (produced using Student's t-test, assuming normally distributed data), is indicated in parenthesis, where \*\* and \*\*\* represent significance at the 1% and 0.1% level respectively.



the Milan strain (MHS), and their age and sex matched normotensive controls (MNS) were obtained (Field Laboratories, University of Sheffield). The blood pressure in these rats could not be measured as the necessary equipment was not available, however a significant decrease in kidney mass (wet weight per body weight) acts as a marker for hypertension in Milan rats (Bianchi *et al.*, 1973b). The mean body weight of the hypertensive rats was 13% greater than that of the normotensives (see table 1). The mean wet weight: body weight ratio of various tissues are shown in table 1. The mean kidney/body weight ratio for MHS was decreased by 28% compared to MNS. The decrease in kidney mass was even larger than that suggested for this strain of rats (ie a 10% decrease as reported in Genest *et al.*, 1977). The tissue wet weight/body weight ratio of the left and right ventricular tissues in MHS showed increases of 6% and 13% respectively compared to normotensive controls. This degree of hypertrophy was slightly less than the 15-20% suggested for the left ventricular myocardium (Genest *et al.*, 1977). The mean atrial myocardial tissue samples of MHS rats also showed a 13% increase in the wet weight/ body weight ratio compared to MNS controls. The increases in wet weight of tissue per body weight, in hypertensive ventricular and atrial tissue were however, not statistically significant.

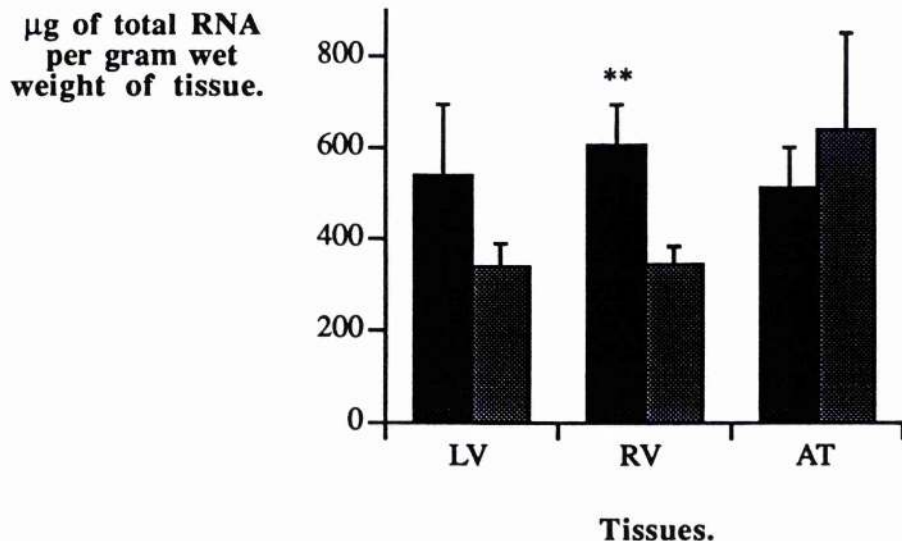


Figure 4. A chart showing the amount of total RNA extracted from hypertensive (■), and normotensive (▨) rat tissues per gram wet weight of tissue. The abbreviations are LV, left ventricle (n = 3); RV, right ventricle (n = 3); and AT, atria (n = 2). The error bars represent standard deviations. The statistical significance of the data was assessed by Students t-test (\*\* = significant at the 1% level), the data was assumed to be normally distributed.



Figure 5. Dot blots of total RNA from Milan normotensive (NT) and hypertensive (HT) rats. Tissues extracted for total RNA and dot blotted were; NT 1) left ventricle (LV), 2) right ventricle (RV) and 3) atria (AT), and; HT 4) LV, 5) RV and 6) AT (all 7.5  $\mu$ g respectively). Samples of total RNA from NT 7) kidney (0.75  $\mu$ g), 8) brain (0.75  $\mu$ g), and 9) lung (30  $\mu$ g), as well as *E. coli* (7.5  $\mu$ g) were also used. Standard dots are illustrated in 11, the highest concentration dot (left) represents 500 CPM, with subsequent dots radioactivity (left to right) decreasing by 60% per dot. Dot blots were hybridised at 48°C (in 50% formamide) with Na,K-ATPase isoform-specific probes as illustrated. Filters were finally washed in 0.1 x SSC, 0.1% SDS at 65°C for 10 minutes.

	$\alpha 1$	$\alpha 2$	$\alpha 3$	$\beta 1$
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				

11



In order to extract significant amounts of total RNA and limit losses, total RNA was isolated from pooled samples of two ventricles and three atria. This reduced the number of independent tissue samples from the 12 rats used. For each of the normotensives and hypertensives groups, 3 tissue samples were obtained from pooled left ventricle, 3 samples from pooled right ventricle and 2 samples from pooled atrial tissues. The mean amount of total RNA extracted per mg wet weight of tissue, from these pooled samples was as illustrated in figure 4. In figure 4, it can be seen that in both of the ventricular tissue samples, the amount of RNA present (per mg of tissue) in hypertensive rats was 25-28% lower than in normotensive rats. However only the amounts of RNA extracted from the normotensive and hypertensive right ventricle samples were significantly different (the non-significance of the left ventricular samples was probably due to greater variation in the amount of RNA extracted from these samples). In the atrial tissue samples the position was reversed with the amount of RNA (per mg of tissue) increased in hypertensive rats, although the difference between normotensive and hypertensive samples was not significant.

The total RNA from the normotensive and hypertensive rat tissues was then used for the dot blotting experiments. Dot blots were produced with 2 duplicate dots of each sample. The 8 hypertensive and 8 normotensive myocardial samples were blotted together with normotensive RNA samples from brain, kidney and lung, as well as E.Coli

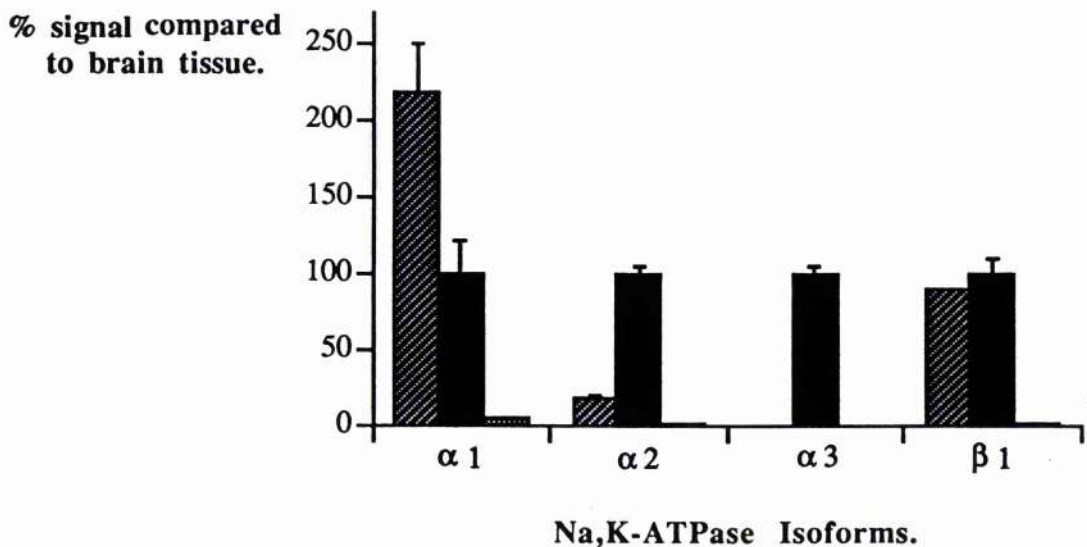


Figure 6. The relative abundance of Na,K-ATPase isoform mRNA's in normotensive rat brain (■), kidney (▨), and lung (▩) tissues. Abundances are quantified per  $\mu\text{g}$  of total RNA and as a percentage of the abundance in brain tissue. The error bars represent  $\pm$  the % relative abundance of two RNA dots per tissue.

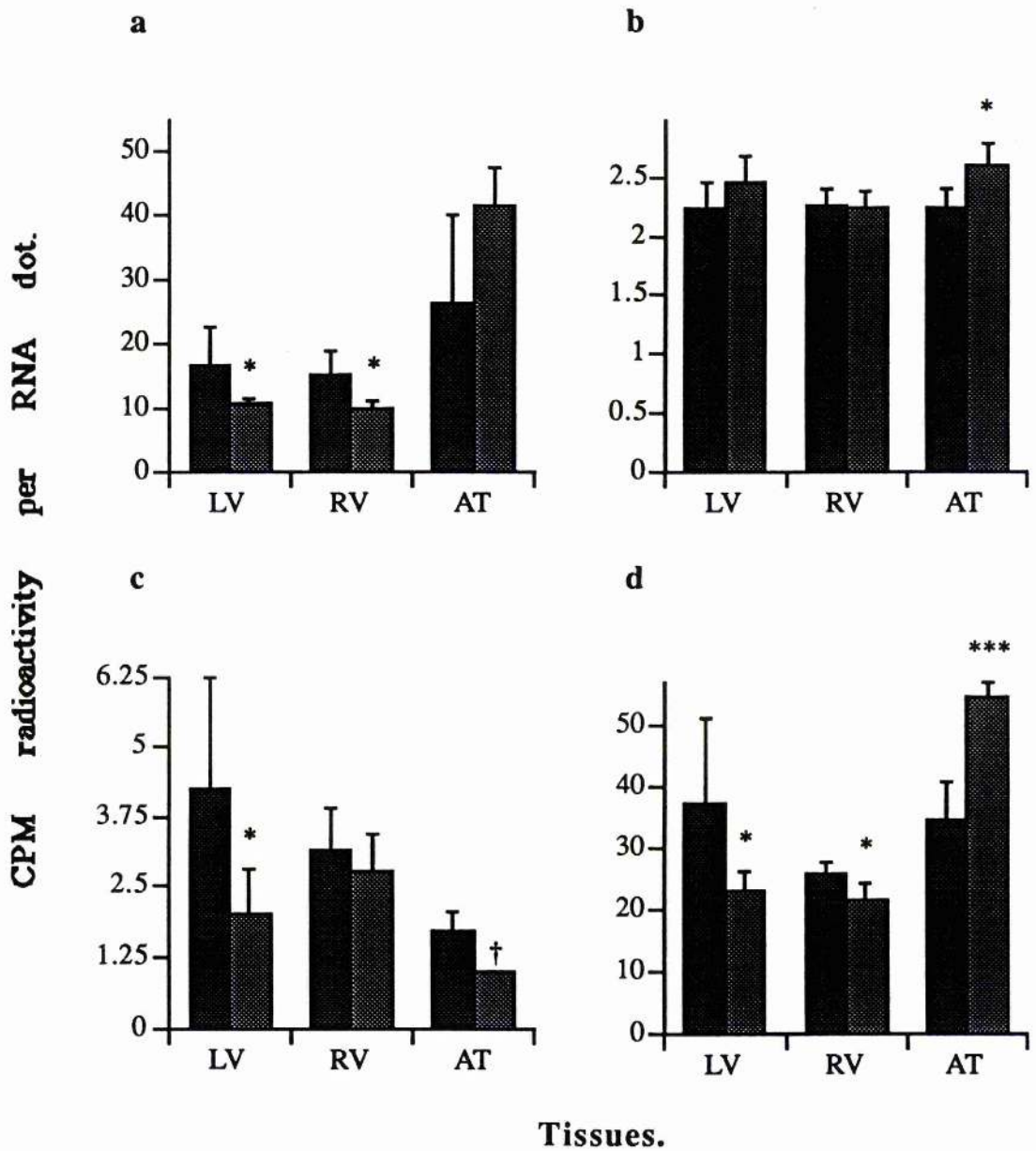


Figure 7. The relative abundance of the mRNA for the a,  $\alpha 1$ ; b,  $\alpha 2$ ; c,  $\alpha 3$ ; d,  $\beta 1$  isoforms of Na,K-ATPase from the myocardium of normotensive (■) or hypertensive (▨) rats. The abundances are expressed relatively per 7.5  $\mu\text{g}$  dot of total RNA. The abbreviations represent LV, left ventricle; RV, right ventricle; and AT, atria. The error bars are standard deviations of 2 dots of RNA from each of 3 left or right ventricle (ie.  $n = 6$ ) and 2 atrial (ie.  $n = 4$ ) samples. The \* symbols indicate the statistical significance of a Student's t-test comparison between normotensive and hypertensive rat tissues, where \* = significant (at the 5% level) and \*\*\* = very highly significant (at the 0.1% level). The column indicated with a † symbol had no detectable hybridisation signal. This column therefore indicates the maximum possible level of radioactivity which would produce no hybridisation signal under the film exposure conditions used.



total RNA. E.Coli total RNA was used as a marker for the level non-specific hybridisation background as Na,K-ATPase does not exist in prokaryotes. Four identical filters were produced which were hybridised to each of the 4 isoform-specific DNA probes ( $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$  and  $\beta 1$  isoform; see section 4.I.iii.), under identical conditions. The resulting autoradiographs of these dot blots are illustrated in figure 5. Some non-specific adsorption of probe to the filters had occurred especially in the  $\alpha 1$  isoform hybridisation, although quantification of dots was still possible. The intensity of the dots on the autoradiograph were quantified by scanning densitometry (see section 2.III.ii.c.2.V.), and are illustrated in figures 6 and 7. The relative abundance of isoform mRNA's in normotensive rat brain, kidney, and lung tissues were as shown in figure 6. The relative abundance (compared to brain) of the  $\beta 1$  isoform in the tissues in this blot were similar to the values determined previously by Northern blotting. However, the relative abundance of the  $\alpha 1$  isoforms were considerably different to the values determined by Northern blotting (kidney  $\alpha 1 = 2.2 \times$  brain  $\alpha 1$  abundance, and lung  $\alpha 1 = 0.04 \times$  brain  $\alpha 1$  abundance). The reason for the discrepancies in  $\alpha 1$  mRNA abundance is not known, non-specific background may contributed to a higher level of signal in the dots of brain RNA, hence decreasing the relative proportion in other tissues. The abundance of the isoform mRNA's in the myocardial tissues of the normotensive and hypertensive rats were as shown in figure 7. The measurements of mRNA abundance between rat strains were analysed statistically using Student's t-test. The significant values from this analysis are also illustrated in figure 7. The abundance of mRNA for the  $\alpha 1$ ,  $\alpha 3$  and  $\beta 1$  isoforms in hypertensive rat ventricles was significantly lower than in the normotensive controls (except the  $\alpha 3$  isoform in right ventricle samples). Whereas, the abundance of the  $\alpha 2$  isoform mRNA, in the ventricles of both strains of rats was approximately equal, although the dots were very faint due to poor hybridisation with this probe and were consequently difficult to quantify. In the atrial tissue samples the relative abundance of the isoform mRNA's was reversed compared to ventricular tissue samples. The relative amounts of  $\alpha 1$ ,  $\alpha 2$  and  $\beta 1$  isoform signals were increased in hypertensive atrial tissue samples (although the increase in abundance of the  $\alpha 1$  isoform was not significant). Whereas, the abundance of the  $\alpha 3$  isoform mRNA was lower in hypertensive atria. The exact abundance of  $\alpha 3$  mRNA in hypertensive atrial tissues could not be assessed as, the extent of hybridisation with the  $\alpha 3$  probe was too low to produce a measurable autoradiographic signal. The highest level of radioactivity failing to produced a hybridisation signal under the conditions used was used to illustrate the maximum



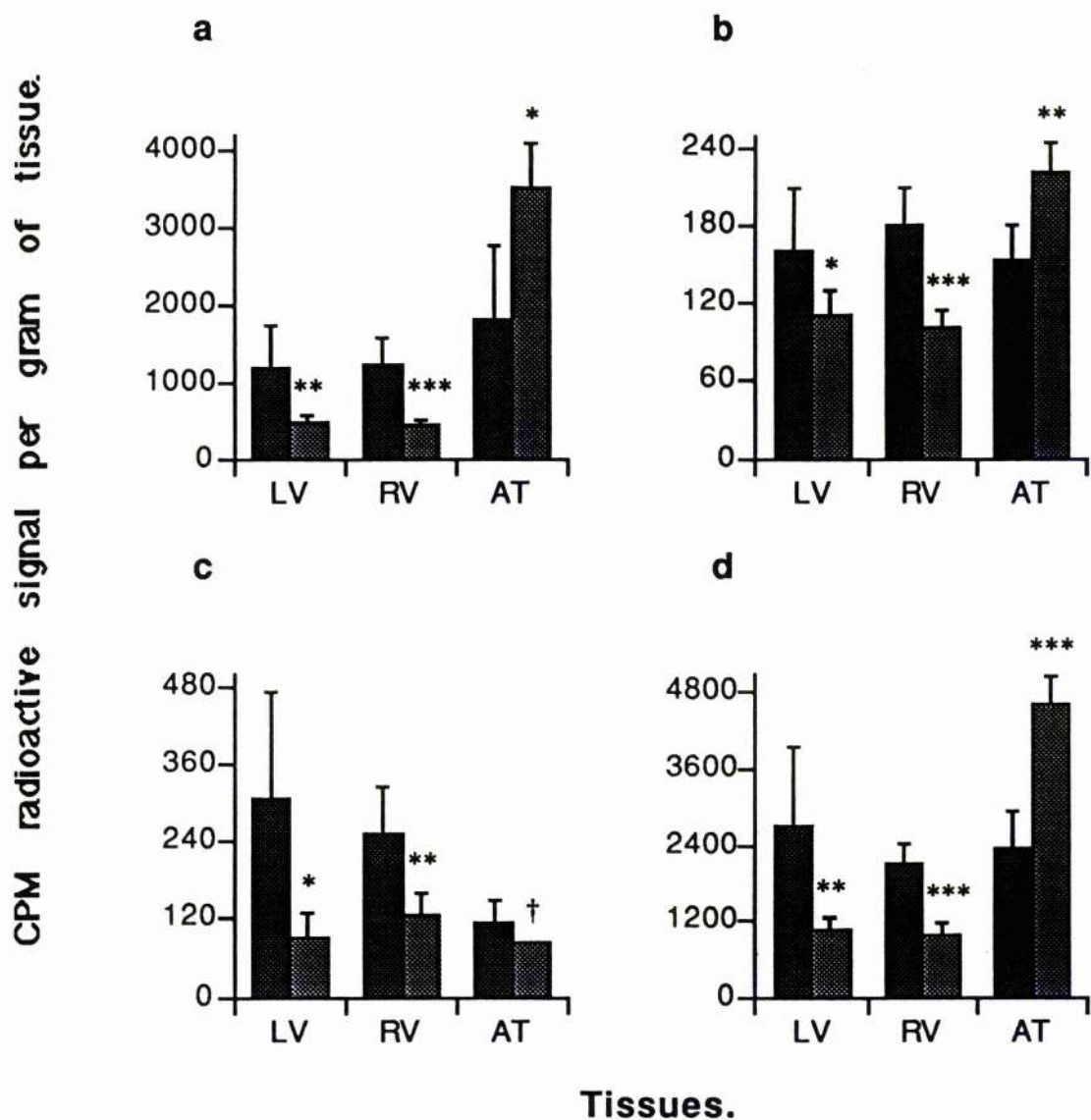


Figure 8. The relative abundance of the mRNA for the a,  $\alpha 1$ ; b,  $\alpha 2$ ; c,  $\alpha 3$ ; d,  $\beta 1$  isoforms of Na,K-ATPase from the myocardium of normotensive (■) and hypertensive (■) rats. The abundances are expressed relatively per gram of tissue extracted for total RNA. The abbreviations represent LV, left ventricle; RV, right ventricle; and AT atria. The error bars are standard deviations of 2 dots of RNA from each of 3 left or right ventricle (ie. n = 6) and 2 atrial (ie. n = 4) samples. The column indicated with a † symbol had no detectable hybridisation signal. This column therefore indicates the maximum possible level of radioactivity which would have produced no signal under the film exposure conditions used. The \* symbols indicate statistical significance of a Student's t-test comparison between normotensive and hypertensive rat tissues, where \* = significant (at the 5% level), \*\* = highly significant (at the 1% level) and \*\*\* = very highly significant (at the 0.1% level).

possible atrial  $\alpha 3$  mRNA abundance (in figures 7 and 8). The method of expressing the relative abundance of individual mRNA species, per  $\mu\text{g}$  of total RNA, can sometimes give an erroneous estimate of the changes in abundance of a mRNA species per cell. This is because, the amount of total RNA in cells may also be variable (ie., all RNA species in a cell may also increase or decrease to a similar extent), producing changes in the abundance of specific mRNA's per cell, which would not be detected by measurements made per  $\mu\text{g}$  of total RNA. The proportion of different RNA species such as rRNA within total RNA may also alter giving erroneous values for the abundance of a specific mRNA per  $\mu\text{g}$  of total RNA. The relative levels of the expression of specific mRNA species in the cells of a tissue can not be estimated directly, because the number and the type of cells in an intact tissue is unknown. However, changes in the mean amount of total RNA extracted from tissues can be accounted for by expressing the hybridisation signal relatively per unit weight of tissue. This value, providing the efficiency of total RNA extraction is consistent between tissue samples, should yield a better estimate of the mean concentration of mRNA species within the cells of a tissue. The estimates of the relative abundances of the isoform mRNA's per gram wet weight of tissue are presented in figure 8. It can be seen that the effect of expressing the data per gram of tissue was to increase the significance of differences between the levels of expression of the isoform mRNA's in hypertensive and normotensive tissues. Using the per gram wet weight figures, all of the isoform mRNA's were apparently decreased by between 1.5-3 fold in both hypertensive rat left and right ventricles, compared to controls. In contrast the abundance of the mRNA for the  $\alpha 1$ ,  $\alpha 2$  and  $\beta 1$  isoforms in the atria of hypertensive rats showed an apparent increase of 1.5-2 fold over controls. The exception to this was the abundance of the  $\alpha 3$  isoform mRNA, where, levels in hypertensive atria showed an apparent decrease of  $> 27\%$  compared to controls. The actual size of the decrease was undetermined, because the level of this mRNA in hypertensive atria was too low to be measured and the value given for  $\alpha 3$  hypertensive atria is the maximum possible value. The results expressed per gram wet weight of tissue, are still not entirely accurate as a measure of the mean mRNA expression per cell because no account of the extent of hypertrophy was made. The effect of accounting for cellular hypertrophy, would decrease the magnitude of the differences found between hypertensives and controls in ventricular tissues, but would increase the differences found for the  $\alpha 1$ ,  $\alpha 2$ , and  $\beta 1$  isoforms in atrial tissues.

Herrera *et al.* (1988), reported a 2-3 fold increase in the abundance of mRNA for the  $\alpha 1$  and  $\beta 1$  isoforms of Na,K-ATPase in the left ventricular myocardium of hypertensive rats. Results from this group suggested that the increases in  $\alpha 1$  and  $\beta 1$  mRNA abundance were due to increased sodium influx. This was presumably implying that the  $[Na]_i$  of cells was increased, causing an up regulation of Na,K-ATPase mRNA's, as has been shown to occur by other groups (see section 1.V.ii.a.). The hypertension found in their rat models was induced by treatment with deoxycorticosterone (DOC; a mineralocorticoid agonist) and a high salt diet or angiotensin II (A II) treatment. The action of the latter treatment is also thought to be due to the secondary increase in the level of circulating mineralocorticoid hormone (aldosterone). These treatments (mineralocorticoid agonists and a high salt diet) may, as suggested, be responsible for the increases in the abundance of the  $\alpha 1$  and  $\beta 1$  isoform mRNA's, via an increase in both Na influx and  $[Na]_i$ . The same group also reported a 3-15 fold decrease in the abundance of the  $\alpha 2$  and  $\alpha 3$  isoform mRNA's in the left ventricle of hypertensive rats (Herrera *et al.*, 1988); these decreases were similar although larger than those found in this study. The decrease in  $\alpha 2$  and  $\alpha 3$  mRNA abundance was suggested to be related to increases in vascular pressure in hypertensive rats, by an unknown mechanism. This was because, the aortas and ventricles of hormone treated (DOC treated, without a high salt diet, or sub-pressor doses of A II), but non-hypertensive rats did not show these decreases. Furthermore, skeletal muscle, which was not exposed directly to increased blood pressure, did not show the down regulation of these isoforms in hypertensive animals. In the Milan hypertensive rat model, hypertension was thought to develop due to a decreased rate of ultrafiltration in the kidney glomeruli. Ultrafiltration was subsequently increased to normal levels by a compensatory increase in blood pressure. After the onset of hypertension (about 7 weeks after birth), Na excretion, plasma volume and plasma renin levels returned to normal levels (Genest *et al.*, 1977), suggesting that circulating mineralocorticoid concentrations and consequently Na balance, had also returned to normal. The increase in the  $\alpha 1$  and  $\beta 1$  isoform mRNA abundance in the ventricles of the DOC-salt rat model, was thought to be independent of the developing hypertension and to be due directly to the action of DOC-salt treatment on  $[Na]_i$ . The absence of these treatments from the Milan hypertensive model, suggested that the  $\alpha 1$  and  $\beta 1$  isoform mRNA abundances (just as with the  $\alpha 2$  and  $\alpha 3$  isoform mRNA abundances in the ventricles of the DOC-salt and AII models), were then (in the absence of increased mineralocorticoids or  $[Na]_i$ ) regulated by changes in vascular pressure and were

consequently decreased in hypertensive rats. This hypothesis, if true, would suggest that the decreases caused by the effect of vascular pressure on the  $\alpha 1$  and  $\beta 1$  isoforms in the DOC-salt and A II models were masked by the increases in  $[Na]_i$ . The magnitude of the decreases in isoform mRNA's (in the ventricles) in the Milan hypertensive model were less than those reported in the DOC-salt and A II models ( $\alpha 2$  and  $\alpha 3$  isoforms). The difference in magnitude could possibly be related to the degree of hypertension. Milan hypertensive rats have systolic blood pressures in the range 160-170 mmHg, whereas the blood pressures of DOC-salt and A II hypertensive rats were reported to be within 168-220 mmHg (Herrera *et al.*, 1988). The reduction in the abundance of the mRNA for the isoforms of Na,K-ATPase in hypertensive ventricular tissues was likely to cause a decrease in the number of sodium pump units. A decrease in sodium pump numbers could result in a reduction in total Na,K-ATPase activity. This would effectively have the same result on the cell as cardiac glycoside action. In myocardial tissues inhibition of Na,K-ATPase activity has been shown to produce positive inotropy. Positive inotropy was associated with an increase in  $[Ca]_i$ , which was achieved by the stimulation of Na-Ca exchange caused by an increase in the  $[Na]_i$ . The increase in the  $[Na]_i$  was itself caused by a reduction in Na,K-ATPase activity (see section 1.V.i.b.2.). The decreases in the abundance of the mRNA for the isoforms of Na,K-ATPase, may therefore be part of the adaptive response of ventricular tissues to increased blood pressure in hypertensive rats. The reason for the increases in mRNA abundance of the  $\alpha 1$ ,  $\alpha 2$  and  $\beta 1$  isoforms in the atria of hypertensive rats was unknown.

#### 5.III.iii. Isoforms of Na,K-ATPase in MDCK cells.

The optimum hybridisation conditions for the annealing of isoform-specific probes to MDCK RNA was previously determined (see section 4.III.ii.) to be 33°C in 50% formamide. However, even at the higher hybridisation temperature of 43°C, as much as 70% of the signal found for the optimum hybridisation temperature (33°C) was detected under low temperature washing conditions (0.1 x SSC at 35°C). In order to use the most stringent hybridisation conditions available and to reduce the level of background, initial experiments were carried out at 43°C in 50% formamide. To act as positive controls samples of rat brain total RNA were blotted simultaneously with MDCK total RNA. Rat brain total RNA was used as a control because it was known to contain abundant levels of mRNA for all of the isoforms of Na,K-ATPase (Young and Lingrel, 1987). Canine



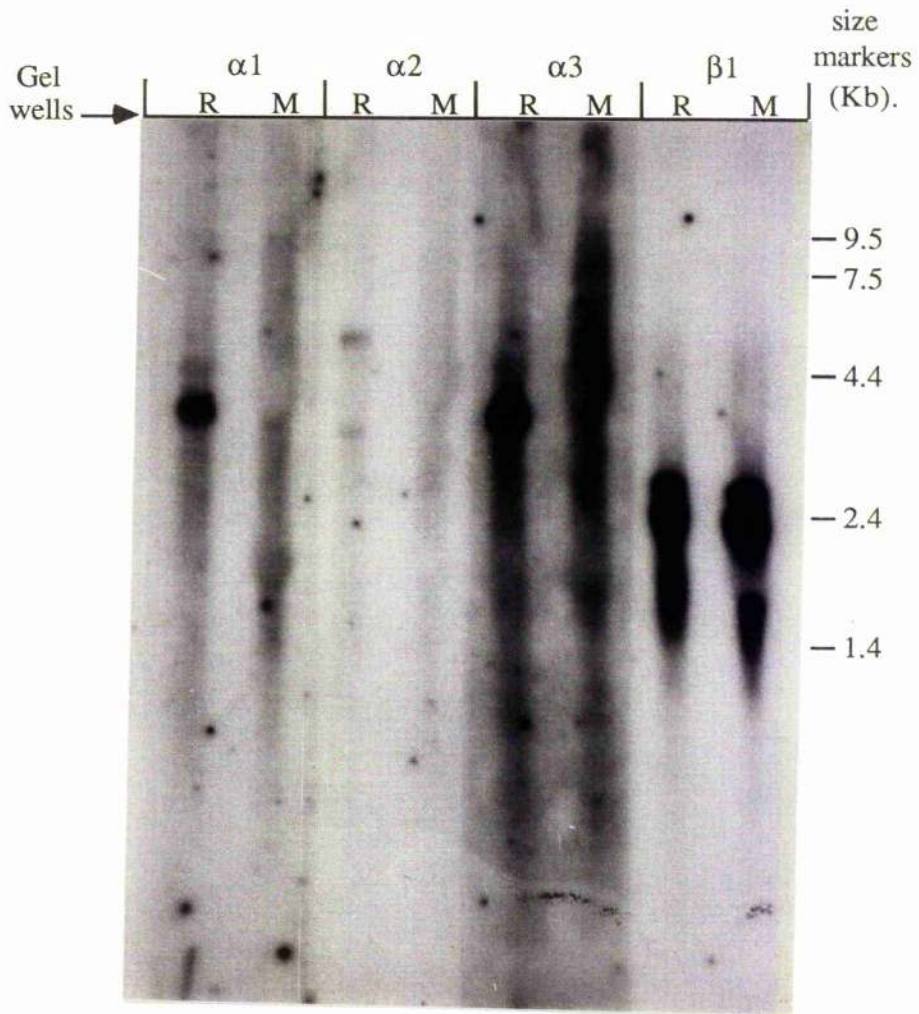
brain total RNA, should have mRNA's for all the isoforms of Na,K-ATPase with identical nucleotide sequences to those possibly present in MDCK cells. This source of total RNA would therefore have served as a better control than rat brain total RNA however, a suitable source of canine brain total RNA was not found. Of the isoform specific probes ( $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ , and  $\beta 1$ ; see 4.I.iii.), the  $\alpha 1$  specific probe had the lowest homology compared to the sequences of equivalent nucleotide regions in the  $\alpha$  isoforms of humans, which is the only other species for which the isoform sequences have been fully characterised. Therefore, if the cross hybridisation of the rat  $\alpha 1$  isoform specific probe to MDCK cell RNA was successful, it was likely that, the hybridisation conditions should be sufficiently relaxed (ie of low enough stringency) to allow the successful cross hybridisation of all the other isoform specific probes to any complementary mRNA present in MDCK cell RNA.

Northern blots of 2 $\mu$ g of rat brain total RNA and 20 $\mu$ g of MDCK total RNA were hybridised with the isoform specific DNA probes at 43°C in 50% formamide (see figure 9). As can be seen in figure 10, virtually no  $\alpha 1$  specific hybridisation signal was present in the MDCK cell RNA. However, a specific signal could be seen in the  $\beta 1$  isoform blot. At least three sizes of  $\beta 1$  isoform mRNA (2.7, 2.35, 1.7 Kb) were present in both the rat brain and MDCK cell total RNA blots. These bands were thought to represent initiation and termination of mRNA transcripts, at different initiation and poly adenylation sites, and the sizes of these bands were comparable to those reported by Omori *et al.* (1988) and Young *et al.*, (1987). No  $\alpha 3$  mRNA signal was detected in the MDCK cell total RNA, although hybridisation to the equivalent isoform in the rat brain RNA sample was apparent. In the case of the  $\alpha 2$  isoform specific blot, no hybridisation signal was detected in rat brain or MDCK cell total RNA after short exposure times (not shown). A long exposure of the  $\alpha 2$  isoform blot (see figure 9) however, revealed the presence of two bands of 3.6 and 5.6 Kb in size in the rat brain total RNA sample. The  $\alpha 2$  specific DNA probe had consistently radiolabelled poorly (5-25% incorporation) and this was thought to be the main reason for the poor hybridisation and the low intensity of the  $\alpha 2$  mRNA bands in figure 9. As the  $\alpha 2$  isoform specific probe was the shortest of the DNA probes (142 bp long), it was possible that it may have been too small for efficient radiolabelling of the DNA by the Klenow fragment of DNA polymerase I (the enzyme used in the Multiprime radiolabelling kit).

Following gel electrophoresis of  $\alpha 1$  and  $\alpha 2$  cDNA plasmid restriction endonuclease digests, both the  $\alpha 1$  and  $\alpha 2$  isoform-specific bands were in close proximity to other non



Figure 9. A series of Northern blots showing the presence of Na,K-ATPase isoform mRNA's in R) 2  $\mu$ g of rat brain total RNA or M) 20  $\mu$ g of MDCK total RNA. RNA samples were separated on a 1.2% agarose formaldehyde denaturing gel and electroblotted onto Zeta probe nylon membranes. The blots were hybridised at 43 °C (in 50% formamide) and were finally washed in 0.1 x SSC, 0.1% SDS at 30°C for 5 minutes.

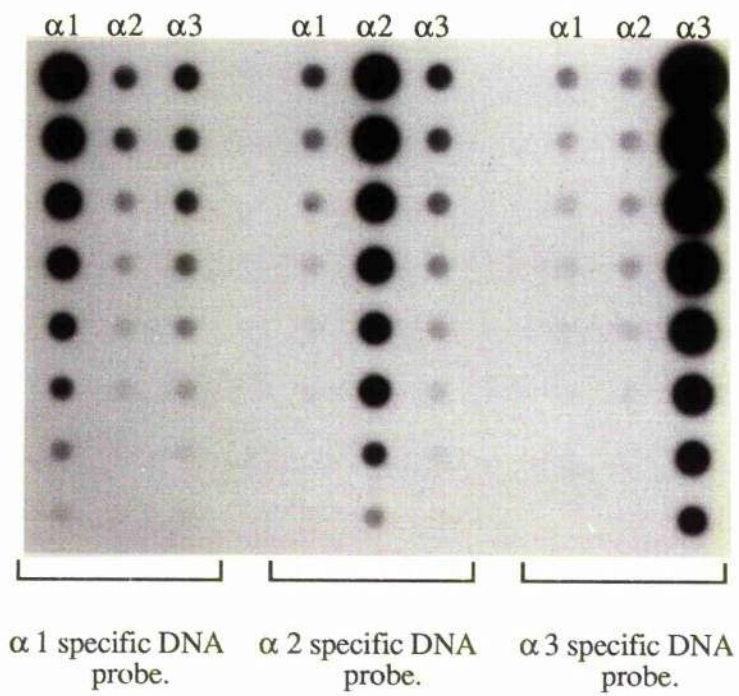


isoform-specific restriction fragments from the digested cDNA's (see Chapter 4; figure 1). The isoform-specific DNA fragments isolated, were tested for the presence of cross contamination by the other restriction fragments. This was done by hybridising filters containing dots of the  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$  plasmid DNA with each of the isoform-specific probes (see figure 10). Each of the isoform-specific DNA restriction fragments, if free of other contaminating fragments should only have hybridised with its own plasmid DNA under the high stringency conditions used (hybridisation in 50% formamide, at 42°C, and washing in 0.1% SDS, 0.1 x SSC at 70°C). This was because the homology of each probe to the other isoforms or vector sequences was theoretically too low (ie less than 66%) to result in cross hybridisation. Hybridisation to the other isoform plasmid DNA dots was therefore likely to be an indication of the presence of contaminating DNA restriction fragments from other regions of the cDNA's. The extent of cross hybridisation was estimated by comparing the relative intensity of the signal obtained from hybridisation of a probe to its own plasmid, to that obtained with the other plasmids. The results showed a greater cross hybridisation of the  $\alpha 1$  specific (~3.5%) and  $\alpha 2$  specific DNA probes (~7.0%) to the other isoform plasmid dots, than the  $\alpha 3$  specific probe (~0.2%). As a result of the possible problems with cross hybridisation and poor radiolabelling of small fragments it was decided to reselect the restriction fragments used as  $\alpha$  isoform-specific probes in order to 1, increase their length to >200bp, 2, produce probes which would result in better separation from other bands during isolation on gels, and 3, make quantification of isoform mRNA's more comparable by making the lengths of all the probes as similar as possible. It was also decided for the latter reason, to produce a  $\beta 1$  isoform DNA probe which was of similar length to the  $\alpha$  isoform probes and, which shared maximum homology with the  $\beta 1$  isoform sequence reported from canine species (Brown *et al.*, 1987).

With the above considerations in mind, the following new isoform specific probes were isolated, 1, the  $\alpha 1$  isoform; a 219 bp NcoI-AvaI fragment corresponding to nt's 237-455 (inclusive) of the cDNA sequence (Shull *et al.*, 1986b; see figure 11), 2, the  $\alpha 2$  isoform; a 202 bp EcoO109 fragment corresponding to nt's 72-273 (inclusive) of the cDNA sequence (Shull *et al.*, 1986b; see figure 12), and 3, the  $\beta 1$  isoform; a 208 bp PstI-SpeI fragment corresponding to nt's 771-978 (inclusive) of the cDNA sequence (Mercer *et al.*, 1986; see figure 13). The  $\alpha 3$  isoform specific fragment was as detailed previously (a 202 bp SstII-SmaI fragment). As the bands were now well separated from other possible contaminating DNA bands during gel electrophoresis (see figures 11 to

Figure 10. Shows dot blots of Na,K-ATPase  $\alpha$  isoform cDNA containing plasmids. Three identical filters containing plasmid DNA dots were hybridised with the three  $\alpha$  isoform-specific probes, in order to detect contamination of the probes (with other cDNA or vector fragments) occurring during their isolation. The series of plasmid dots represent ~500 ng of DNA double diluted. The dot blots were hybridised at 42 °C (in 50% formamide) and were finally washed in 0.1 x SSC, 0.1% SDS at 70°C for 10 minutes.

Isoform Plasmid dots.



Radioactive standard dots.

CPM  
1000  
600  
360  
216  
130  
78  
47  
28

$\alpha 1$  specific DNA probe.

$\alpha 2$  specific DNA probe.

$\alpha 3$  specific DNA probe.



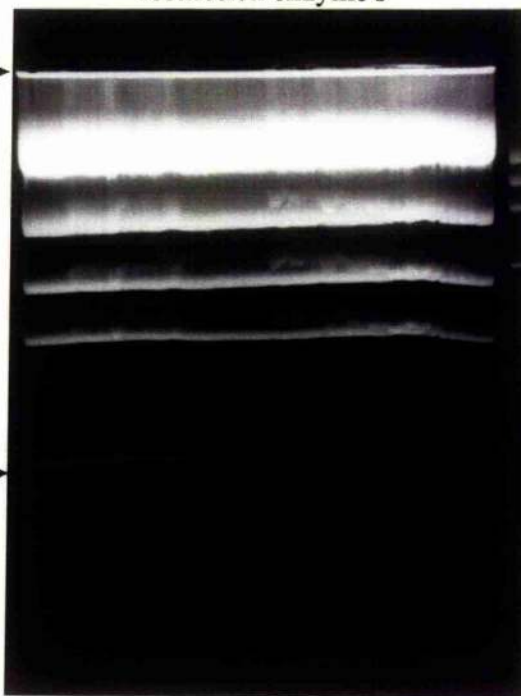
Figure 11. A preparative agarose gel of Nco I and Ava I restriction endonuclease digested rat Na,K-ATPase  $\alpha$ 1 cDNA plasmid, stained with ethidium bromide. The size standards illustrated represent  $\phi$ X-174 Hae III restriction digested DNA. The arrows indicate the positions of the gel wells and the  $\alpha$ 1 isoform-specific fragment band isolated.

$\alpha 1$  cDNA/ pBR322 DNA digested  
with Nco1 and Ava 1.  
restriction enzyme's

size  
standards  
(bp).

Gel wells →

→  $\alpha 1$  Nco1-Ava1  
DNA fragment  
(219 bp).



1353  
1078  
872

603

310  
281  
271  
234  
194

118

72

Figure 12. A preparative agarose gel of EcoO109 restriction endonuclease digested rat Na,K-ATPase  $\alpha 2$  cDNA plasmid, stained with ethidium bromide. The size standards illustrated represent  $\phi X-174$  Hae III restriction digested DNA. The arrows indicate the positions of the gel wells and the  $\alpha 2$  isoform-specific fragment band isolated.

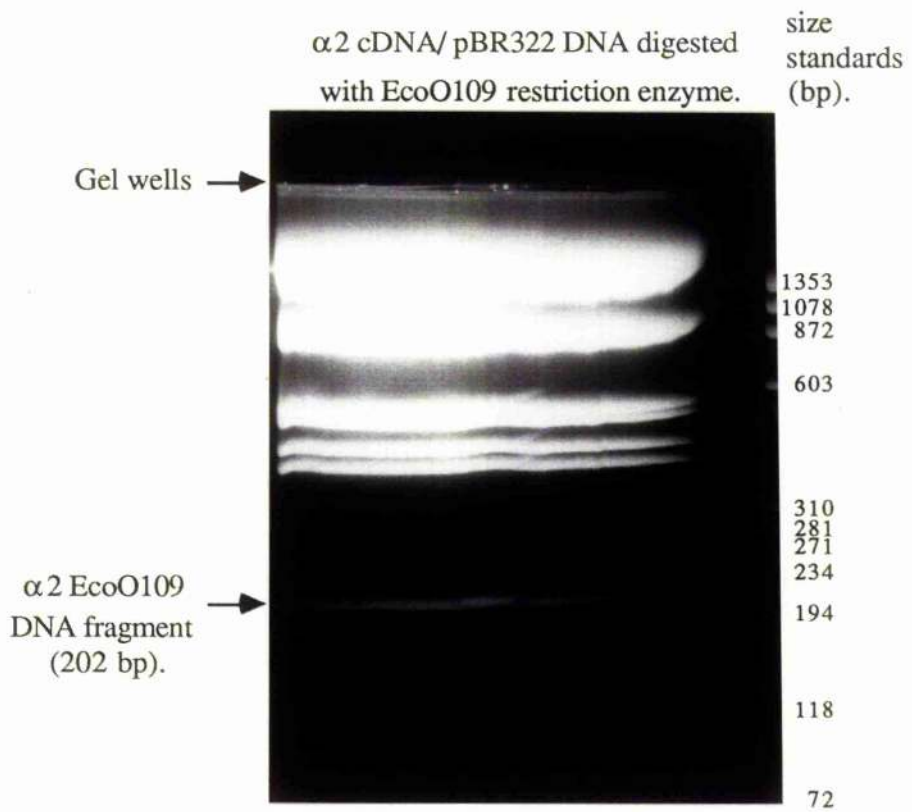
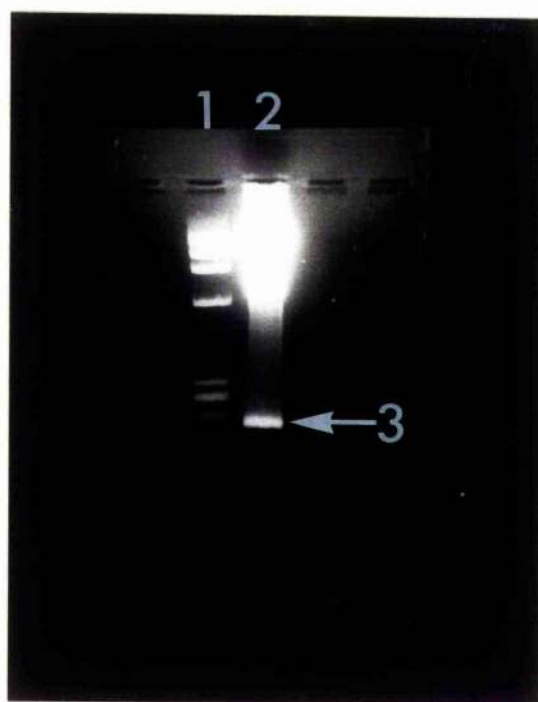


Figure 13. A preparative agarose gel of Pst I and Spe I restriction endonuclease digested rat Na,K-ATPase  $\beta$ 1 cDNA plasmid (2), stained with ethidium bromide. The size standards illustrated (1) represent  $\phi$ X-174 Hae III restriction digested DNA. The arrow (3) indicates the positions of the gel wells and the  $\beta$ 1 isoform-specific fragment band isolated.

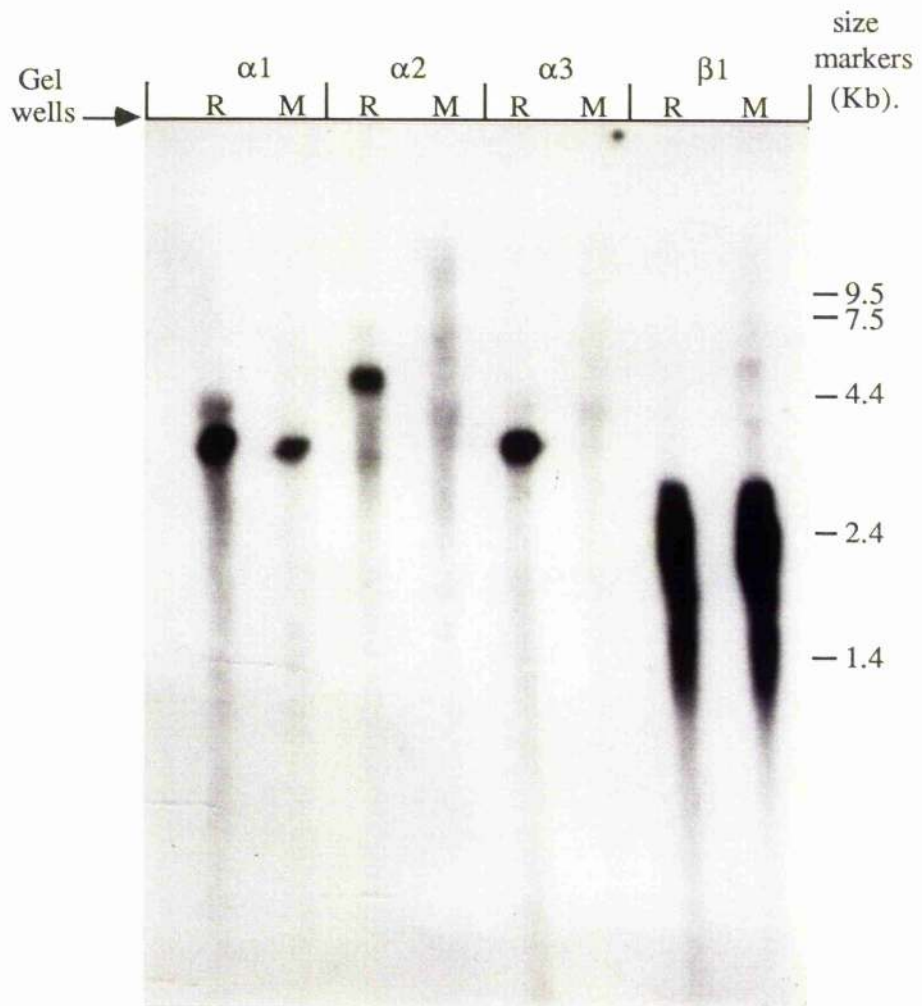




13), it was deemed unnecessary to test these probes for the presence of cross contamination. The new  $\alpha 1$  isoform specific DNA probe was 53.9% and 50.2% homologous to the comparable regions of the  $\alpha 2$  and  $\alpha 3$  cDNA sequences respectively. The new  $\alpha 2$  isoform specific probe was 49.5% and 41.6% homologous to the comparable regions of the  $\alpha 1$  and  $\alpha 3$  cDNA sequences respectively. The new rat  $\beta 1$  isoform specific probe isolated was 92.8% homologous to the reported  $\beta 1$  sequence of canine species. A comparison of the new rat isoform specific probes to their counterparts in human species (the only species for which all of the isoform sequences were available) showed homologies of 83.6%, 92.6%, 92.1% and 91.9% for the  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ , and  $\beta 1$  isoforms respectively. This indicated that the  $\alpha 1$  isoform specific probe was probably still the least homologous of the probes between species. Thus experimental conditions that allowed the hybridisation of this probe to its equivalent mRNA in MDCK cells, were likely to allow the hybridisation of all the probes to their equivalent mRNA's.

The hybridisation of the original  $\alpha 1$  isoform-specific probe to MDCK total RNA (see figure 9) at 43°C, resulted in a very low signal on Northern blots compared to that found with rat brain RNA. To overcome the low signal: noise ratio, a series of Northern blots were produced, using MDCK poly A<sup>+</sup> mRNA rather than total RNA. Blots were also hybridised at lower stringency than previously, at the optimal conditions determined for the rat  $\alpha 1$  probe (at 33°C in 50% formamide; see section 4.III.ii.), using the new isoform-specific probes (see figure 14). All of the new isoform-specific probes hybridised well to the rat brain total RNA suggesting that hybridisation had occurred successfully. Significant levels of specific hybridisation of the  $\alpha 1$  and  $\beta 1$  probes to MDCK cell poly A<sup>+</sup> mRNA were also apparent. The mRNA for the  $\alpha 1$  isoform in MDCK cells was approximately the same size as the equivalent  $\alpha 1$  isoform mRNA in the rat brain RNA sample, which was estimated to be 3.65 Kb in size. This result was identical to that reported by Bowen and McDonough (1987), who also found a single  $\alpha 1$  isoform mRNA species of 3.65 Kb in MDCK cell total RNA. The mRNA for the  $\beta 1$  isoform in MDCK cells also showed a similar number and size of mRNA's compared to those previously reported for rat brain (ie 4 or more mRNA species of approximately 2.85, 2.25, 1.75 and 1.45 Kb in size). This result was in direct contrast to that of Bowen and McDonough (1987) who found only a single  $\beta 1$  mRNA species of 2.75 Kb in MDCK cell total RNA. The reason for this discrepancy is not known, but may possibly be due to the use of a short autoradiographic exposure time in the study by Bowen and McDonough (1987). The hybridisations that used the  $\alpha 2$  and  $\alpha 3$  specific probes

Figure 14. A series of Northern blots showing the presence of Na,K-ATPase isoform mRNA's in R) 4  $\mu$ g of rat brain total RNA or M) 5  $\mu$ g of MDCK poly A<sup>+</sup> RNA RNA samples were separated on a 1.2% agarose formaldehyde denaturing gel and electroblotted onto Zeta probe nylon membranes. The blots were hybridised at 33 °C (in 50% formamide) and were finally washed in 0.1 x SSC, 0.1% SDS at 30°C for 5 minutes.



however, indicated that no detectable  $\alpha 2$  or  $\alpha 3$  mRNA's existed in the poly A<sup>+</sup> mRNA of MDCK cells. The presence of these isoform mRNA's was not examined in the study by Bowen and McDonough (1987).

As all the isoform-specific DNA probes hybridised well to the rat brain total RNA in figure 14, and another identical blot (not shown), estimation of the size and relative abundances of the individual mRNA's in rat brain tissues was also possible. The size of specific mRNA bands was determined to be as follows :-  $\alpha 1 = 3.65$  Kb;  $\alpha 2 = 5.05$  and  $3.45$  Kb;  $\alpha 3 = 3.55$  Kb;  $\beta 1 = 2.90, 2.40, 1.90$  and  $1.50$  Kb. These sizes were in good agreement with the previously reported values (Young and Lingrel, 1987; Omori *et al.*, 1988; and Young *et al.*, 1987). Utilizing only the signals obtained within the specific bands, from 2 gels, the abundance of the isoform mRNA's in brain tissue relative to those of the  $\alpha 1$  isoform mRNA were as shown in table 2. The relative abundances of the isoforms in this study were similar to those reported by Young and Lingrel (1987) and Orłowski and Lingrel (1988), with the exception that the  $\alpha 2$  isoform mRNA was only

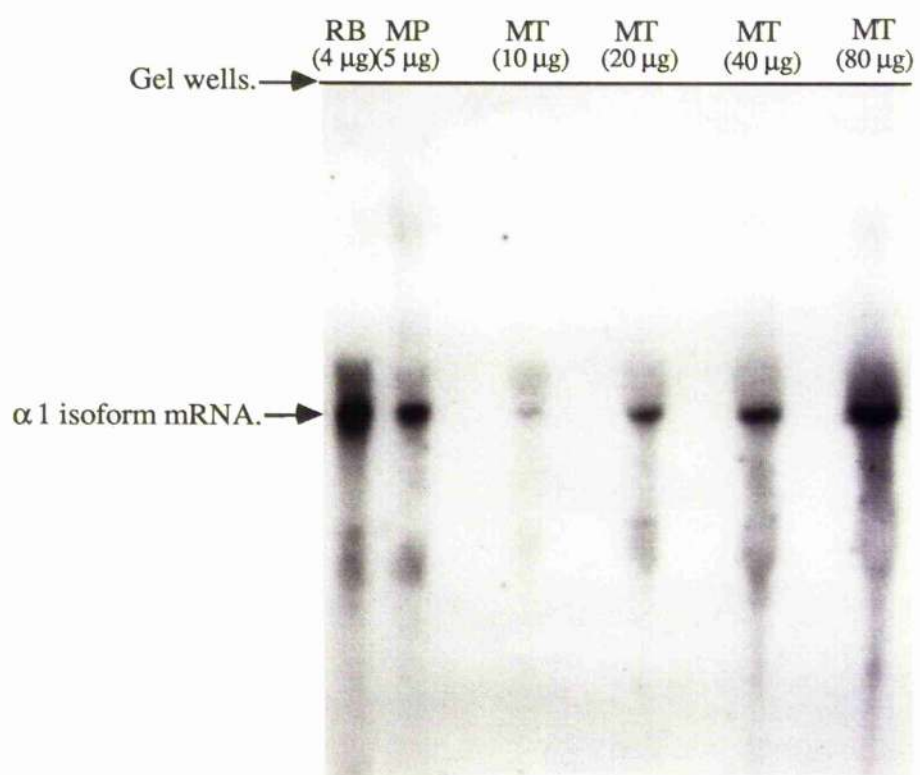
Source.	Isoforms.			
	$\alpha 1$	$\alpha 2$	$\alpha 3$	$\beta 1$
This study.	1.00	0.39	0.87	5.14
Young.	1.00	1.20	0.80	5.20
Orłowski.	1.00	0.80	1.20	4.50

Table 2. The relative abundance of the Na,K-ATPase isoform mRNA's in rat brain from this study, and that of Young and Lingrel (1987) and Orłowski and Lingrel (1988).

30-50% as abundant in this study, compared to the relative levels reported by the other groups. The reason for this discrepancy is not known, however, the relative comparisons reported by Young and Lingrel (1987) and Orłowski and Lingrel (1988), were



Figure 15. A Northern blot showing the abundance of Na,K-ATPase  $\alpha$ 1 isoform mRNA in 4  $\mu$ g of rat brain total RNA (RB), 5  $\mu$ g of MDCK poly A<sup>+</sup> RNA (MP) and 10, 20, 40, 80  $\mu$ g of MDCK total RNA (MT). RNA samples were separated on a 1.2% agarose formaldehyde denaturing gel and electroblotted onto Zeta probe nylon membranes. The blot was hybridised with the  $\alpha$ 1 isoform-specific probe at 33 °C (in 50% formamide) and were finally washed in 0.1 x SSC, 0.1% SDS at 30°C for 5 minutes.



determined from slot blots where, the level of any background was unknown and this may have contributed to an overestimate of the signal obtained. The level of hybridisation signal of the  $\alpha 1$  isoform mRNA in MDCK poly A<sup>+</sup> mRNA was very low compared to the equivalent level in rat brain total RNA. An experiment was needed to determine whether this mRNA could be detected in total RNA (see figure 15) and if so, how much total RNA would be required for an adequate level of signal. The minimum amount of MDCK total RNA necessary to produce a significant level of hybridisation signal from the  $\alpha 1$  isoform mRNA was found to be around 20  $\mu$ g. However, the blot in figure 15 also demonstrated that at such a low level of stringency, a considerable amount of lane or RNA specific background was present. This suggested that at this level of stringency the isoform specific DNA probes should not be used for the purposes of dot or slot blot hybridisation, as specific RNA levels would be over estimated. These results indicated that future experiments utilizing the cross hybridisation of the rat isoform specific probes to MDCK cell total RNA, should incorporate the use of Northern blot, rather than dot or slot blotting techniques.

CHAPTER 6. THE MORPHOLOGY OF AND Na,K-ATPase EXPRESSION IN MDCK CELLS.

## 6.1 Introduction.

The general background information on Na,K-ATPase in MDCK cells has been outlined in Chapter 1, however, additional information on blister formation and Na,K-ATPase expression in MDCK cells is outlined below.

Leighton *et al.* (1969) were the first to demonstrate that MDCK cell monolayers viewed under the light microscope formed hemispherical vesicles or blisters. These blisters were suggested to be comprised of interstitial collections of fluid which had accumulated in the plane between the cell monolayer and the impermeable glass substrate (Leighton *et al.*, 1969). The properties of the cell monolayers in blistered areas has been shown not to be different from non-blistered areas as the morphology, polarity, and relative ionic permeabilities of the monolayer in these regions were similar (Rabito *et al.*, 1978). The composition of the fluid contained within blisters has been shown not to be significantly different from culture medium in terms of its sodium and potassium contents (Rabito *et al.*, 1978). The fluid has therefore been suggested to represent reabsorbed components of the growth medium, probably accumulated by the vectorial transport of salts (Leighton *et al.*, 1969). Blister formation has been shown not to occur on permeable collagen or Millipore filter substrates (Cereijido *et al.*, 1981a). This was a function of the substrate permeability, rather than its composition, as blisters form on collagen coated glass substrates (Rabito *et al.*, 1978).

A model of blister formation on impermeable substrates has been proposed by Cereijido *et al.* (1981a). The model predicts that in blistered areas, the tight junctions formed between cells are stronger than the cell-substrate interactions. As fluid accumulates under the monolayer, pressure on both the tight junctions and the cells adhesion to the substrate increases. In some areas, fluid pressure results in the detachment of the cell monolayer from the substrate and blister formation. While in other regions the tight junctions are burst open allowing the interstitial fluid to return to the medium. The lateral diffusion of fluid through the interspace between cells, was thought to be negligible, or else blister formation would not occur (Rabito *et al.*, 1978). The formation of blisters on permeable substrates is prevented by diffusion of fluid through the substrate, leaving the cells tight junctions intact and allowing the formation of electrically resistant cell monolayers. As evidence in support of the model of blister formation, Cereijido *et al.* (1981a) have shown that [<sup>125</sup>I]-lactoperoxidase labelling of cell surface proteins, was no different in cell monolayers grown on impermeable



substrates, before or after cell tight junctions were dissociated with EGTA. Lactoperoxidase has also been used to label proteins from the apical side of MDCK strain I and II cell monolayers, grown on permeable Millipore filters (Richardson *et al.*, 1981) or permeable collagen coated nylon substrates (Cereijido *et al.*, 1978), respectively. These experiments have demonstrated that the lactoperoxidase enzyme could not penetrate the tight junctions of MDCK cells, to label proteins on the basolateral side of the monolayer, when cells were grown on permeable substrates. Major cell proteins (such as Na,K-ATPase; Caplan *et al.*, 1986; Lamb *et al.*, 1981) are known to be polarised to the basolateral membrane domain (Na,K-ATPase >90%; Caplan *et al.*, 1986). When cells were grown on impermeable substrates, lactoperoxidase must have had access to the basolateral domain, because otherwise a larger set of proteins would have been labelled when the tight junctions were dissociated with EGTA. As a consequence of the model of blister formation, only the relatively small percentage of cells associated with blistered areas of the monolayer would have access to their basolateral membranes restricted. Thus the number of [<sup>3</sup>H]-ouabain binding sites (Na,K-ATPase units) should be determinable on cells grown on permeable or impermeable substrates, with only a small underestimate of ouabain binding sites occurring in monolayers with blisters (Cereijido *et al.*, 1981a).

There is some evidence from this laboratory (unpublished data; see appendix 4) and others (Kennedy and Lever, 1984; Nakazato *et al.*, 1989) that the amount of cellular ouabain binding and Na,K-ATPase activity declines as cell cultures approach confluence. The level of [<sup>3</sup>H]-ouabain binding on plastic supports has been reported to be lower than that of cells grown on filter supports (Cereijido *et al.*, 1981a; Kennedy and Lever, 1984). Wide differences in the number of [<sup>3</sup>H]-ouabain binding sites per cell and hence Na,K-ATPase units, have been reported in work on MDCK cells. The numbers estimated range from  $2.33 \times 10^5$  (Lamb *et al.*, 1981) to  $8 \times 10^7$  sites per cell (Kennedy and Lever, 1984). Those variations may reflect differences in culturing conditions or cell density when measurements of ouabain binding were made. However, Nakazato *et al.* (1989) have shown that different cloned lines of MDCK cells exhibit altered morphologies and Na,K-ATPase activities during growth. They (Nakazato *et al.*, 1989) have characterised the Na,K-ATPase activity of clonal cell lines which were either motile or non-motile during growth, the two morphological types having different Na,K-ATPase activities. The motile clones, which were judged to be motile during repeated microscopic observations, had extended and flattened cytoplasms and a relatively low level of Na,K-ATPase activity in the first 2 days of growth. This was compared to the non-motile clones, which were

cuboid with few if any cell processes and had a relatively high level of Na,K-ATPase activity. The data determined by Nakazto *et al.* (1989) suggests that some of the differences in the number of enzyme units found by several authors may also be due to the use of different subclones of MDCK cells. In support of this idea, Richardson *et al.* (1981) also found differences in the number of enzyme units between two strains of MDCK cells known as Strain I and II.

The purpose of this study was to determine factors affecting the surface expression of Na,K-ATPase units (ouabain binding sites) in MDCK strain I cells, and to correlate alterations in enzyme abundance with changes in Na,K-ATPase  $\alpha$  subunit mRNA abundance. In view of the above information, the factors investigated were; 1, changes in the seeding density of cells (ie alteration in the level of cell-cell contacts), 2, different types of growth surfaces (ie changes in cell-substrate interactions), and 3, the effect of changes in the volume of growth medium. To confirm and extend the work of others (Cereijido *et al.*, 1981a; Rabito *et al.*, 1978), experiments were also performed to test the possible restriction of access of ouabain to the basolateral surface of MDCK strain I cell monolayers grown on impermeable supports.

## **6.II. Methods.**

### **6.II.i. Cell morphology.**

Changes in MDCK cell morphology during culture resulting from different seeding densities was examined by phase contrast light microscopy. The MDCK cells were grown on coverslips at two different seeding densities; a high seeding density of  $4.17 \times 10^4$  cells/cm<sup>2</sup> (the normal seeding density used for cell culture), and a low seeding density of  $4.9 \times 10^3$  cells/cm<sup>2</sup>. Cells were observed after 1, 2, 4, and 6 days in culture. Phase contrast light micrographs were also taken of cells growing at high density (as above) on permeable collagen culture plate inserts (ICN Biomedicals; see Chapter 2). Micrographs were taken using a Leitz Dialux 20 microscope and 32 ASA Kodak Pan-X film. An increased number of cells appeared to attach to the central portion of the coverslips and collagen inserts used for the light micrographs. The light micrographs illustrated in figures 1-3 were all taken from the central portion of the cover slip or collagen insert except where otherwise stated.

### 6.II.ii. Na,K-ATPase expression.

The other methods used were as outlined in Chapters 2. Na,K-ATPase  $\alpha$  subunit mRNA abundance was determined by the dot blotting technique. This was because large numbers of samples were to be blotted, making Northern blotting unsuitable. The rat  $\alpha$  isoform-specific DNA probes were unsuitable for use in dot blotting (see section 5.III.iii), as the low stringency hybridisation and washing conditions required for their use, resulted in high level of background signals. As the high stringency conditions used previously with the pHANK probe (partial human  $\alpha$ 1 cDNA; see section 4.I.ii) showed no signs of background signals on Northern blots and as only  $\alpha$ 1 isoform mRNA was detected in MDCK cell RNA, this probe was chosen in preference to the rat DNA probes for use in dot blotting.

### 6.III. Results and Discussion.

#### 6.III.i. Light micrographs of MDCK cells during growth on glass supports.

Figure 1 shows the effect of high and low seeding density on the subsequent morphology of MDCK strain I cells grown in culture. The light micrographs showed that after 24 hours of culture, there was a considerable difference in the morphology between high and low density seeded cultures. As expected, the low density seeded cells appeared mostly as single cells often connected by cell processes (figure 1a), whereas, the high density seeded cells were mostly grouped into islands of cells (figure 1b). The high density seeded cells showed evidence of blisters being present well before confluence was established, even after only one day of culture (figure 1b). The culture of high density seeded cells reached confluence after only two days (figure 1d), whereas, confluence was only achieved after 4 days culture at the low seeding density (figure 1e). The MDCK cells exhibited long cellular processes at low density (figure 2a), and often had several cell processes connecting an individual cell to several of the surrounding cells (figure 2b). Micrographs of the cells at low density (figure 1a, 1c, 2a, and 2b), showed that many of the cells had cell processes suggesting that the cell population consisted largely of the 'motile' class of MDCK cells, as suggested by Nakazato *et al.* (1989). However, at the edges of cover slips where the cells were originally less densely populated, the edge of the cell monolayer surrounding large gaps (in the monolayer), had

Figure 1 a-g. Shows phase contrast light micrographs of MDCK cells grown on glass supports. Cells were grown for up to six days after seeding at high or low density (see Chapter 2).

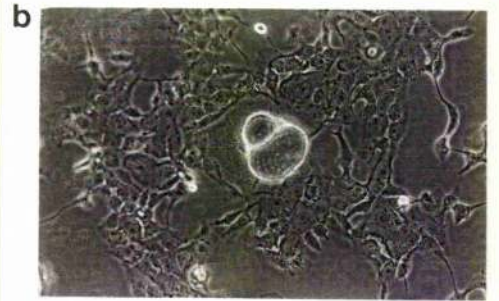
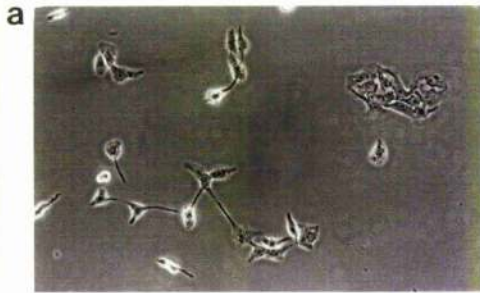


Incubation  
(Days).

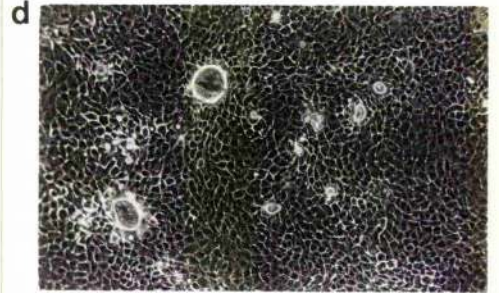
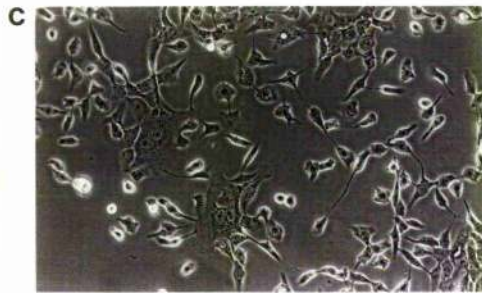
Low density  
seeded.

High density  
seeded.

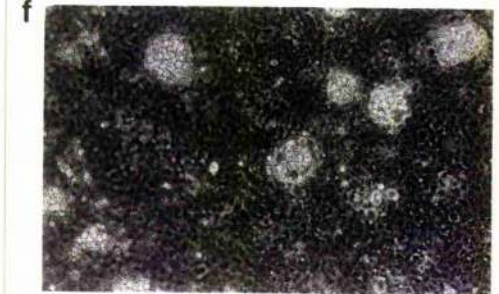
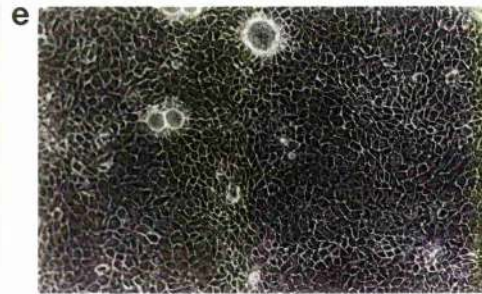
1



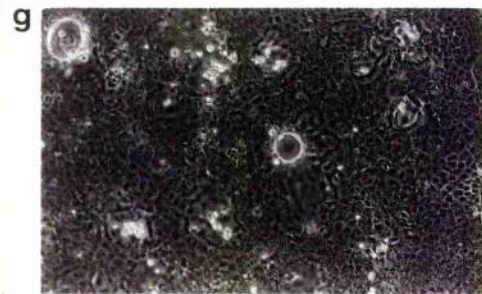
2



4



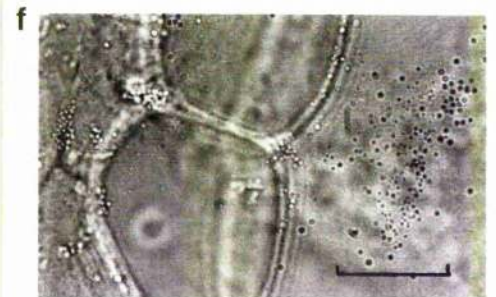
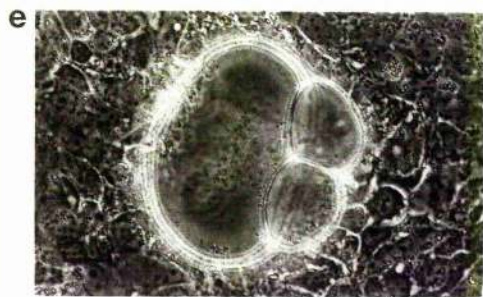
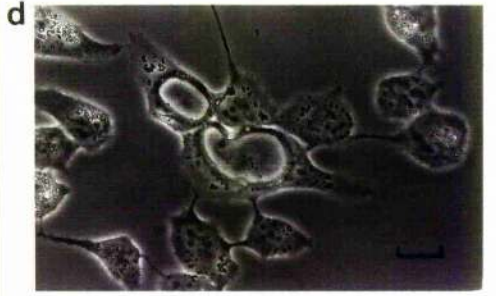
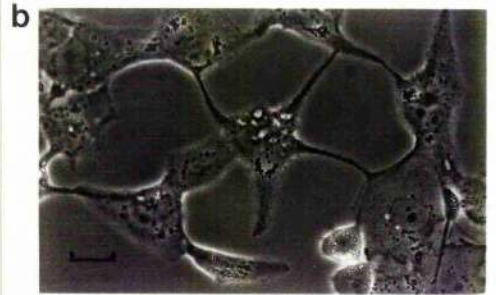
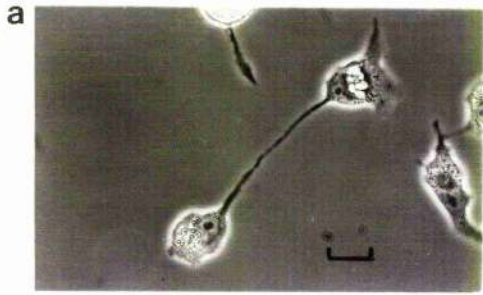
6



200  $\mu$ m



Figure 2 a-f. Shows phase contrast light micrographs of various morphological of MDCK cells grown on glass supports. Typical cell morphological features include a) single cells connected by a long cell process, b) a cell connected to three other cells by processes, c) the edge of a confluent cell monolayer, where few cell processes were apparent, d) blisters or domes formed on single cells. e) overlapping domes or blisters, f) magnified view of e). The bars illustrated on micrographs represent 20  $\mu\text{m}$ .



very few cell extensions (see figure 2c). Cells with few extensions were characterised by Nakazato *et al.* (1989) to be 'non-motile' (see introduction). Therefore after seeding at low density, it was possible to find in the population, cells exhibiting both motile and non-motile morphological characteristics. Blisters in this strain of MDCK cells were formed underneath single cells and did not appear to require the formation of a confluent cell monolayer (figure 2d). When blisters were formed in close proximity to each other, they were also capable of overlapping (see figure 2e and 2f). This suggested that cells were capable of vectorial transport before the complete formation of a confluent monolayer or tight junctions (a marker for the polarisation of cells).

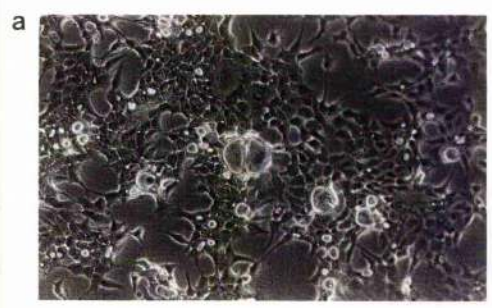
#### 6.III.ii. Light micrographs and properties of MDCK cells during growth on collagen supports.

MDCK strain I cells were also grown on type I collagen permeable culture plate inserts, at high seeding density, ( $4.1 \times 10^4$  cells/cm<sup>2</sup>) as shown in figures 3. Unexpectedly, although the type I collagen membranes were supposedly permeable (information supplied by ICN Biomedicals Inc.), the MDCK cell monolayers grown on this substrate (see figure 3a-f) exhibited blister formation. This prompted the question as to whether the collagen culture plate inserts were really permeable. The permeability of collagen membranes was assessed qualitatively by raising the level of liquid (growth medium) inside the inserts (cell free) 2-3 mm above that outside, in the culture plates, and vice versa. After an overnight incubation at 37°C, the level of medium inside and outside the culture plate insert was still 2-3 mm higher than that outside and vice versa. The permeability of the collagen inserts was then assessed quantitatively, using similar conditions to ouabain binding assays on culture plate inserts (see section 2.III.i.b.). This was done by adding the 2.5 ml of [<sup>3</sup>H]-ouabain (0.1 μCi/ml) in ordinary Krebs (with 5 mM K; see appendix 3) buffer to the culture plate and ordinary Krebs buffer to the culture plate insert. After 20 minutes of incubation at 37°C, the buffer from the insert was sampled and the radioactivity determined. The results showed that the concentration of [<sup>3</sup>H]-ouabain within the insert had risen to ~16% of the initial concentration in the culture plate. This showed that although liquid appeared not to be forced through the collagen membrane by hydrostatic forces (such as exist during blister formation), ouabain could still readily diffuse through the membrane.

Figure 3 a-c. Shows phase contrast light micrographs of MDCK cells grown on collagen inserts supports. Cells were grown for up to six days after seeding at high density (see Chapter 2).

Incubation  
(Days).

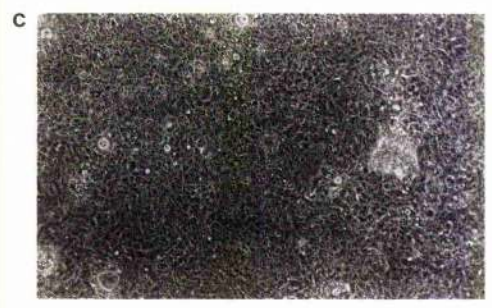
2



4



6



200  $\mu$ m



6.III.iii. The growth characteristics, specific ouabain binding sites and abundance of Na,K-ATPase  $\alpha$  subunit mRNA of MDCK cells grown on plastic supports.

Changes in the number of Na,K-ATPase enzyme units (as assessed by [ $^3$ H]-ouabain binding sites) on the surface of MDCK cells were determined during the growth of MDCK cells in culture. Cells were grown after seeding at high density ( $4.2 \times 10^4$  cells/cm $^2$ ) under the normal conditions used for subculturing (ie 0.57 ml of medium/cm $^2$ ; see section 2.III.i.a.). The cells used for all the experiments were grown under identical conditions for at least 2 passages prior to use, and were passaged after 5 days in culture. The cells were grown on plastic cell culture plates for the purposes of ouabain binding assays, and in plastic culture flasks for RNA experiments. Cells were grown under similar conditions to the previous passages, where they were normally subcultured after 5 days. The cell volume, the number of ouabain binding sites, the amount of total RNA, and the abundance of the Na,K-ATPase  $\alpha$ 1 isoform mRNA, at time 0 were all assumed to be equivalent to the levels present after 5 days in culture.

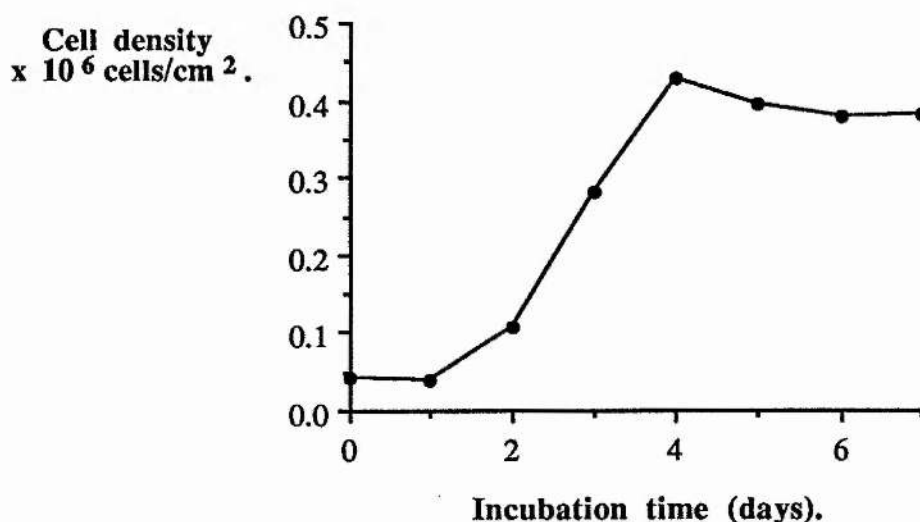


Figure 4. The density of MDCK strain I cells during growth. The cells were seeded at high density and were grown under normal conditions in plastic culture flasks.

The cells appeared to have a lag phase of approximately 1 day (by virtue of the fact that cell density had not increased over the first 24 hours; see figure 4) before the cell density rapidly increased to a maximum after 4 days. The number of cells then reduced slightly during the plateau phase after 4 days in culture. Changes in cell volume also

occurred during culturing (see figure 5). During the 24 hour lag phase the mean cell volume increased by ~50% to ~2100 fl. During the rapid growth phase (days 1-4) the mean cell volume was continually reduced until it reached its initial day 0 value of around 1400 fl. The mean cell volume then increased slightly during the stationary or plateau phase, this was paralleled by an equally small decrease in cell numbers. The number of Na,K-ATPase units ( $[^3\text{H}]$ -ouabain binding sites) present on the MDCK cells grown at high density was determined by the procedure outlined in section 2.III.i.b. The number of enzyme units, the amount of total RNA per cell, and the abundance of Na,K-ATPase  $\alpha$  subunit mRNA at time 0 were all assumed to be approximately equivalent to the levels present after 5 days of culture (the point at which cells were normally subcultured). The number of enzyme units increased initially from up to 2 days, but then declined rapidly to a very low level at maximum cell density (day 4; see figure 6). One possible explanation is that tight junctions may have remained intact between adjacent MDCK cells in cell monolayers, and therefore prevented the access of  $[^3\text{H}]$ -ouabain to basolateral cell surfaces (where Na,K-ATPase was thought to be located; see the introduction and section 1.IV.). If this explanation was correct then the model proposed by Cereijido *et al.* (1981a) for the formation of blisters in MDCK cell monolayers (strain II) grown on impermeable substrates would not apply to MDCK strain I cells.

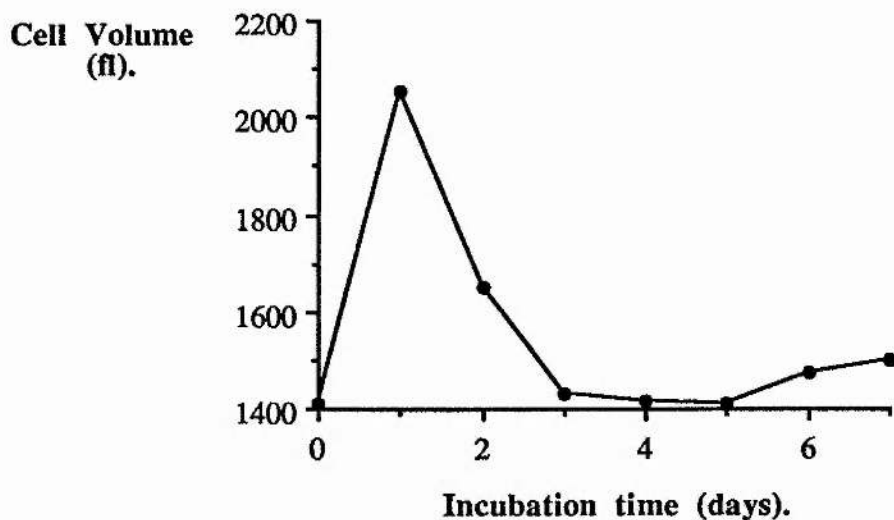


Figure 5. Shows the changes in cell volume of MDCK strain I cells during growth. The cells were seeded at high density ( $4.17 \times 10^4$  cell/cm<sup>2</sup>) and were grown under normal culturing conditions (0.57 ml culture medium/cm<sup>2</sup>) in plastic culture flasks.

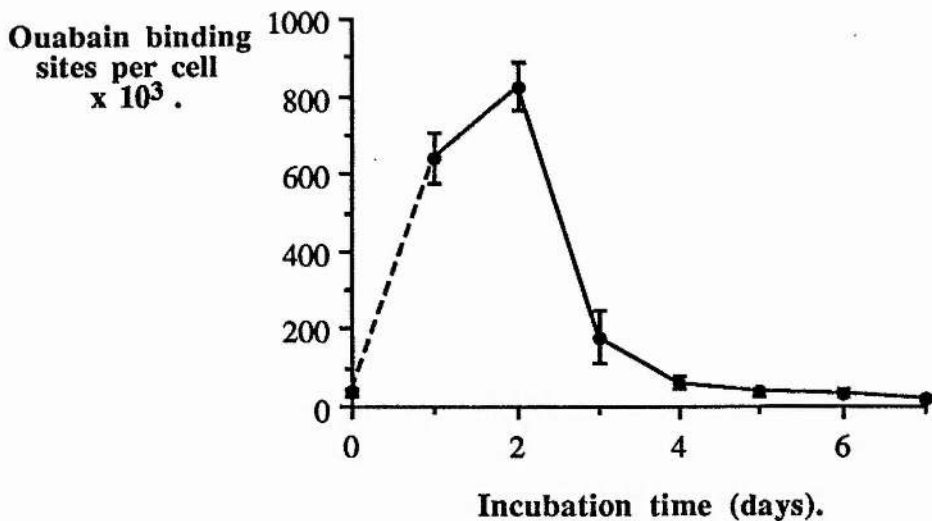


Figure 6. Shows the number of specific ouabain binding sites present on the surface of MDCK strain I cells during growth after seeding at high density. Specific ouabain binding sites were determined as the difference between the amount of ouabain bound in the presence and absence of 15 mM K ions. The error bars represent standard deviations where  $n = 6$  plates.

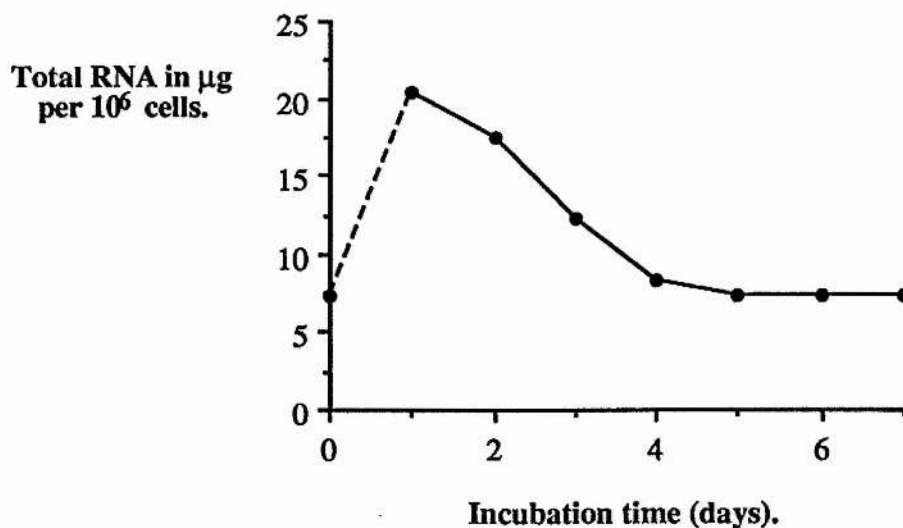
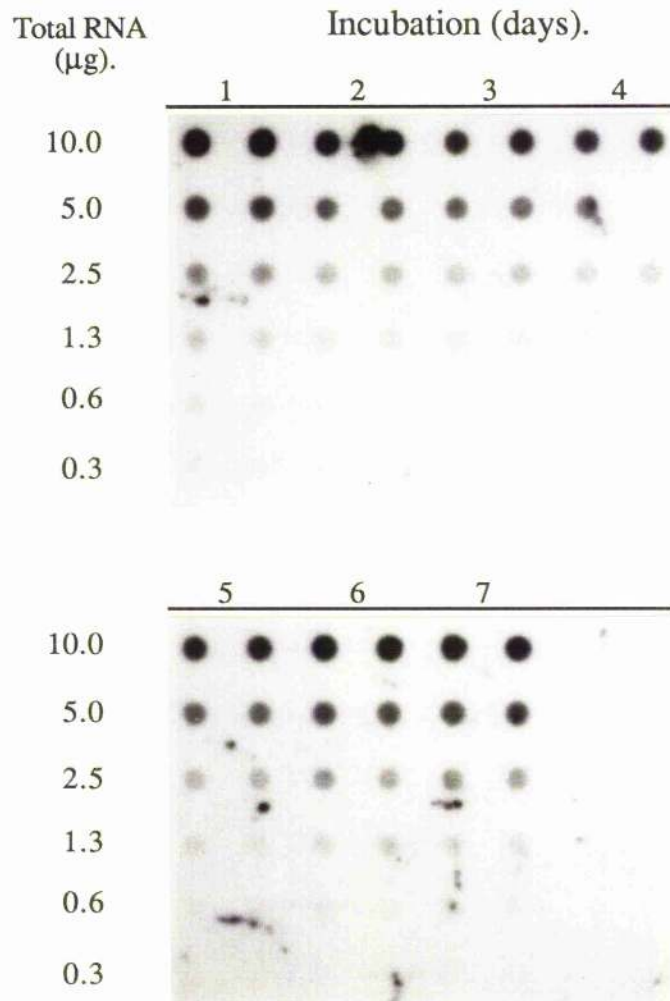


Figure 7. Shows the abundance of total RNA in  $\mu\text{g}$  per  $10^6$  MDCK strain I cells, grown in plastic culture flasks after seeding at high density.

Figure 8. A RNA dot blot of MDCK total RNA hybridised with the Na,K-ATPase  $\alpha$ 1 subunit pHANK probe. The total RNA was extracted from cells after a given number of days growth. Each concentration of total RNA was dotted in duplicate. The dot blot was hybridised at 42 °C (in 50% formamide) and finally washed in 1 x SSC, 0.1 % SDS at 55 °C for 30 minutes.





The RNA from cells grown in plastic roux's was extracted as described in the general methods (section 2.III.ii.b.). The amount of total RNA recovered was as illustrated in figure 7. The amount of total RNA extracted per cell increased by 3 fold after 24 hours in culture, this elevated level subsequently returned to the initial value after approximately 4 days. The increase in total RNA per cell took place during the lag phase and coincided with the transient increase in cell volume. The actual change in the concentration of the total RNA per fl of cell H<sub>2</sub>O was therefore not as dramatic as that suggested by the increase in the amount of total RNA per cell. Whether the increases in the abundance of total RNA and cell volume were related, remains undetermined. However, both increases are likely to be related to the importation and utilisation of nutrients acquired from the fresh growth medium. Amino acids, sugars and inorganic phosphate are all cotransported into the cell with Na ions (see section 1.II). Without an equivalent increase in Na,K-ATPase activity, Na ions will accumulate within the cell, increasing the osmolarity and attracting H<sub>2</sub>O into the cell, which causes an increase in cell volume. The likely increase in Na influx due to the uptake of nutrients is also likely to cause a compensatory increase in Na,K-ATPase enzyme units (and/or enzyme activity) during lag phase (Lechne, 1988; see section 1.V.II.a.), and this was also found to be the case (see figure 6).

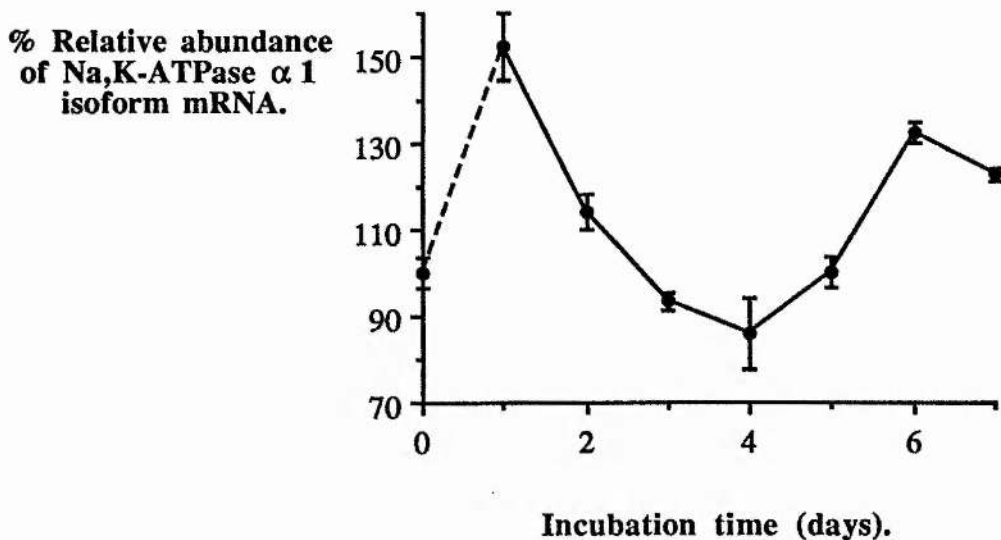


Figure 9. Shows the relative abundance of Na,K-ATPase  $\alpha$  subunit mRNA during the growth of MDCK strain I cells. The relative abundance (signal per 10  $\mu$ g RNA dot) was determined by comparison to a standard curve produced from the dots of serially diluted total RNA extracted from cells after 1 day (see figure 8). The abundances were then re-scaled relative to the level at time 0. The error bars represent  $\pm$  the % relative values obtained from 2 RNA dots (10  $\mu$ g RNA dots) per sample of total RNA

The total RNA extracted from the MDCK cells was then used for dot blotting and was hybridised with the pHANK cDNA probe for the  $\alpha 1$  isoform of Na,K-ATPase (see figure 8). The resulting autoradiograph of the MDCK total RNA dot blot was quantified by scanning densitometry (as outlined in section 2.III.ii.c.2.V.). Changes in the relative abundance of  $\alpha 1$  isoform mRNA were calculated (see figure 9). The relative abundance of (signal per 10  $\mu\text{g}$  RNA dot) Na,K-ATPase  $\alpha 1$  isoform mRNA appeared to have increased after 24 hours by  $\sim 50\%$ . The level then subsequently decreased to a low level after 4 days in culture, before returning to within 10% of the level after 6 days. The 50% increase in the abundance of  $\alpha 1$  isoform mRNA coupled with the 3-fold increase in the amount of total RNA per cell, showed that there was a large increase in the abundance of the mRNA for  $\alpha 1$  isoform per cell, within 1 day of the passaging of the cells. This was correlated to the increase in the number of Na,K-ATPase units ( $[^3\text{H}]$ -ouabain binding sites) from that at time 0 (equivalent to that at day 5),  $3.8 \times 10^4$  sites per cell, to  $6.4 \times 10^5$  sites after 1 day and  $8.2 \times 10^5$  sites after 2 days. However, caution must be observed when relating the increase in  $\alpha$  isoform mRNA abundance and Na,K-ATPase units as, the abundance of the  $\alpha 1$  isoform mRNA also increased between 4 and 6 days of growth, with no apparent simultaneous increase in the number of Na,K-ATPase units.

#### 6.III.iv. Specific ouabain sites in the presence or absence of Ca ions.

In order to determine whether the model of Cereijido *et al.* (1981a) for the formation of blisters in MDCK strain II cell monolayers (which when grown on impermeable substrates have no restriction of access to the basolateral side of monolayers) also extended to MDCK strain I cells, the restriction of access of ouabain to the basolateral side of the cell monolayer was assessed. A  $[^3\text{H}]$ -ouabain binding assay was performed on MDCK strain I cell monolayers in the presence or absence of calcium ions. Calcium is reported to be required for the formation of tight or occluding junctions, which have been postulated to 1, maintain the polarisation of Na,K-ATPase (to the basolateral surface) and 2, to seal access to the lateral surfaces off from the apical side of the cell monolayer, conferring a high level of electrical resistance to cell monolayers grown on permeable filters (Cereijido *et al.*, 1981b; Martinez-Palomo *et al.*, 1980). The cells used to determine the number of Na,K-ATPase enzyme units ( $[^3\text{H}]$ -ouabain binding assay) were grown on plastic plates (4 days) and were then pre-incubated in Krebs buffer with or without 2.8 mM  $\text{CaCl}_2$  for 20 minutes prior to the assay. Removal of extracellular calcium by

incubation in Ca free medium (even in the absence of EDTA) has been reported to dissociate tight junctions within 20 minutes of incubation (Gumbiner and Simons, 1986). The results (see figure 10) showed that even at a lower cell density, ( $0.2 \times 10^6$  cells per  $\text{cm}^2$ , equivalent to 2.5 days growth in the previous experiment; see figure 4), the cells had only a maximum (Ca free) of  $3.2 \times 10^5$  Na,K-ATPase units per cell. The difference between the Ca plus and Ca free [ $^3\text{H}$ ]-ouabain binding experiments was only  $0.6 \times 10^5$  enzyme units, and this difference was not significant (as assessed by Students t-test). The pre-incubation in calcium free medium had failed to detect any significant increase in the number of Na,K-ATPase units, over the values in normal calcium Krebs. These results demonstrated that there was no restriction of access to the basolateral surfaces of MDCK strain I cells grown on plastic supports. This result was entirely consistent with the model and results reported by Cereijido *et al.* (1981a) for blister formation and the accessibility of basolateral membranes in MDCK strain II cell monolayers.

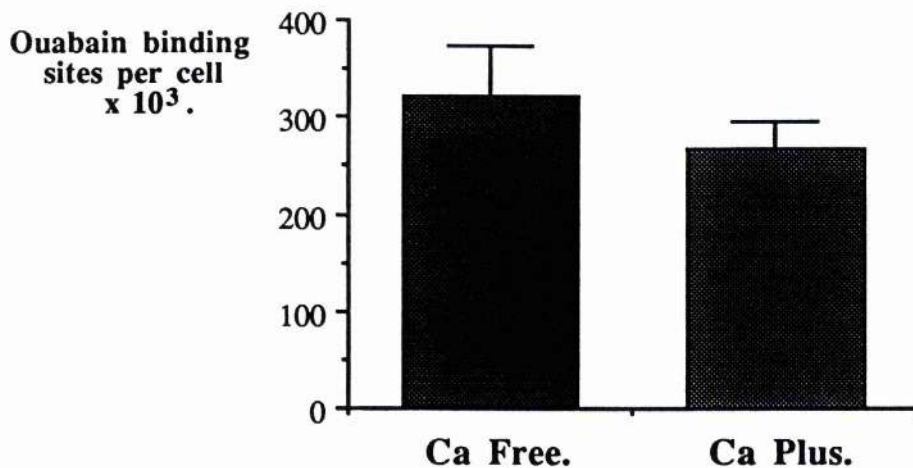


Figure 10. The level of specific ouabain binding on MDCK strain I cells grown on plastic culture plates, and incubated in Ca-free (■) or normal Krebs buffer (containing 2.8 mM  $\text{CaCl}_2$ ; Ca plus (▣)). The error bars represent standard deviations where  $n = 3$  plates.



#### 6.III.v. The specific ouabain binding sites of MDCK cells grown on Millipore HA filters.

The effect of growth of MDCK cells on permeable Millipore filters, on the expression of Na,K-ATPase units at the cell surface was assessed. The cells were grown under identical conditions to the experiments on plastic culture plates. Cells were seeded at high density ( $4.2 \times 10^4$  cells/  $\text{cm}^2$  of growth area), and were grown for 4 days in culture. Experiments were performed to determine whether a larger number of Na,K-ATPase units were accessed by the addition of [ $^3\text{H}$ ]-ouabain to the basal surface of the cell monolayer during [ $^3\text{H}$ ]-ouabain binding assays.

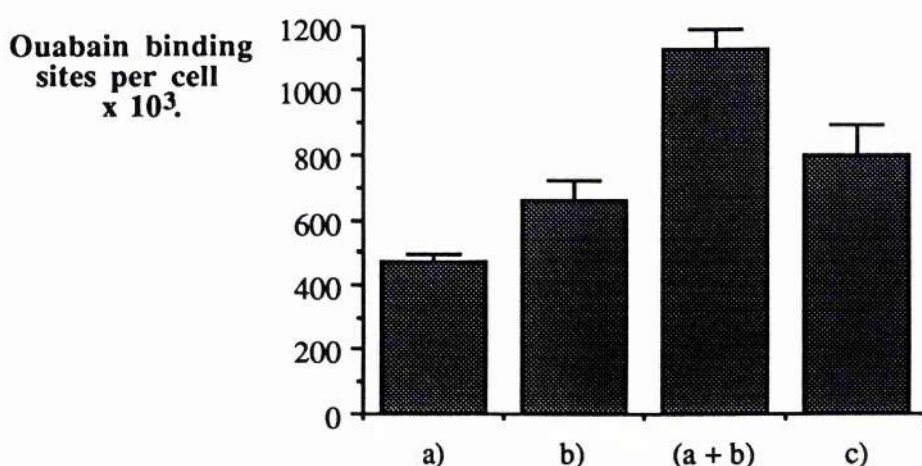


Figure 11. The amount of [ $^3\text{H}$ ]-ouabain binding (number of Na,K-ATPase units) to MDCK strain I cells grown on Millipore filter inserts, when radiolabel is added from only a) the apical, or b) the basal side of the cell monolayer. The sum of these apical and basal components (a+b) is compared to c) the number of units found when [ $^3\text{H}$ ]-ouabain is added to both sides of the monolayer. The error bars represent standard deviations where  $n = 3$  plates, except for the apical estimation where  $n = 2$  plates.

The number of Na,K-ATPase units accessible from the apical side of the cell monolayer was assessed by adding [ $^3\text{H}$ ]-ouabain to only the inside of the culture plate insert. Similarly, the number ouabain binding sites accessible from the basal side of the cell monolayer was assessed by adding [ $^3\text{H}$ ]-ouabain to the wells of the multiwell plate containing the inserts. The total number of Na,K-ATPase units was assessed by adding [ $^3\text{H}$ ]-ouabain to both sides of the cell monolayer. The non-specific binding of ouabain was assessed as usual, in the presence of 15 mM K. The number of Na,K-ATPase units found on the apical, basal and apical plus basal membrane domains, are shown in figure 11. The number of Na,K-ATPase units determined by [ $^3\text{H}$ ]-ouabain binding to the apical

or basal surfaces separately, when added together, was larger than the total number of enzyme units (where apical and basal [ $^3\text{H}$ ]-ouabain binding sites were determined simultaneously). This indicated that a proportion of the ouabain had penetrated the cell monolayer to bind sites on the other membrane domain. However this proportion was not large enough to bind all the Na,K-ATPase enzyme units available. The cell density (mean  $> 0.19 \times 10^6$  cells/cm $^2$ ; an estimate as not all the cells were removed from the filters by trypsinisation) was such that confluence of the cell culture had been achieved (cells reach confluence at  $\sim 0.1 \times 10^6$  cells/cm $^2$ : Kennedy and Lever, 1984). This suggested that after 4 days of culture at least, the presence of tight junctions was not totally occluding the diffusion of ouabain through the cell monolayer. This was contrary to reported results, as on permeable substrates tight junctions were not dissociated, restricting access to the basal surface from the apical side of the monolayer (Cereijido *et al.*, 1981a). This may have been due to the cells not having reached maximum cell density (normally  $0.45\text{-}0.5 \times 10^6$  cells/cm $^2$ ). As not all the Na,K-ATPase units could be accessed from the apical side of the monolayer, future experiments using filters would require [ $^3\text{H}$ ]-ouabain binding to both sides of the monolayer if the total number of Na,K-ATPase units was to be assessed correctly. The total number of Na,K-ATPase units was also much greater ( $1.05 \times 10^6$  enzyme units per cell) than the numbers previously determined (after 4 days) from growth on plastic substrates ( $6.2 \times 10^4$  enzyme units per cell). This showed that the cell surface expression of Na,K-ATPase units was dependent on the type of growth substrate used.

Differences in the cell surface expression of Na,K-ATPase units on MDCK cells grown on different substrates were then investigated further. The level of [ $^3\text{H}$ ]-ouabain binding to cells, during growth on Millipore filters was determined. The number of Na,K-ATPase units that were present (see figure 12) on cells grown on Millipore filters showed a qualitatively similar trend to those grown on plastic plates. The number of Na,K-ATPase units per cell again showed increases between days 1 and 2 before falling to much lower levels at days 3-7. However, the number of enzyme units was on average  $5.5 \times 10^5$  sites per cell higher than when the MDCK cells were grown on plastic plates. This suggested that, either MDCK cells grown on plastic had far fewer Na,K-ATPase units, or that the enzyme units located on the basolateral membranes of these cells were occluded. The latter of these conclusions is unlikely as there was no significant increase in enzyme units under Ca-free conditions. The results indicated that the composition of the substrate could have a profound effect on the level of cell surface expression of



Na,K-ATPase units on these cells, although the profile of enzyme abundance during growth was similar on both substrates.

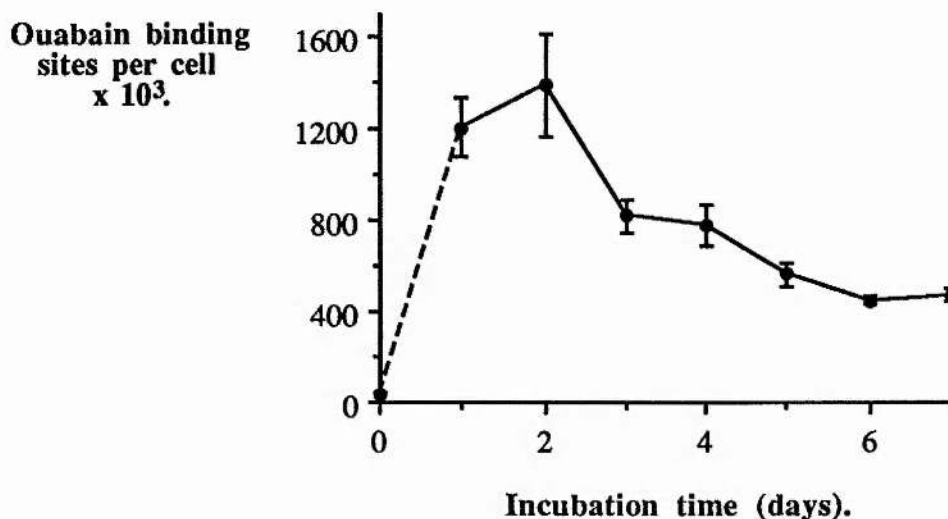


Figure 12. Shows the abundance of specific ouabain binding sites present on the surface of MDCK strain I cells during growth on Millipore filters. Specific ouabain binding sites (Na,K-ATPase units) were determined as the difference between the amount of ouabain bound to cells both apically and basally in the presence or absence of potassium ions. The error bars represent standard deviations where  $n = 3$  plates.

#### 6.III.vi. A comparison of the specific ouabain binding sites of MDCK cells grown on Millipore HA filters and collagen culture plate inserts.

As both plastic and Millipore, mixed cellulose ester filters were both unnatural growth substrates for MDCK cells, experiments were also conducted on a more natural, collagen substrate. In order to have some kind of reference to the previous experiments, a direct comparison was made between the growth of cells on ICN collagen inserts and the Millipore filters used previously. Unfortunately, the growth conditions could not be reproduced exactly as the collagen inserts required a greater volume of both medium (during growth) and radioactive ouabain solution (for the ouabain binding assay). This was because the height of collagen inserts was raised by 2 mm stilts, to prevent collagen membranes from adhering to the base of multiwell dishes. An increased volume of growth medium (0.83 ml of medium/  $\text{cm}^2$  of growth area; see section III.i.a.), was used to completely cover the collagen membranes. To make the comparison with Millipore

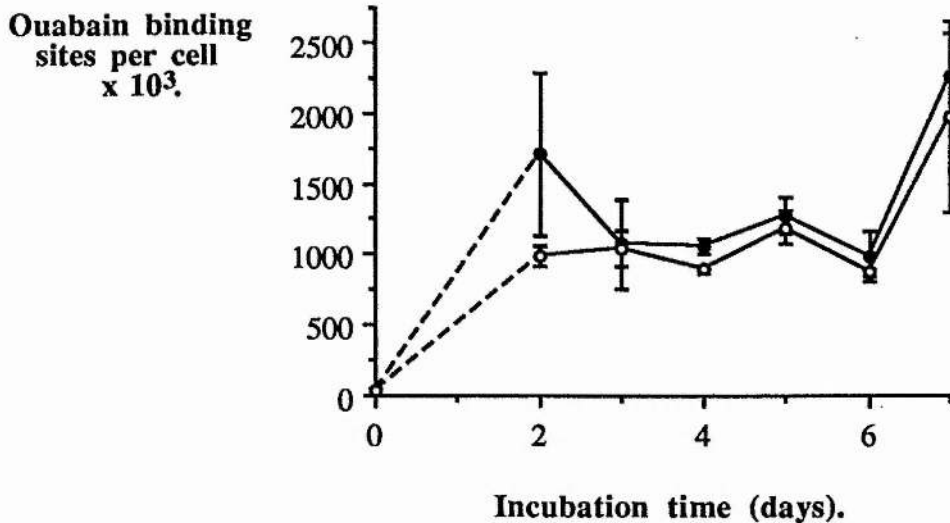


Figure 13. The number of specific ouabain binding sites (Na,K-ATPase units) on MDCK strain I cells during the growth on either Millipore HA (●) or Type 1 collagen culture plate inserts (○). The error bars represent standard deviations where  $n = 3$  inserts.

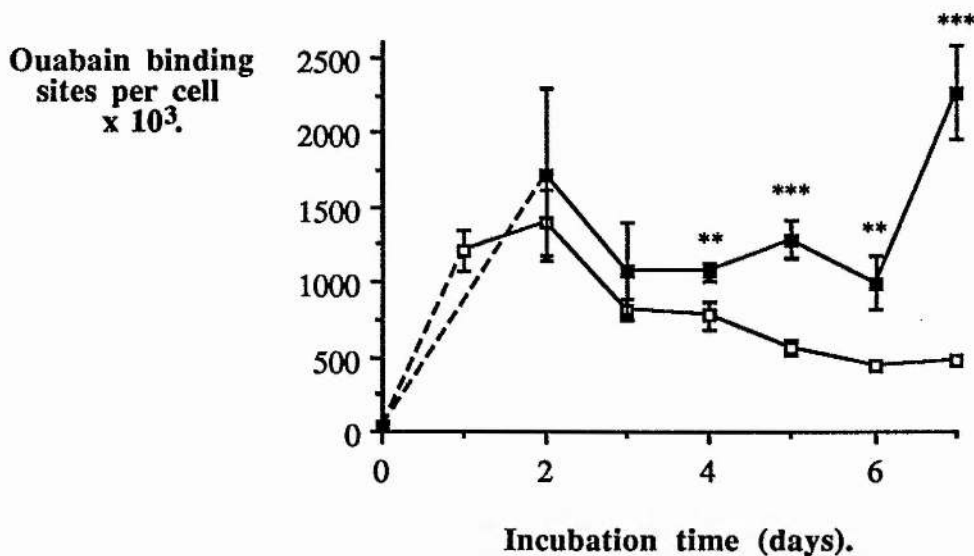


Figure 14. A comparison between the number of specific ouabain binding sites per cell, of cells grown in varying volumes of growth medium. MDCK strain I cells were grown on Millipore HA culture plate inserts containing either 0.83 (■) or 0.57 (□) ml of growth medium per  $\text{cm}^2$  (see Chapter 2). Error bars represent standard deviations where  $n = 3$  filters. Statistical significance was tested by the use of Student's t-test, where \*\* = highly significant at the 1% level and \*\*\* = very highly significant at the 0.1% level. The results at each time point were assumed to be normally distributed.

filters similar, MDCK cells grown on this substrate were also grown with an identically increased volume of growth medium, and both types of filter were seeded as on plastic, at  $4.2 \times 10^4$  cells/cm<sup>2</sup>. The number of Na,K-ATPase units found on MDCK cells during growth was very similar on either Millipore or collagen filters (see figure 13). The numbers of Na,K-ATPase units was on average  $10^5$  greater on cells grown on Millipore rather than collagen filters between days 3-6 of growth, although this difference was too small to be statistically significant. However, there was a considerable difference between the number of enzyme units on cells grown on Millipore filters in this experiment (figure 13) and those found in the previous experiment (figure 12). The number of Na,K-ATPase units appeared to drop between days 2-3, but remained fairly constant thereafter (a mean level of  $1.1 \times 10^6$  units per cell), and did not decline, as found in earlier experiments (see figure 14). The number of Na,K-ATPase units between days 4-7 in the latter experiment was significantly higher, with the number of units between days 2-6 increased by on average  $4.3 \times 10^5$  sites per cell. As the number of sites on the collagen filters (also with larger volumes of medium) was also significantly higher than the experiment with cells grown in lower medium volumes on Millipore filters (see figure 13), the difference in the number of enzyme units was likely to be due to the increased volume of growth medium used.

#### 6.III.vii. The effects of seeding density on the number of ouabain binding sites on MDCK cells.

To assess the effect of seeding density on the number of ouabain binding sites, the cells were grown on collagen after initial seeding at high ( $4.2 \times 10^4$  cells/cm<sup>2</sup>) and low ( $4.9 \times 10^3$  cells/cm<sup>2</sup>) densities. The cells were grown in the presence of  $0.83$  ml/cm<sup>2</sup> and were assayed for ouabain binding as before. The experiment was performed twice at each density, and together with the results of the previous experiment on collagen inserts (see figure 13), the results were as illustrated in figure 15. The number of Na,K-ATPase units at time 0 should have been equivalent to the number of units present on cells grown on plastic after 5 days (approximately  $3.8 \times 10^4$  enzyme units per cell), as these cells were equivalent to the cells used to seed collagen inserts in this experiment. If indeed this was the case, the number of Na,K-ATPase units expressed had increased between time 0 and day 1, 52 and 42-fold on average in low and high density seeded cells respectively. In the next 24 hours, the number of Na,K-ATPase units continued to increase in the low density seeded cultures reaching a peak of  $5.4 \times 10^6$  sites per cell on average (a further

2.8 fold increase) after 2 days. Thereafter, the number of enzyme units declined gradually, eventually (days 6 to 7) reaching a similar level to that of high density seeded cultures. The number of enzyme units in high density seeded cultures however, decreased slightly from the peak at day 1 ( $1.6 \times 10^6$  sites per cell), reaching a low of  $0.9 \times 10^6$  units per cell by day 5. The number of enzyme units was at minimum level of around  $0.9$  to  $1 \times 10^6$  units per cell when cultures were at maximum cell density (after 4-5 days in high, and 6-7 days in low density seeded cultures). The number of Na,K-ATPase units was significantly greater in low compared to high density seeded cultures from day 2 until day 4. The difference between cultures was maximal after 2 days of culture, when on average low density seeded cultures had 3.9 fold greater numbers of enzyme units than high density cultures.

When cells are seeded in culture they are initially single cells, in time, cell-cell contacts are established between neighbouring cells, before cell growth and division produces enough cells to form a confluent cell monolayer. During the period when cells were subconfluent (below  $0.1 \times 10^6$  cells per  $\text{cm}^2$ ) and consequently had the lowest

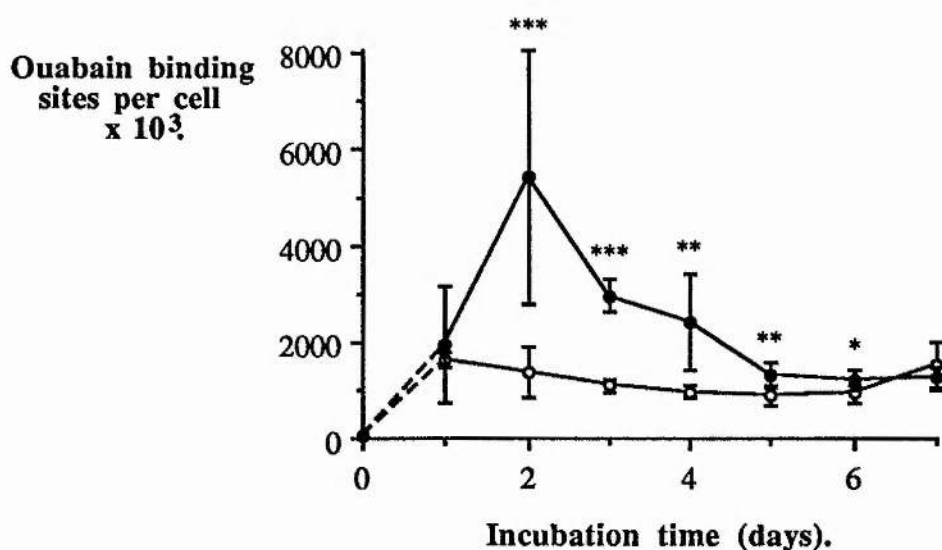


Figure 15. Specific ouabain binding (Na,K-ATPase units) during the growth of MDCK strain I cells on collagen inserts after seeding at high (○) or low (●) density. The error bars represent standard deviations where  $n = 9$  (3 experiments) for high and  $n = 6$  (2 experiments) for low density seeded cells, with the exception that  $n = 6$  (2 experiments) for the day 1 time point of high density seeded cells. Statistical significance was tested by the use of Student's t-test, where \* = significant at the 5% level; \*\* = highly significant at the 1% level; and \*\*\* = very highly significant at the 0.1% level. The results at each time point were assumed to be normally distributed.

levels of cell-cell contacts, the number of enzyme units increased rapidly (40 - 50 fold within 24 hours in both cultures). The main difference between high and low density seeded cultures is the length of time taken for cultures to establish cell-cell contacts, and achieve a confluent monolayer (see figure 1 to 3). The length of time for low density cultures to establish cell-cell contacts is obviously likely to be longer than in high density cultures. This correlates with the extended period of increase in enzyme units in low density compared to high density seeded cultures (day 1 to 2). If this correlation is correct then it suggests that cell-cell contact may be inhibiting the number of enzyme units expressed on the cell surface. An alteration in the number of enzyme units implies that there are changes in either the rate of synthesis of new Na,K-ATPase units and/or the rate of insertion or removal of new enzyme units into or from the plasma membrane. Nelson and Hammerton (1989), have demonstrated that Na,K-ATPase in MDCK cells is associated with elements of the cytoskeleton (ankyrin and foderin; see section 1.VI.). As this association into high molecular weight complexes appears to correspond with the insolubility Na,K-ATPase, Nelson and Hammerton (1989) have suggested that cell cultures without cell-cell contact have considerably less (>30%) Na,K-ATPase enzyme associated with the cytoskeleton. This observation, together with the results of the seeding experiments suggests that a possible explanation for the decrease in Na,K-ATPase units after cell-cell contacts are established, is that enzyme units are removed from the membrane due to their association with the cytoskeleton.

#### 6.III.viii. The effect of seeding density on the abundance of Na,K-ATPase $\alpha$ subunit mRNA in MDCK cells.

In order to study the effects of seeding density on the abundance of total RNA and Na,K-ATPase  $\alpha$ 1 isoform specific mRNA in MDCK cells, cells were grown only in plastic culture flasks. Unfortunately due to cost considerations, cells could not be grown on Millipore filters or collagen culture plate inserts. To obtain sufficient quantities of total RNA, extraction of  $10^7$  cells or greater was required. In order to achieve this number after one day of growth in a low density seeded culture, twelve 175 cm<sup>2</sup> culture flasks were required. This was assuming 100% viability of the MDCK cells used for subculturing. The viability of the MDCK cells in experiments involving RNA extraction proved to be highly variable, especially for low density seeded cells. The experiment on the effect of seeding density on the abundance of Na,K-ATPase  $\alpha$  subunit mRNA, was



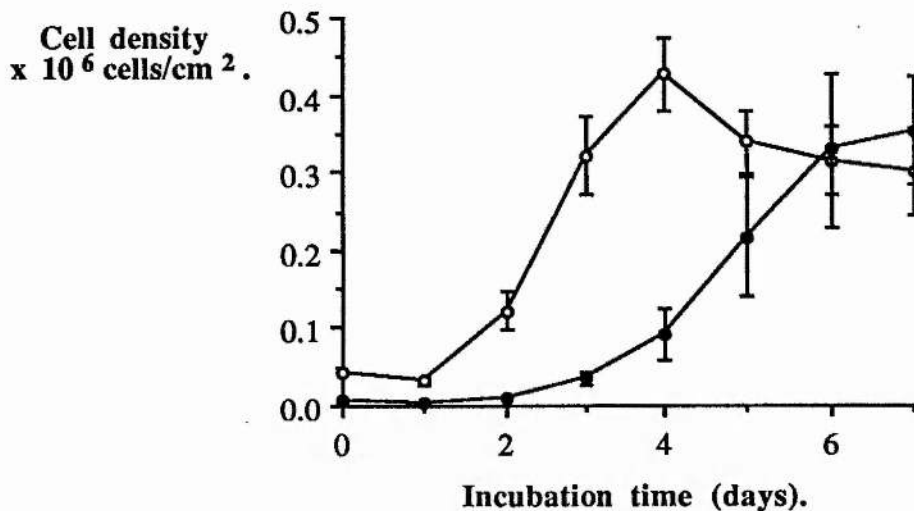


Figure 16. Shows the density of MDCK strain I cells during growth after seeding at high (○) or low (●) density. The cells were grown in plastic culture flasks with 0.57 ml of medium per cm<sup>2</sup> of growth area. The error bars represent standard deviations where  $n = 5$  experiments for high density and  $n = 3$  experiments for low density seeded cultures.

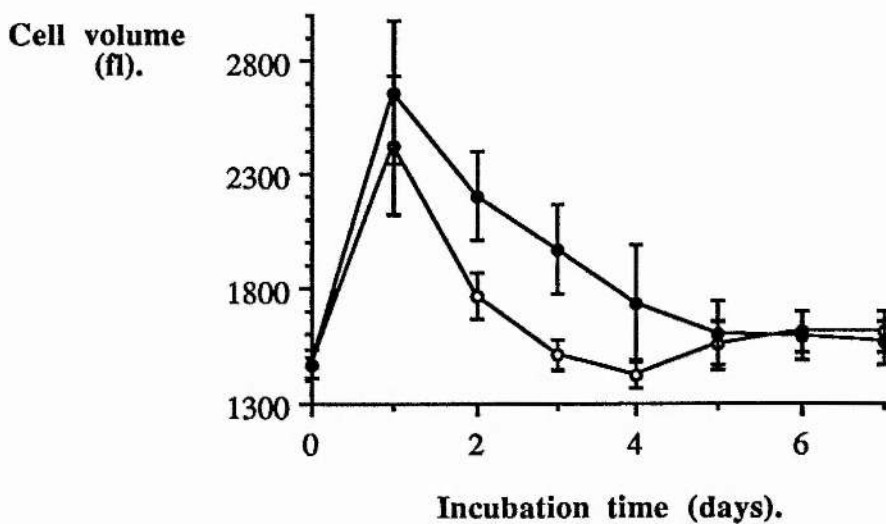


Figure 17. Shows the changes in cell volume of high (○) and low (●) density seeded cultures of MDCK strain I cells. The error bars represent standard deviations where  $n = 5$  experiments for high density and  $n = 3$  experiments for low density seeded cultures.

repeated 3 times. The rate of growth of cells in high and low density seeded cultures was as illustrated in figure 16. The high density seeded cells reached confluence after 4 days and the low density seeded cells after 6-7 days. The rate of growth of the high density cultures was similar to the experiments reported previously. The cell volumes of the two cultures were as illustrated in figure 17. This shows that the cell volumes of both high and low density seeded cultures had increased considerably 1 day after seeding. The volume of individual cells in low density cultures, was slower to reduce to its original value. The increase in total RNA found previously with high density seeded cultures (see figure 7), was also found in the high and low density seeded cultures (figure 18). In both cultures, the amount of total RNA per cell had approximately doubled after 1 day.

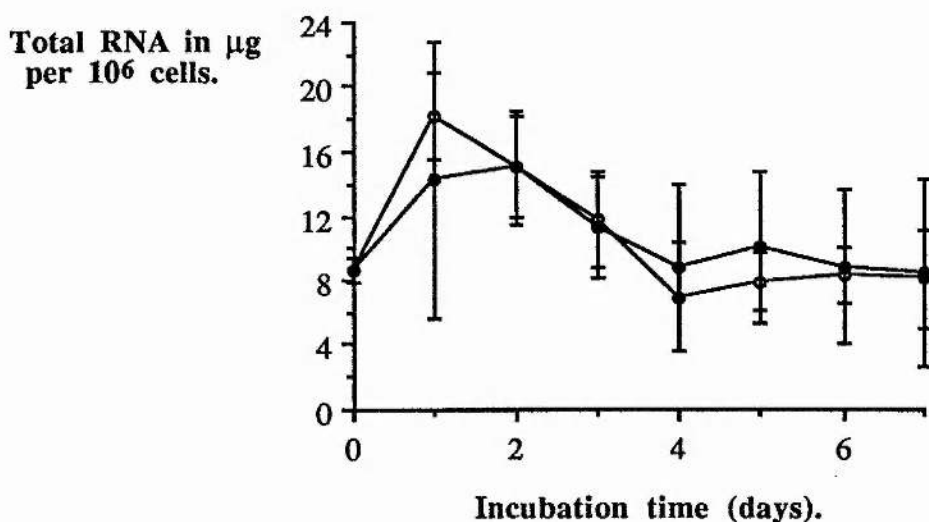


Figure 18. Shows the changes in the amount of total RNA per cell, during the growth of MDCK strain I cells seeded at high (○) and low (●) density. The error bars represent standard deviations where  $n = 3$  experiments.

Thereafter, the total RNA level reduced, returning to original levels by day 4. In one of the experiments, extra culture flasks were used to investigate the effect of re-newing the medium during the growth of low density cultures. The medium in these culture flasks was changed after either 3 or 5 days of growth. The effect of changing the medium was similar at both 3 or 5 days (see figures 19 to 21). The cells initially did not divide as rapidly as the control cells (figure 19). The cell volumes, instead of decreasing 1 day after the medium was changed, showed an increase (figure 20). The level of total RNA per

cells 1 day after the medium was changed, also increased by 50-65 % over the control cells (low density cultures without medium renewal; see figure 21). This indicated that at

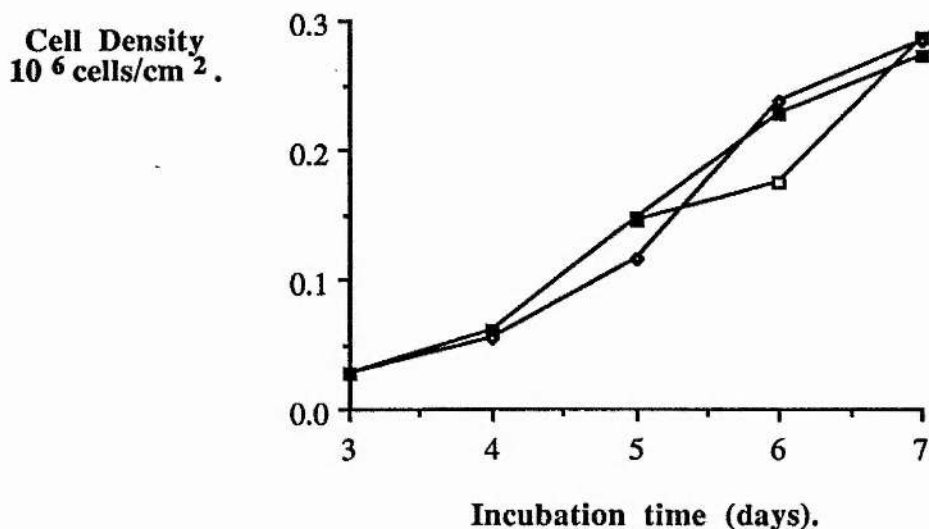


Figure 19. The alterations in the cell density of low density seeded MDCK strain I cell cultures (■), and similarly seeded cultures in which the growth medium was renewed after 3 days (◇) or 5 days (□) incubation.

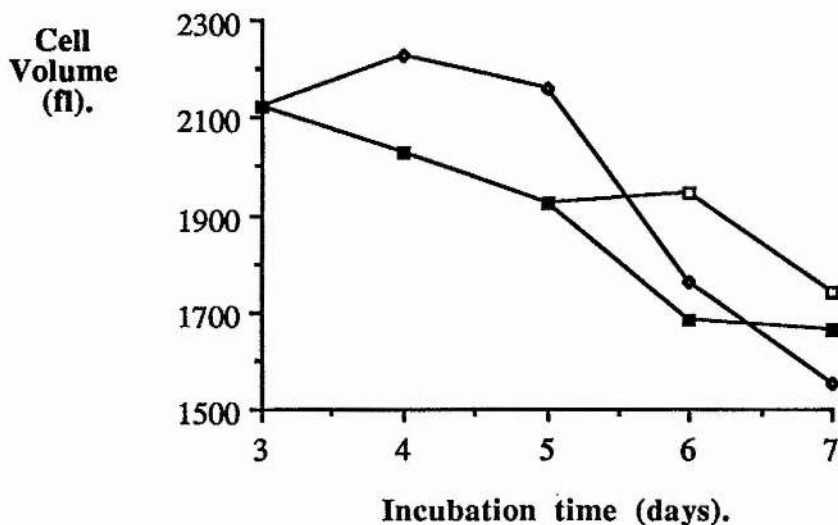


Figure 20. Alterations in the volume of cells in low density seeded MDCK strain I cell cultures (■), and similarly seeded cultures in which the growth medium was renewed after 3 days (◇) or 5 days (□) incubation.

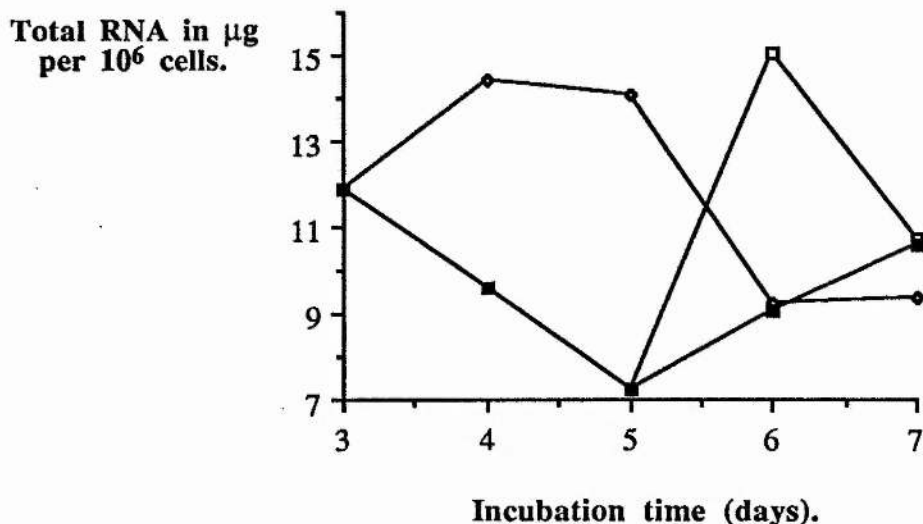


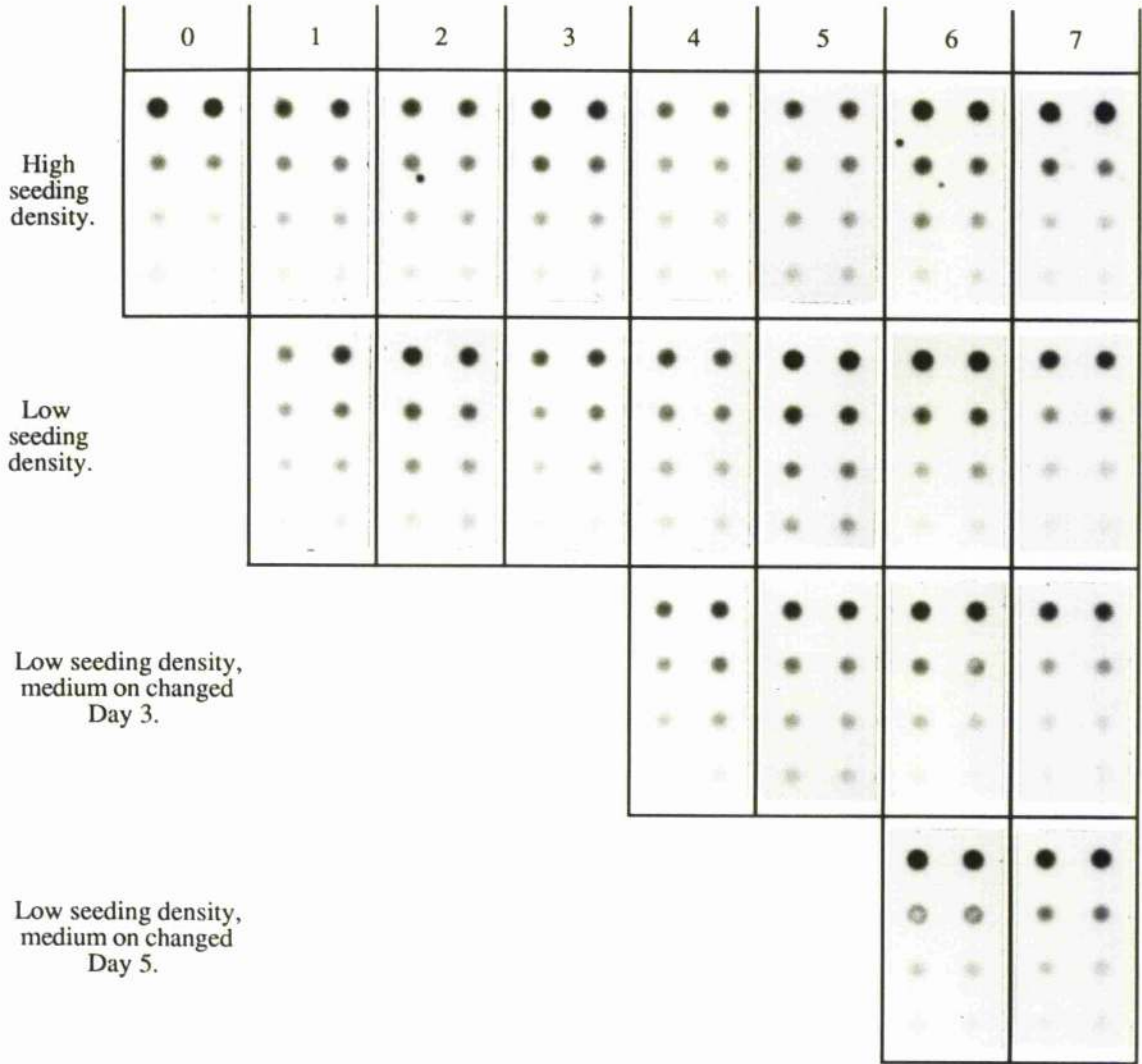
Figure 21 Alterations in the amount of total RNA per cell in low density seeded MDCK strain I cell cultures (■), and similarly seeded cultures in which the growth medium was renewed after 3 days (◇) or 5 days (□) incubation.

least part of the increase in the cell volume and the amount of total RNA in cultures when they were seeded initially was due, to the replenishment of nutrients, and other factors in the medium. The total RNA extracted from cells was then dot blotted, and hybridised with the pHANK and actin cDNA probes (figures 22 and 23 respectively). There was a large variation both within and between experiments measuring the abundance of specific mRNA's. This may have been due to variability in both the viability of cells and the length of time cultures spent in lag phase, as well as experimental error. However, the results showed (figures 24 and 25) that on average, there was no significant change in specific mRNA's in the cells during the growth, following seeding at low or high densities. The relative abundance of Na,K-ATPase  $\alpha$ 1 isoform mRNA increased on average by a maximum of 55% during growth compared to the abundance at the onset of the experiment (time 0). The abundance of the  $\alpha$  isoform mRNA was generally higher in low density seeded cells (except for days 6 and 7), but this was within the error of the experiments. The variation in the abundance of the  $\alpha$ 1 isoform mRNA during growth can be further seen as the mean 55% increase found in these experiments after 1 day of culture in low but not high density seeded cells was also previously found in high density seeded cells (see figure 9.). The small changes in the  $\alpha$ 1 isoform mRNA were also not

Figure 22. Shows a RNA dot blot of MDCK total RNA probed with the  $\alpha 1$  subunit pHANK DNA probe. Total RNA was extracted cells grown at high or low seeding density with changed or unchanged growth medium, at various intervals during growth. RNA was serially diluted from 3  $\mu\text{g}$  per dot (top dot), and dotted in duplicate pairs. The blot was hybridised at 42 °C (in 50% formamide) and finally washed in 0.1 x SSC, 0.1% SDS at 55°C for 5 minutes. Standard dots containing known amounts of radioactivity are also illustrated.



Incubation (days).



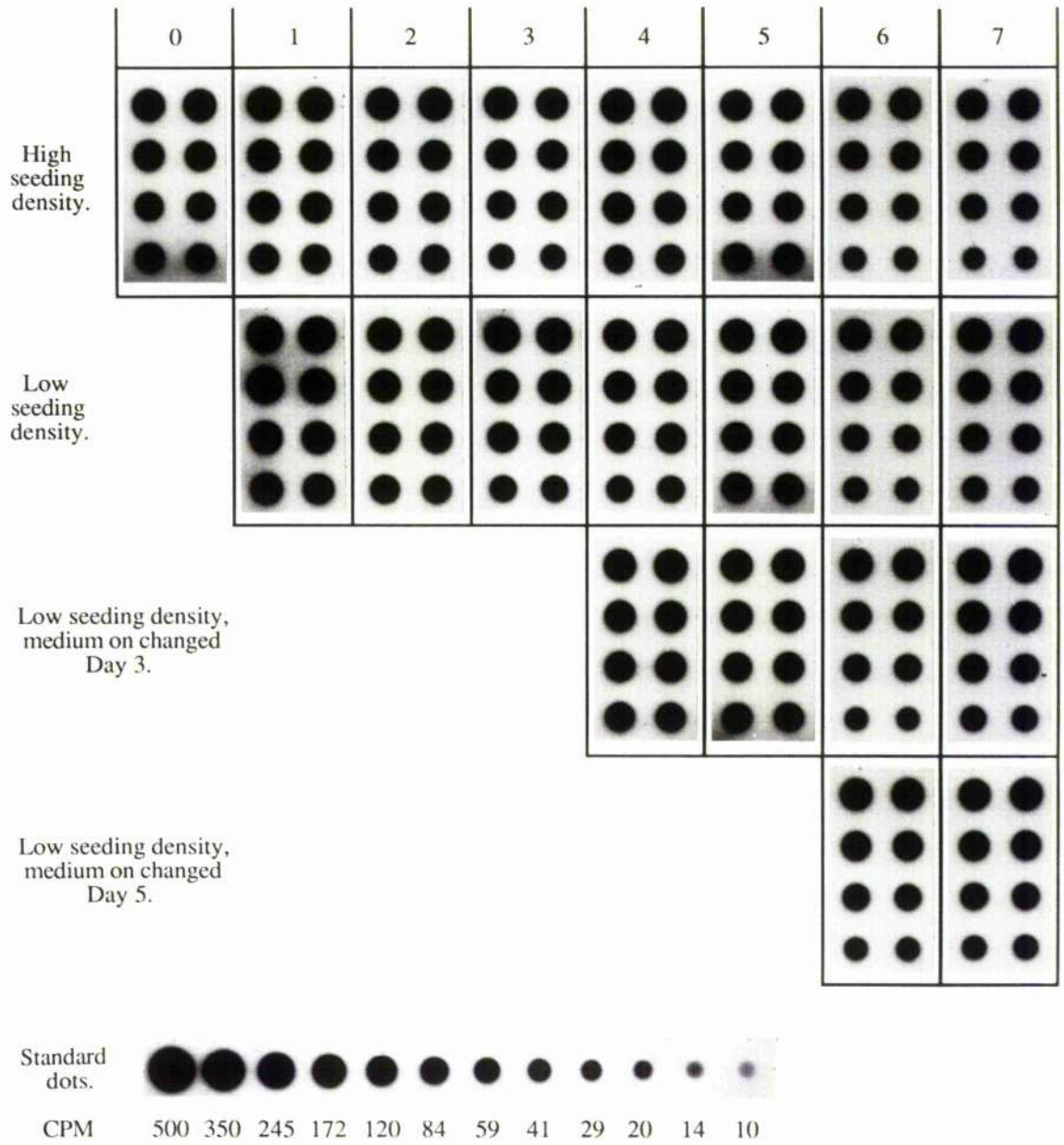
Standard dots.



CPM 500 350 245 172 120 84 59 41 29 20 14 10

Figure 23. Shows a RNA dot blot of MDCK total RNA probed with the mouse  $\alpha$  actin cDNA probe. Other details are as described in the legend for figure 22.

Incubation (days).



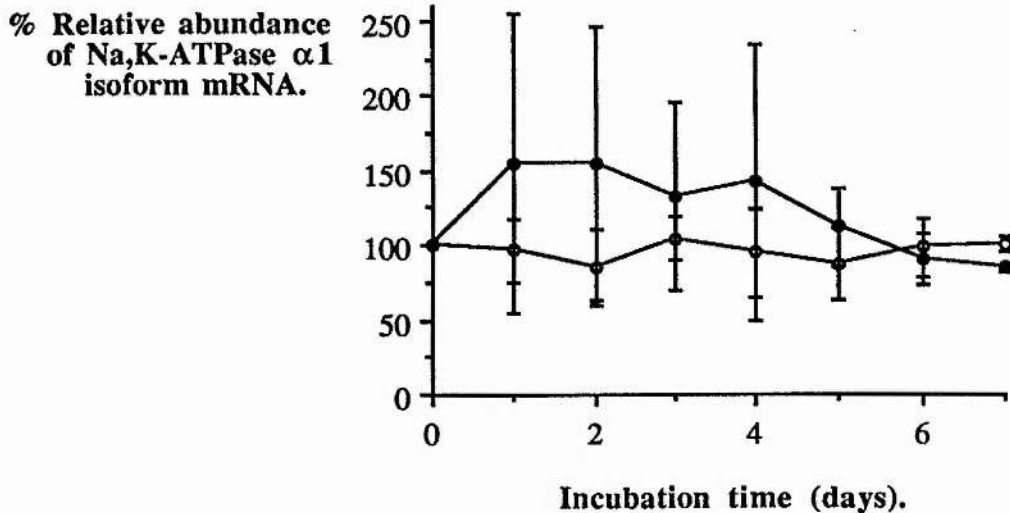


Figure 24. Shows the relative abundance of Na,K-ATPase  $\alpha$  subunit mRNA per  $\mu$ g of total RNA, in high (○) and low (●) density seeded MDCK strain I cells during growth. The results were expressed relative to the abundance of this mRNA in cells at time 0. Error bars represent standard deviations where  $n = 3$  experiments.

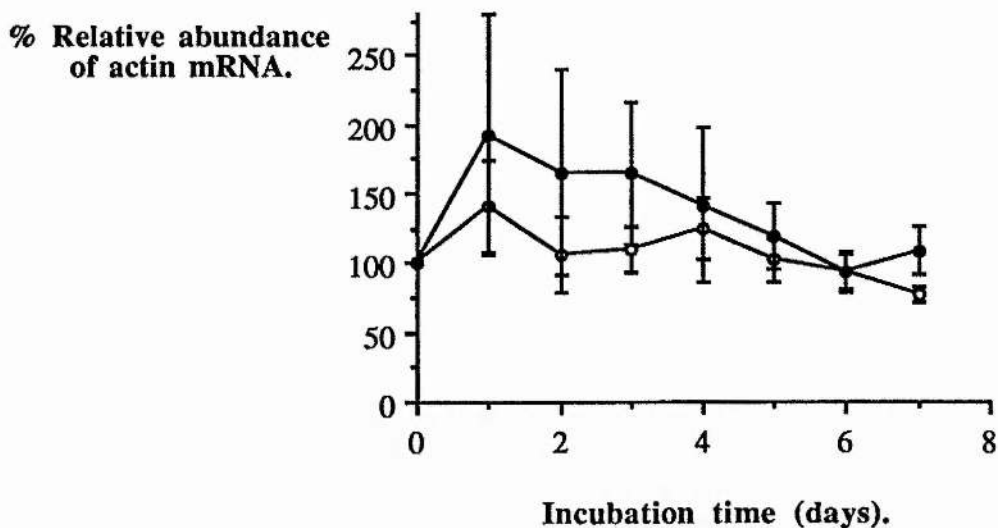


Figure 25. Shows the relative abundance of actin mRNA per  $\mu$ g of total RNA, in high (○) and low (●) density seeded MDCK strain I cells during growth. The results were expressed relative to the abundance of this mRNA in cells at time 0. Error bars represent standard deviations where  $n = 3$  experiments.

specific, as the relative abundance of the mRNA for actin showed a similar trend, but with a maximum increase in abundance of as much as 93% of that at the onset of the experiment (time 0). These results suggested that although a small increase in the abundance of  $\alpha 1$  isoform mRNA in low density compared to high density cells may have existed, this was not significant and probably did not account for the 3.9 fold increase in the number of Na,K-ATPase units found between the two experimental conditions. As stated previously, the amount of total RNA per cell was not significantly different between low and high density seeded cultures. This suggested that the effect of different seeding densities on the control of the cell surface expression of Na,K-ATPase in MDCK cells was mostly mediated during or after translation of polypeptides from the  $\alpha 1$  isoform mRNA.

Bowen and McDonough (1987) have shown that the levels of  $\alpha 1$  and  $\beta 1$  isoform mRNA's and the rate of polypeptide synthesis and abundance, are all increased in response to a low extracellular potassium concentration. However, the 3 fold increase in the abundance of mRNA was only transient and was at a maximum after only 1 hour. The mRNA abundance was reduced to only a 1.8 fold increase after 4 hours. The increase in enzyme abundance lagged considerably behind this transient increase in the amount of mRNA, with maximum expression having increased by 70% after 24 hours. If the response to differences in seeding density, involved a similarly transient increase in mRNA abundance after cell culture passaging, this would probably not have been detected by measuring the level of mRNA after 24 hours. However, the difference in the number of Na,K-ATPase units between low and high density seeded cells was much larger than the value reported by Bowen et al., and did not reach a maximum until the 48 hour time point (see figure 15). Furthermore, significant differences between low and high density seeded cells did not appear until the 48 hour time point. This suggested that if increased levels of mRNA were responsible for the increase in Na,K-ATPase expression in low density cells, then there must have been a considerable delay in the expression of enzyme at the cell surface. Otherwise, an increase in mRNA abundance would have been detected after 24 hours in low density seeded cultures. No such significant increase was found.



### 6.III.ix. Future experiments.

The results from the experiments in this chapter have demonstrated that the cell surface expression of Na,K-ATPase in MDCK cells is dependent on the type of growth substrate, the initial seeding density, and to a lesser extent the volume of culture medium used in the experiments. Future experiments could be carried out to determine whether the differences in Na,K-ATPase expression found in this study correlate with the level of total cellular enzyme activity, or whether the basal increases in the surface expression of the enzyme merely serve to increase the cells potential Na and K pumping capacity. Experiments could also be performed to investigate the initial increase in enzyme abundance within 24 hours after cell seeding. These experiments would be used to determine when the extra enzyme units, found 24 hours after cell seeding, are inserted into the plasma membrane. It could also be determined whether the increases in enzyme units are in response to transient increases in the abundance Na,K-ATPase mRNA. The role if any of the  $\beta$  subunit in the regulation of Na,K-ATPase expression of could also be investigated. Does the abundance of  $\beta$  subunit mRNA control the expression of the enzyme as has recently been proposed (Geering *et al.*, 1989; Noguchi *et al.*, 1990; Geering, 1990; McDonough *et al.*, 1990), and is the recently discovered  $\beta 2$  isoform of Na,K-ATPase expressed in MDCK cells. In the advent of cheaper collagen culture plate inserts or, collagen coated plastic ware, RNA blotting experiments on cells grown at different seeding densities could be performed. These experiments would show whether the lack of an increase in Na,K-ATPase mRNA abundance in low compared to high density seeded cells was due to them being grown on plastic rather than collagen substrates. Further studies on the role of the growth substrate in determining the cell surface expression of Na,K-ATPase could also be performed, as recently plastic ware coated with other natural substrates have become available. The substrates available include fibronectin, laminin and basement membrane (a mixture of collagen, laminin and other minor components; Collaborative Research Inc.). Interaction of the MDCK cells with these substrates may alter the expression of Na,K-ATPase. Finally, the effect of the growth substrate, the initial seeding density and the amount of medium used in experiments could also be investigated in other strains of MDCK cells. Cell lines such as Strain II MDCK cells, have been reported to have significantly higher levels of expression of Na,K-ATPase in subconfluent or confluent cell monolayers grown on plastic substrates (Richardson *et al.*, 1981).

CHAPTER 7. THE EFFECT OF HORMONES ON Na,K-ATPase ENZYME AND mRNA ABUNDANCE IN MDCK CELLS.

## **7.I. Introduction.**

The effect of various hormones such as mineralocorticoids, glucocorticoids and thyroid hormones on the Na,K-ATPase enzyme has already been well documented, and this was reviewed in Chapter 1. The effect of these hormones on Na,K-ATPase in MDCK cells has however not been studied extensively. The mineralocorticoids, aldosterone (Al) and deoxycorticosterone (Do) have been shown to stimulate transepithelial Na transport in MDCK cells (Simmons, 1977). The action of Al was found at concentrations of 200 nM or 1  $\mu$ M, after 4 hours of incubation. The action of Do was found only at high concentrations (such as 10  $\mu$ M, but not 100 nM) and over a longer timespan (60 hours). A separate steroid receptor type (type IV) has been postulated to exist in MDCK cells (Ludens *et al.*, 1978). This novel receptor is thought to be a low affinity receptor ( $K_d = 70$  nM) which is present in high concentrations in MDCK cells (25000 fmol/mg protein; Fanestil and Park, 1981) with binding affinities in the following order of potency, Do > Al > Corticosterone (Co) > Dexamethasone (De). The possibility of the regulation of MDCK cell Na,K-ATPase by these hormones was investigated.

## **7.II. Methods.**

The level of [ $^3$ H]-ouabain binding and Na,K-ATPase isoform mRNA abundances were investigated in MDCK cells. Cells were seeded at high density ( $4.2 \times 10^4$  cells/cm $^2$ ) on collagen culture plate inserts, using high medium volumes (0.83 ml/cm $^2$  of growth area). Cultures were grown for 3 days, to provide a sufficient number of cells for the extraction of total RNA. After 3 days growth (Time 0), various hormone treatments diluted in 100% ethanol were added to the cells (final ethanol concentration = 1%; see later). An identical quantity of ethanol alone was added to control cell cultures. The cells used in both the ouabain binding and RNA extraction experiments were grown for approximately 24 hours after the initiation of hormone treatment. They were then assayed for ouabain binding and extracted for total RNA as described in Chapter 2. Northern blots of total RNA were prehybridised and hybridised at relatively low stringency, at 33°C in 50% formamide. Blots were then washed in 5 x SSC, 0.1% SDS at 50°C and in 0.1 x SSC, 0.1% SDS at 30°C. The DNA probes used for hybridisation were the isoform specific DNA probes as described in Chapter 5. These were, the  $\alpha 1$  NcoI-AvaI,

the  $\alpha 2$  EcoO109, the  $\alpha 3$  SstII-SmaI, and the  $\beta 1$  PstI-SpeI, DNA restriction enzyme fragments respectively. After autoradiography, the blot was stripped of its radioactive probe for subsequent hybridisation with another probe. This probe was removed from the filter by boiling twice in 500 ml of 0.1 x SSC, 0.5% SDS for 20 minutes. Overnight autoradiographs confirmed the removal of the probe.

In the case of the cultures used for the ouabain binding assay, hormones were added at the following concentrations:- Al, De, Co, and Do, 300 nM; tri-iodo-thyronine (T3), 10 nM; spironolactone (Sp), and  $17\alpha$ -hydroxy progesterone (Hp), 300  $\mu$ M. The concentrations of Al, De, Co, and Do were decided upon, as this concentration of Al had been shown to increase the level of Na,K-ATPase enzyme and mRNA's in *Xenopus laevis* A6 kidney cells (Verrey J Cell Biol). Furthermore, this concentration should have been sufficient for each agonist to bind their own respective receptors types almost completely (Al, Type I,  $K_d = 0.5-3.7$  nM; De, Type II,  $K_d = 3-5$  nM; Co, Type III,  $K_d = 3$  nM and; Do, Type IV,  $K_d = 70$  nM; Fanestil and Park, 1981; Ludens *et al.*, 1978). The concentration of T3 was chosen as it had been suggested to represent the optimum for the stimulation of ouabain binding sites in chick embryo ventricular and rat neonatal myocardial cells (Kim and Smith, 1984; Orłowski and Lingrel, 1990). Sp and Hp were used as they are competitive antagonists of mineralocorticoid and glucocorticoid receptor binding respectively (Fanestil and Park, 1981; Norman and Litwack, 1987). The concentrations of Sp and Hp chosen for use, were the same as those reported by Verry *et al.* (1987). The concentration of Sp however was very high and near its solubility limit in ethanol (Reynolds, 1982). When the Sp was added to the aqueous growth medium, it formed a significant precipitate which could be seen on cell monolayers. This resulted in the death of most of the cells in Sp treated monolayers. For this reason, when the RNA extraction experiments were performed, the concentrations of Sp and HP were reduced to 300 nM. The concentrations and dilutions of the other hormones used for the RNA extraction experiments were as those used for the ouabain binding assays.

### **7.III. Results and Discussion.**

#### **7.III.i. The effects of hormones on cell density.**

The cell density of the MDCK cells from both the ouabain binding (15 mM K incubated cells only) and the RNA extraction experiments, after treatment with the



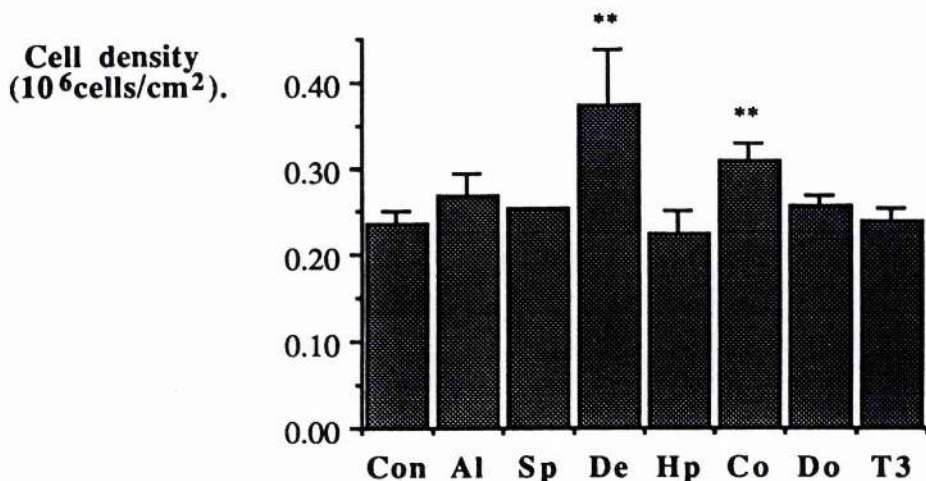


Figure 1. Shows the density of MDCK cells grown on collagen substrates after various hormone treatments. The cells were grown from high density seeding for 3 days, followed by 24 hours growth with various hormone supplements. The conditions or treatments were:- control (Con; ethanol only; final concentration 1%), Al (300 nM), Sp (300 nM), De (300 nM), Hp (300  $\mu$ M or 300 nM), Co (300 nM), Do (300 nM) and T3 (10 nM). Stock hormone solutions were made up in ethanol. The error bars represent standard deviations where  $n = 4$  except for Sp where  $n = 1$ . Statistical significance was tested by Student's t-test, assuming normally distributed data. \*\* = significant at the 1% level.

various hormone agonists and antagonists was determined (see figure 1). Twenty four hours growth in the presence of several of the hormones resulted in increases in cell numbers (density) compared to control cells. The increases were in the order De (+60%) > Co (+31%) > Al (+14%) > Do (+9%), although only the increases produced by De and Co were statistically significant. The order of potency of hormones at increasing in cell numbers was similar to the order of potencies reported for the steroid binding to type II glucocorticoid receptors (De > Co > Do  $\geq$  Al; Fanestil and Park, 1981). This circumstantial evidence suggested that glucocorticoid receptors were present in MDCK cells, and that their activation in these cells led to a mitogenic response. The effect of glucocorticoids on the growth of MDCK cells was similar but of larger magnitude than the response reported by Taub *et al.* (1979). Taub and co workers found increases in cell numbers of 15-25% after treatment with the less potent glucocorticoid, hydrocortisone (concentrations in the range 1 nM - 1  $\mu$ M). The affinity of Al for glucocorticoid receptors was such ( $K_d = 25-60$  nM; Fanestil and Park, 1981) that a concentration of 300 nM might be expected to elicit a response similar to that of 300 nM De. This suggested that



besides the known mechanism which prevents activation of Type I receptors by converting glucocorticoids to their inactive 11-keto analogs (see section 1.V.ii.c.1; Funder *et al.*, 1988), a mechanism might exist to prevent the activation of Type II receptors by mineralocorticoids.

### 7.III.ii. The effects of hormones on ouabain binding per cell.

The extent of ouabain binding to cells after approximately 24 hours growth in the presence of the various hormone treatments was as shown in figure 2. The number of Na,K-ATPase units in the control cells was not significantly different to those determined previously for the growth of MDCK cells at a high seeding density on collagen substrates (see section 6.III.vi). The number of ouabain binding sites in the Al, De, Co and Do treated cells showed small increases compared to the control. The increases were in the order Do (+19.0%) > Al (+16.5%) > Co (+14.6%) > De (+8.1%). However, of these increases, only those produced by Al and Co were statistically significant, due to the large variation in the results from Do treated cells. The order of increases in Na,K-

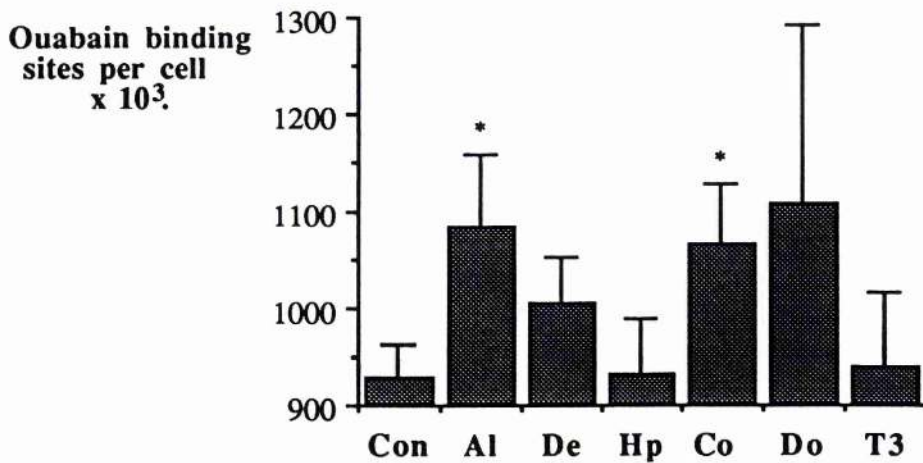


Figure 2. Shows the number of ouabain binding sites (Na,K-ATPase units) present on MDCK cells after 24 hours growth in various hormone containing media. Cells were grown from high density seeding for 3 days, prior to the addition of hormone supplements. The conditions or treatments were:- Con (ethanol only; final concentration 1%), Al (300 nM), De (300 nM), Hp (300  $\mu$ M), Co (300 nM), Do (300 nM) and T3 (10 nM). Stock hormones solutions were made up in ethanol. The error bars represent standard deviations, where n = 3 culture plates. Statistical significance was tested by Student's t-test, assuming normally distributed data. \* = significant at the 5% level.

ATPase units cited above, was similar to the order of potencies of binding to the type IV receptors that has been postulated to exist in MDCK cells (Ludens *et al.*, 1978). The order was also similar to that of binding to type I mineralocorticoid receptors (Fanestil and Park, 1981). The relatively small increase in Na,K-ATPase abundance, may have been due to the postulated slow onset of action of the major agonist of type IV receptors (Do) on Na transport (after 60 hours). Further experiments would be necessary to determine whether Do actually produces a significant increase in Na,K-ATPase abundance, and whether the increases produced by Do, Al and Co would be larger after 60 hours treatment.

### 7.III.iii. The effects of hormones on total RNA abundance.

The amount of total RNA extracted from cells treated with the various hormones for 24 hours was as in figure 3. The amount of total RNA per cell was only increased after treatment with Al. With all the other hormone treatments the amount of total RNA was equivalent or reduced compared to control cultures. However the significance of the alterations in the abundance of total RNA per cell could not be determined. The reduction in total RNA produced by Sp may have been due to the antagonism of the action of endogenous Al probably present in the serum of the growth medium. The level of total RNA might have been expected to be reduced in cells that were rapidly dividing such as

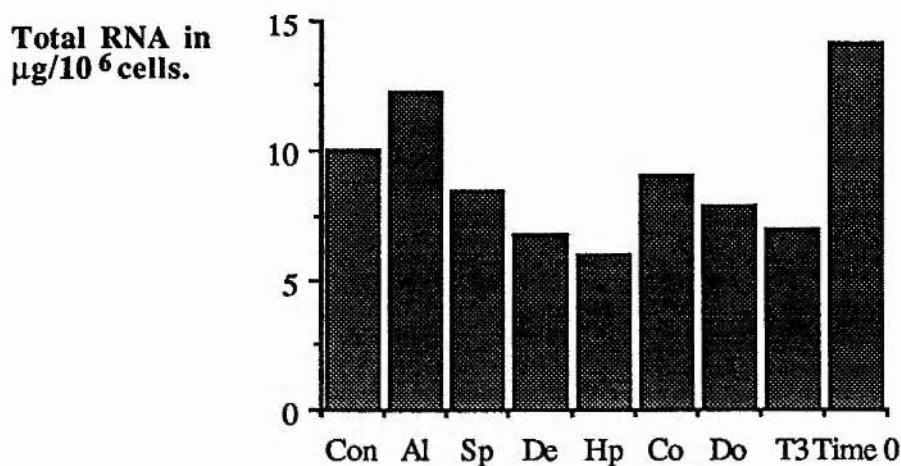


Figure 3. Shows the amount of total RNA extracted from cells grown for 24 hours in the presence of various hormone treatments. Cells were grown at high density with high levels of medium for 3 days, prior to the addition of hormone supplements. For details of hormone treatment see figure 2.

Figure 4. Shows an autoradiograph of a Northern blot hybridised with Na,K-ATPase  $\alpha 2$  isoform-specific DNA probe (the position of the mRNA's are indicated). Samples of rat brain (4  $\mu\text{g}$ ) and MDCK (20  $\mu\text{g}$ ) total RNA were blotted. The MDCK total RNA was obtained from cells grown for 3 days in growth medium (lane 9), and for a further 24 hours with the following additions :- lane 1, control (diluent only); lane 2, Aldosterone (300 nM); lane 3, Spironolactone (300 nM); lane 4, Dexamethasone (300 nM); lane 5, 17 $\alpha$ -Hydroxy progesterone (300 nM); lane 6, T3 (10 nM); lane 7, Corticosterone (300 nM); and lane 8, Deoxycorticosterone (300 nM).

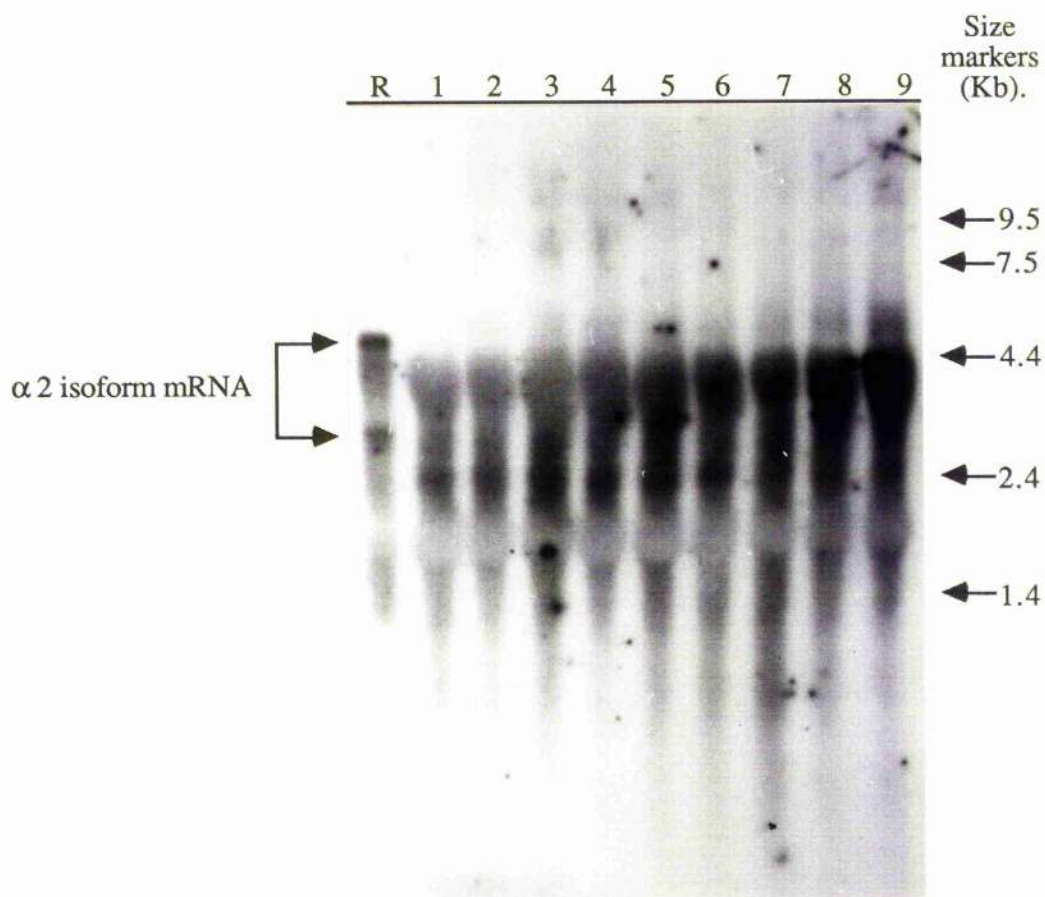


Figure 5. Shows an autoradiograph of a Northern blot hybridised with Na,K-ATPase  $\alpha 3$  isoform-specific DNA probe (the position of the mRNA's are indicated). Other details are as described in the legend for figure 4.



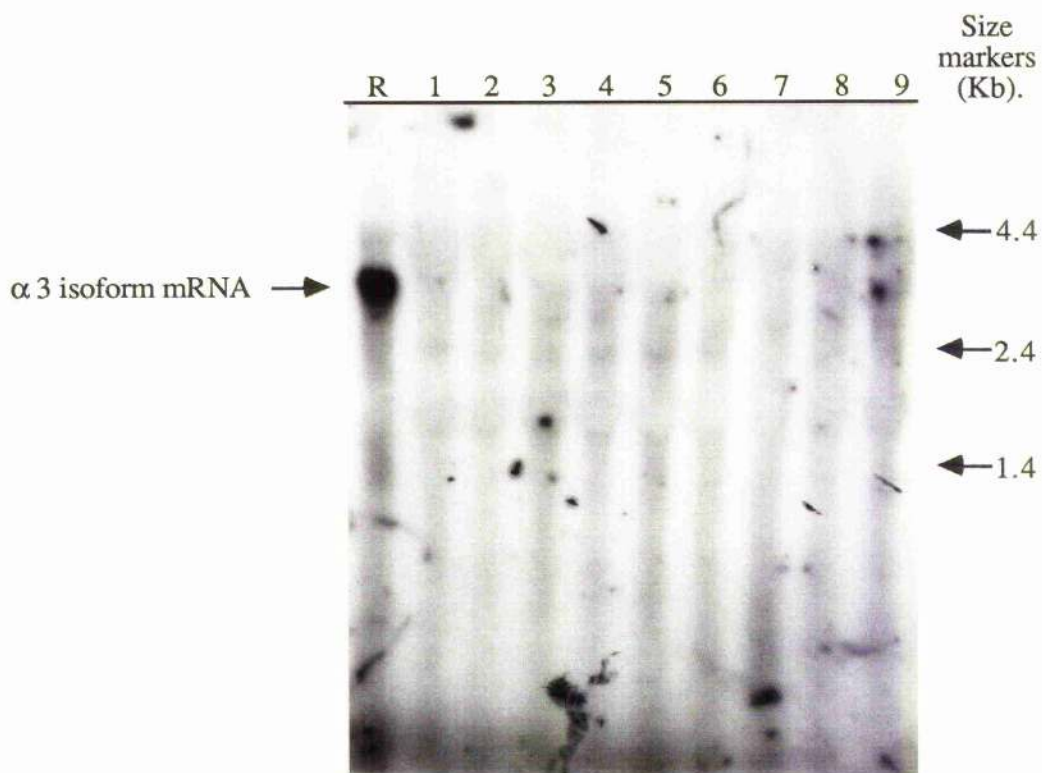


Figure 6. Shows an autoradiograph of a Northern blot hybridised with Na,K-ATPase  $\alpha$ 1 isoform-specific DNA probe (the position of the mRNA's are indicated). Other details are as described in the legend for figure 4.

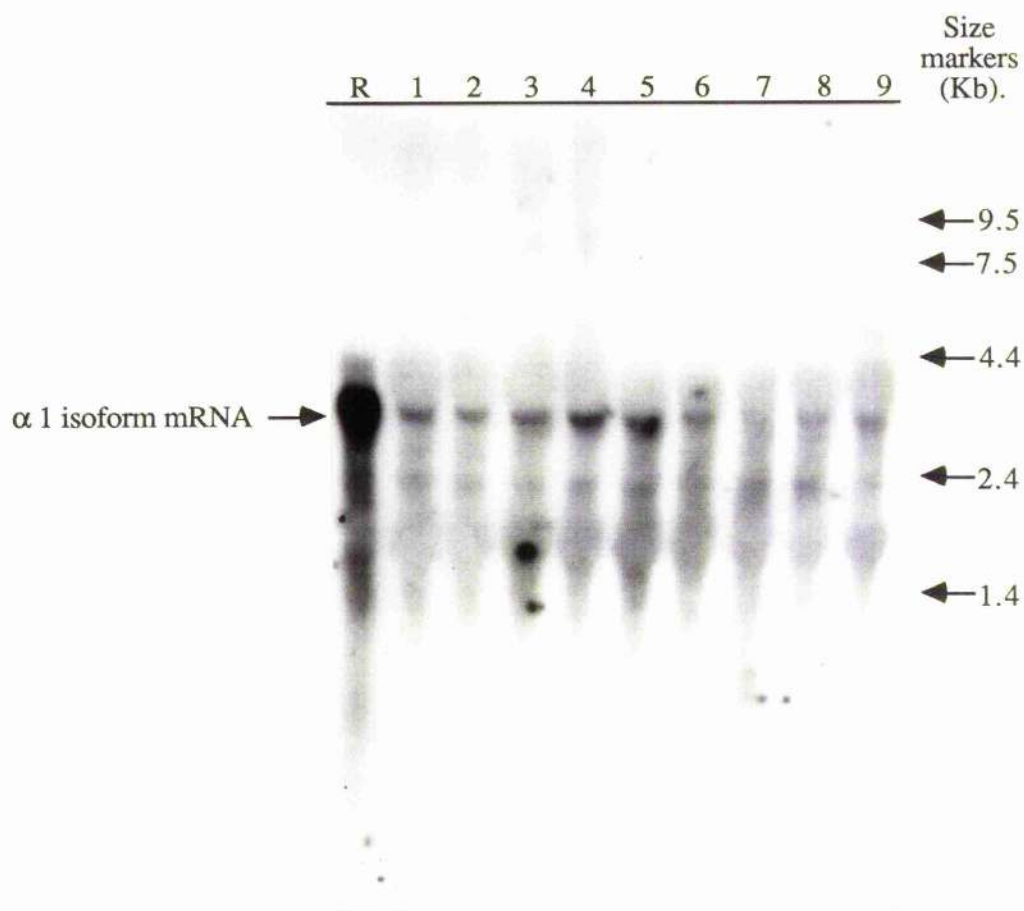
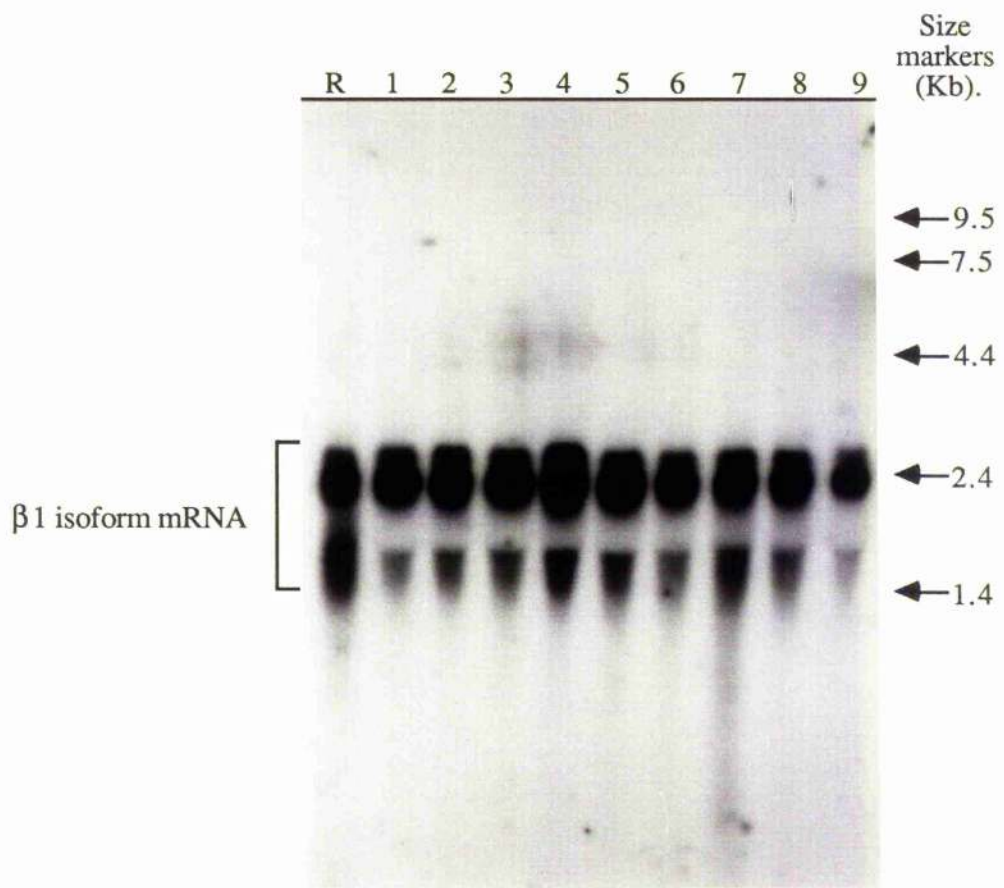


Figure 7. Shows an autoradiograph of a Northern blot hybridised with Na,K-ATPase  $\beta 1$  isoform-specific DNA probe (the position of the mRNA's are indicated). Other details are as described in the legend for figure 4.





those treated with De or possibly Co, as the total RNA per cell is approximately halved at each cell division. The average volume of cells treated with De or Co was also decreased by 10% and 5% respectively compared to control cultures. However the reduced abundance of total RNA in De and Co treated cells may have been a direct consequence of the actions of these hormones as glucocorticoids have been shown to inhibit RNA, DNA and protein synthesis in non-hepatic tissues (McPartland, 1986). The reason for the reduction in the abundance of total RNA produced by the other hormone treatments (Do, Hp and T3) was not known.

7.III.iv. The effect of hormones on the abundance of Na,K-ATPase isoform mRNA's.

Total RNA from the hormone treated cells was electrophoresed, Northern blotted, and hybridised under the conditions determined in section 5.III.iii., using the isoform-specific probes. The Northern blot produced was serially reprobated with each of the  $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 3 and  $\beta$ 1 probes, after removal of the previous probe by boiling for 2 x 20 minutes in 0.1 x SSC, 0.5% SDS. The hybridisations involving the  $\alpha$ 2 and  $\alpha$ 3 isoform-specific probes showed that none of the hormone treatments had induced the production of measurable quantities of  $\alpha$ 2 or  $\alpha$ 3 mRNA's (see figure 4 and 5). The hybridisations involving the  $\alpha$ 1 and  $\beta$ 1 DNA probes (figures 6 and 7 respectively) produced specific

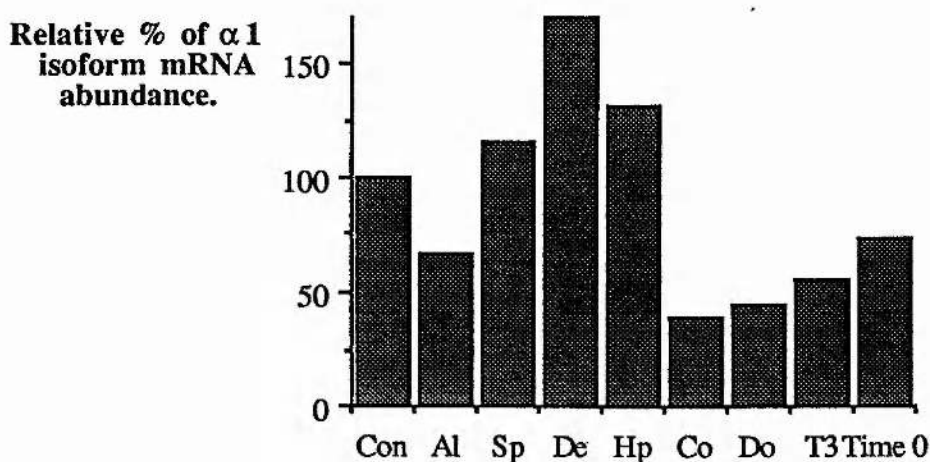


Figure 8. Shows the relative abundance of the Na,K-ATPase  $\alpha$ 1 isoform mRNA in MDCK cells grown after treatment with various hormones (see figure 6). The mRNA abundances were quantified from the autoradiograph of the  $\alpha$ 1 isoform Northern blot, by comparing the area of each band (determined by scanning densitometry) relative to the area of the band(s) of control cell RNA.

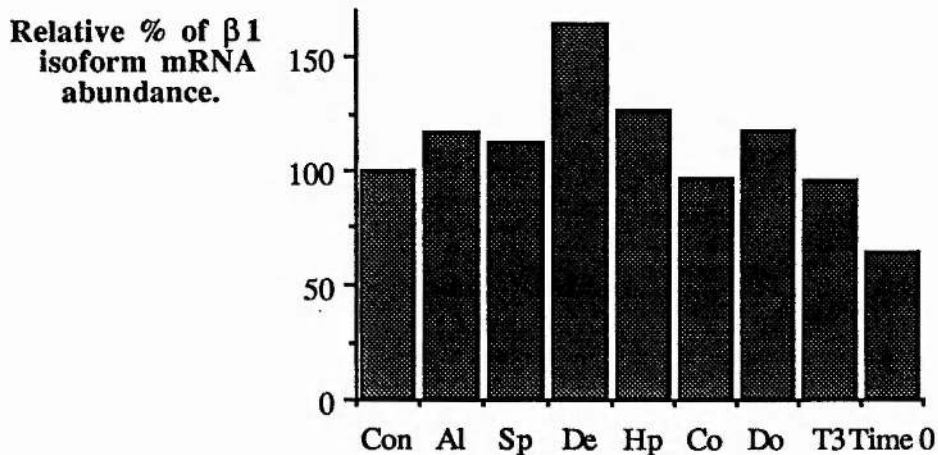


Figure 9. Shows the relative abundance of the Na,K-ATPase  $\beta 1$  isoform mRNA in MDCK cells grown after treatment with various hormones (see figure 7). The mRNA abundances were quantified from the autoradiograph of the  $\beta 1$  isoform Northern blot, by comparing the total area of the bands (determined by scanning densitometry) relative to the area of the bands of control cell RNA.

signals, although as previously shown (see section 5.III.iii.), hybridisations at this level of stringency resulted in high background levels with the  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$  probes. The reason why the  $\beta 1$  probe did not produce a high background at the same stringency was not known. The abundance of mRNA in bands on the Northern blots was quantified by comparing the area of each band (determined by scanning densitometry) with the area of the band(s) of control cell RNA. The resulting estimates of the abundance of mRNA's in hormone treated cells were as illustrated in figure 6 and 7. Only in the De treated cells was the abundance of  $\alpha 1$  and  $\beta 1$  isoform mRNA's increased above the that of the control cells. However the statistical significance of this result could not be tested with only one sample of RNA per hormone treatment. The abundance of both  $\alpha 1$  and  $\beta 1$  mRNA's in De treated cells was also increased compared to cells treated with the glucocorticoid antagonist Hp. Cells treated with Co, Do and T3 all had decreased levels of  $\alpha 1$  mRNA (39%, 44%, and 56% respectively), as well as total RNA per cell (90%, 79%, and 70% respectively). The reason for these decreases was not immediately apparent, especially considering that the Co and Do treatments increased rather than decreased the level of ouabain binding.

#### 7.III.v. General conclusions.

The results obtained suggested that the hormonal regulation of Na,K-ATPase in MDCK cells was highly complicated. Only De and Co treatment increased cell density significantly, suggesting that cells were sensitive to glucocorticoids. However, only Al and Co significantly increased the number of Na,K-ATPase units, suggesting a mineralocorticoid type response. The increase in Na,K-ATPase units might be expected to be caused by a corresponding increase in Na,K-ATPase mRNA abundance for both  $\alpha$  and  $\beta$  subunits, however this was only found after De treatment (per  $\mu\text{g}$  of total RNA; De increased  $\alpha 1$  and  $\beta 1$  mRNA abundance by 70% and 65% respectively). When the increases in Na,K-ATPase mRNA abundance, produced by De treatment, were calculated per cell (compared to control cells) the size of the increases were reduced to around 10% due to the lower level of total RNA per cell in De treated cells. Whereas, calculation of the abundance of Na,K-ATPase isoform mRNA's per cell (compared to control cells) for Al treated cultures, revealed a 44% increase in  $\beta 1$  but not  $\alpha 1$  isoform mRNA's per cell. The changes in Na,K-ATPase mRNA abundances after hormone treatment, when expressed either per  $\mu\text{g}$  of total RNA or per cell, were not correlated to the changes in Na,K-ATPase enzyme abundance. The action of T3 and Do treatment in MDCK cells was if anything inhibitory, in as much as it reduced both the amount of total RNA and the abundance of  $\alpha 1$  isoform mRNA (both per cell and per  $\mu\text{g}$  of total RNA), although the Na,K-ATPase enzyme and  $\beta 1$  isoform mRNA abundances were not altered. The diversity of responses to the hormone treatments may therefore have reflected the possibility of multiple steroid and thyroid hormone receptors. Another complication could also have resulted from the possibility of the cross reactivity of mineralocorticoids and glucocorticoids binding and activating each others receptors. The size of the changes in Na,K-ATPase enzyme abundance produced by hormone treatment was small compared to those produced by alterations in cell density or growth substrates (see Chapter 6), suggesting these may be the overriding factors determining the level of Na,K-ATPase enzyme abundance in MDCK cells.

Further experiments need to be done to determine the significance of changes in Na,K-ATPase mRNA abundances found in this study. Other experiments might be performed to investigate the time course of hormone action both in terms of how long hormones take to elicit a maximal response and whether the magnitude of responses are attenuated during the growth cycle. The effect of the addition of T3 to hormone

treatments might also be investigated as it has been shown to have a permissive role on the actions of Al (Barlet-Bas *et al.*, 1988; Barlet and Doucet, 1987), but not Co (Bartolomei *et al.*, 1983; and Klein *et al.*, 1984b). Furthermore lower, more physiological doses of hormones might be used. Taub *et al.* (1979) have reported maximal increases of 27% and 52% in cell numbers (above controls) after treatment of MDCK cell cultures with 5 nM hydrocortisone (a glucocorticoid) or 5 pM T3 respectively. Treatments with concentrations of T3 above 5 nM produced a reduction in cell numbers compared to control cells. Treatment with 5 pM T3 or 50 nM hydrocortisone, in conjunction with 5 µg/ml transferrin, 5 µg/ml insulin and 25 pg/ml prostaglandin E<sub>1</sub>, also produced increases in blister or hemicyst formation. Blister formation may be a marker for vectorial salt and water transport in MDCK cells, although other factors such as increases in the strength of cell-cell tight junctions, may also increase blister formation without increases in vectorial transport. This evidence suggests that it is possible that these lower hormone concentrations might produce a greater effect on Na,K-ATPase enzyme expression and mRNA abundance in MDCK cells. Future experiments may also be improved. This study involved the growth of MDCK cells in medium supplemented with hormones at known concentrations. However, it is likely that the medium itself already contained some endogenous hormones included in the serum added, although these hormones would probably have been present at lower concentrations. These experiments could therefore be improved by the use of chemically defined medium such as that of Taub *et al.* (1979) mentioned above. This would improve the chances of detecting specific increases in Na,K-ATPase expression as, the basal level of expression of the enzyme in the total absence of hormone agonists, is likely to be lower.

CHAPTER 8. SUMMARY.



The aim of this project was to characterise the cell surface expression of the Na,K-ATPase enzyme in the dog kidney cell line MDCK, and to correlate this with the expression of mRNA's coding for the Na,K-ATPase subunits. The cell surface expression of this enzyme in MDCK strain I cells has previously been shown to decrease during growth (see appendix 4), and to be dependant on the initial seeding density of cultures. The aim of this project was therefore to characterise factors affecting the cell surface expression of Na,K-ATPase during the growth or culturing cycle, and to determine whether alterations in the relative abundance of mRNA's coding for the subunits of the enzyme are responsible for any changes in Na,K-ATPase expression found.

Procedures for cell culture, and the estimation of cell surface Na,K-ATPase enzyme abundance on MDCK cells were already well established in the laboratory. Studies on Na,K-ATPase expression were expanded by developing molecular biological procedures (including RNA extraction and nucleic acid hybridisation), in order to assess the expression of this enzyme at the level of the genome.

### **8.I. RNA extraction.**

Efficient processes for total RNA and poly A<sup>+</sup> mRNA extraction were required, because small percentage variations in the recovery of RNA from low efficiency extractions would result in apparently large changes in the amount of RNA present in samples. A modified version of the method for total RNA extraction reported by Cathala *et al.* (1983) was chosen, because it gave high yields of RNA with recoveries of > 60%. Variation in the yield of RNA extracted was such that within an experiment, differences in the amount of total RNA recovered from cell samples greater than 12.5% (of the mean % RNA recovery) should represent real changes in total cellular RNA. The efficiency of poly A<sup>+</sup> mRNA isolation from total RNA was also assessed. This process was also found to be highly efficient with yields in excess of 70%.

### **8.II. Nucleic Acid Hybridisation Conditions.**

The abundance of mRNA's encoding the protein subunits of Na,K-ATPase were assessed using the standard molecular biological procedures of Northern or dot blotting. The conditions for the use of DNA probes which were specific for the Na,K-ATPase

mRNA were determined. Partial or complete cDNA's were available for the human  $\alpha 1$  isoform, and the rat  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ , and  $\beta 1$  isoforms. Hybridisation of these cDNA 's to RNA from MDCK cells, resulted in cross (species)-hybridisation where, with the exception of the  $\beta 1$  isoform, the level of homology shared by the hybridising nucleic acids was unknown. The conditions for the hybridisation of these cDNA's (or their restriction endonuclease fragments) to MDCK RNA had to be determined empirically. Because of the high level of nt homology between  $\alpha$  isoforms (76-79% in rat species; Shull et al., 1986b) cross hybridisation between  $\alpha$  isoform cDNA probes with the mRNA of the other isoforms in MDCK cells was a possibility. Restriction fragments were therefore chosen, which had such a low level of homology with the other  $\alpha$  isoforms, that very little or no cross hybridisation between isoforms could occur. The level of stringency required for the hybridisation of the  $\alpha 1$  isoform-specific probe to MDCK RNA was likely to be the most relaxed compared to the other isoform-specific probes, because the  $\alpha 1$  probe's nt sequence demonstrated the lowest homology (of the isoform probes) to its complementary sequence in other species (eg humans). The optimum conditions, for the hybridisation of the  $\alpha 1$  isoform-specific probe to MDCK RNA (33°C, with 50% formamide) and for the subsequent washing of filters (0.1 x SSC, 0.1% SDS, at 40°C), were then determined by calculation from values determined empirically, by comparison of the signals produced after hybridisation and the subsequent washing of filters at different temperatures. The amount of formamide required for optimal hybridisation (50%) was also determined empirically, by hybridising in the presence of different formamide concentrations. Hybridisation of the  $\alpha 1$  isoform-specific probe to rat kidney, HeLa cell (human), and MDCK cell RNA extracts, allowed the estimation of the approximate nt homology shared between the probe and MDCK Na,K-ATPase  $\alpha 1$  mRNA. The lower than expected level of homology determined (approximately 77%) suggested that the canine  $\alpha 1$  mRNA sequence might exhibit a 6 nt deletion which exists in the central portion of the complementary (to the rat  $\alpha 1$  isoform-specific probe)  $\alpha 1$  isoform sequences of ovine and porcine species. This region if absent from MDCK  $\alpha 1$  mRNA, would result in destabilisation of DNA-RNA hybrids formed during hybridisation, which would result in the determination of a lower than expected value for the  $T_m$  and the % homology shared with the rat  $\alpha 1$  probe.

### **8.III. Test Hybridisations with rat RNA samples.**

The rat  $\alpha$  isoform-specific probes were then tested for specificity by binding to Northern blots of RNA from various rat tissues. To increase the yield of information on the regulation of Na,K-ATPase obtained from test experiments, the abundance of Na,K-ATPase isoform mRNA's in hypertensive and normotensive Milan rat myocardial tissues was studied. The Milan rat strain has a genetic lesion which is manifested in a renal malfunction associated with the development of hypertension. The Milan hypertensive rats showed a highly significant ( $p \leq 0.01$ ) increase in the amount of total RNA isolated per gram of tissue in right ventricular tissue, compared to control animals, and a similar but insignificant increase in total RNA from hypertensive left ventricular tissue. The specific abundance of the Na,K-ATPase isoform mRNA's present in hypertensive and control rat myocardial tissues was also assessed using a dot blotting technique. The abundance of  $\alpha 1$ ,  $\alpha 3$ , and  $\beta 1$  isoform mRNA's was significantly lower in hypertensive ventricular tissues (with the exception of the  $\alpha 3$  isoform mRNA abundance in right ventricle). In contrast, the abundance of the  $\alpha 2$  and  $\beta 1$  isoform mRNA's in hypertensive atrial tissues was significantly higher. When the abundance of the isoform mRNA's were expressed per gram wet weight of tissue, to take into account changes in the abundance of total RNA, the differences in Na,K-ATPase mRNA abundances between hypertensive and normotensive rat tissues were further accentuated.

### **8.IV. Isoforms of Na,K-ATPase in MDCK cells.**

Due to poor incorporation of radiolabel into, and hybridisation of, the  $\alpha 2$  isoform-specific probe, three of the isoform-specific probes were redesigned. The small size of probes (less than 200 bp) was thought to be responsible for the poor radiolabelling, and so the probes for the  $\alpha$  and  $\beta$  isoforms used in subsequent experiments were greater than 200 bp in size. These new probes were used in experiments to determine the presence or absence of Na,K-ATPase isoform mRNA's in MDCK cell RNA. The presence of  $\alpha 1$  mRNA was demonstrated in MDCK poly A<sup>+</sup> mRNA, as previously reported by Bowen and McDonough (1987). The expression of  $\beta 1$  mRNA in MDCK cells was similar to rat brain total RNA, in that at least 4 bands were present on Northern blots. This was in direct contrast to the results of Bowen and McDonough (1987), who found only 1 major mRNA species in MDCK cells. Although some non-specific background in the

electrophoretic lanes of MDCK cell poly A<sup>+</sup> mRNA was present on Northern blots, no specific signals were obtained using the  $\alpha 2$  and  $\alpha 3$  isoform-specific probes. This evidence strongly suggests, although it does not prove conclusively, that under the conditions used for cell culture there was no significant expression of Na,K-ATPase  $\alpha 2$  or  $\alpha 3$  mRNA in MDCK cells.

#### **8.V. Cell surface expression of Na,K-ATPase in MDCK cells.**

The effect of several environmental factors (such as cell-cell and cell-substrate interactions and the amount of medium used for culturing) on the cell surface expression of Na,K-ATPase was then assessed. As Na,K-ATPase enzyme in MDCK cells was known to be expressed almost exclusively on basolateral membranes, problems with the access of ouabain to this membrane domain from the apical side of cell monolayer were assessed. MDCK cell monolayers have tight junctions which could potentially exclude access to the basolateral side of the monolayer. One feature of MDCK cell monolayers, which suggested restricted access to basolateral membranes was the formation of blisters which result from the build up of fluid under the cell monolayer which then cannot escape due to the integrity of cell-cell tight junctions. Experiments were therefore performed to confirm (or otherwise) the results of Cerejido *et al.*, (1981) and Rabito *et al.*, (1978), who showed that with MDCK strain II cell monolayers grown on impermeable substrates, the lack of blistering in certain regions of cell monolayers was due to the disassociation of tight junctions under the pressure produced by the build up of fluid. Results in this study, using a comparison of the levels of ouabain binding to MDCK strain I cells in the presence or absence of Ca (removal of calcium causing dissociation of tight junctions; Gumbiner and Simons, 1986), showed that the dissociation of tight junctions did not result in a significant difference in the apparent level of ouabain binding to cells. This result suggested that there was no restriction of the access to basolateral membranes from the apical side of MDCK strain I cell monolayers, which was entirely consistent with the results of Cerejido *et al.*, (1981) and Rabito *et al.*, (1978) on MDCK strain II cell monolayers.

MDCK cells grown at high density on plastic substrates showed a similar profile of ouabain binding (Na,K-ATPase expression) during growth as results determined previously (see Appendix 4). The number of Na,K-ATPase units present on cells increased dramatically peaking after two days ( $8.2 \times 10^5$  units per cell), before



subsequently falling to a lower level when cell cultures reach maximum cell density ( $3.8 \times 10^4$  units per cell). To investigate the effect of different cell attachment (growth) substrates on the cell surface expression of Na,K-ATPase, cells were then grown under identical conditions to previous experiments, except that cells were seeded on permeable Millipore culture plate inserts (cellulose ester). The expression of Na,K-ATPase on Millipore inserts during growth had a similar profile to experiments on plastic substrates, but the level of expression was higher with, on average, approximately  $5.5 \times 10^5$  more enzyme units per cell present at all times. As Millipore cellulose inserts were an unnatural substrate for cultured cells, a more natural collagen substrate was chosen for further experiments. As a result of the construction of collagen culture plate inserts, cells were seeded on inserts with an increased volume of growth medium compared to that used for Millipore inserts. The number of Na,K-ATPase units present on cells grown on collagen or Millipore inserts was not significantly different, although there were on average  $10^5$  fewer enzyme units per cell were present on cells seeded on collagen inserts (between 3-7 days of growth). In experiments with cells grown on Millipore or collagen substrates with larger volumes of growth medium, the number of enzyme units found on MDCK cells did not reduce after three days of culture, as found previously with growth in lower volumes of growth medium on plastic or Millipore growth substrates. This resulted in a significantly higher abundance of enzyme on cells grown in larger volumes of medium after 4-7 days growth. This evidence suggested that in the experiments using a substantially lower volume of growth medium, the supply of nutrients or other dependent factors, or the build up of toxic waste products, had limited the expression of Na,K-ATPase on the cell surface. This outcome might be expected, as the cell's Na gradient, provided by Na,K-ATPase is utilised to import many nutrients such as sugars and amino acids into the cell. If this function is reduced or abolished, then the cell's requirement for Na-pumping would also be reduced. One option available to the cell to reduce Na-pumping would be to reduce the number of enzyme units present in the plasma membrane. The evidence above suggests that this option may well be at least one way in which the cell decreases the amount of Na-pumping. The effect of cell density on the expression of Na,K-ATPase was then investigated, by growing cells on collagen inserts after seeding them at high or low density. The pattern of expression of Na,K-ATPase enzyme during the growth of cells seeded at high density was similar to the previous experiments using collagen inserts. However, the number of Na,K-ATPase units expressed on cells seeded at low density was significantly higher after 2-5 days growth.



The maximum level of enzyme abundance on low density seeded cells was found after 2 days growth, this was on average 3.9 fold higher than the level of expression found on high density seeded cells after 2 days. The effect of changes in cell density on the expression of Na,K-ATPase  $\alpha$  subunit mRNA was then investigated. Although a slight increase in  $\alpha$  subunit mRNA abundance was detected on average in low density compared to high density seeded cells during the initial 5 days growth, this difference was not statistically significant. Furthermore any difference obtained was likely to be non-specific, as a similar pattern of expression of actin mRNA was also found in high and low density seeded cell cultures. The level of total RNA per cell was also increased (by approximately 2-fold) in cultures after 24 hours growth, before returning to original levels after 4 days growth. However, there was no difference between the levels of total RNA per cell in low and high density seeded cultures. This suggested that increases in the level of total RNA per cell were not responsible for the differences in the cell surface expression of Na,K-ATPase enzyme found between low and high density seeded cultures. The lack of differences in Na,K-ATPase mRNA abundance between differently seeded cultures indicated that the higher level of expression of the enzyme found in low density seeded cells was probably mediated by post-transcriptional processes. Several other possible explanations of these results exist; one of these is that there was a transient increase in Na,K-ATPase mRNA abundance which remained undetected after 24 hours growth. However, the most likely alternative explanation is that high and low density seeded MDCK cells grown on plastic substrates (used for assaying Na,K-ATPase cell surface expression), do not exhibit the same differences in the expression of Na,K-ATPase enzyme between low and high density seeded cultures, as cells grown on collagen substrates (used for the determination of Na,K-ATPase mRNA abundance). This may be the reason why there was no significant difference in Na,K-ATPase  $\alpha$  subunit mRNA abundance between high and low density seeded cultures.

The main morphological difference between high and low density seeded cultures was the time taken for cell-cell contacts to become established. The results suggest that the extra time spent as single cells in low compared to high density seeded cultures was responsible for the increase in Na,K-ATPase units found between 1 and 2 days growth. Nelson and Hammerton (1989), have demonstrated that Na,K-ATPase is associated with the cytoskeleton in MDCK cells and they have suggested that single cells have considerably less (<20% compared to 50% in confluent cells) enzyme associated with the cytoskeleton than cells in confluent monolayers. This observation together with the

results of cell seeding density experiments, suggests that one possible explanation for the decline in enzyme abundance after cell-cell contacts are established, is that Na,K-ATPase, due to its increased association with elements of the cytoskeleton, is withdrawn from the membrane. The involvement of the cytoskeleton with Na,K-ATPase suggests that the use of an actin cDNA as a control probe to represent the general abundance of mRNA's in cells may represent a poor choice as, the regulation of its expression might be correlated with that of Na,K-ATPase.

#### **8.VI. The effect of hormones on Na,K-ATPase expression in MDCK cells.**

The effect of various hormone agonists and antagonists (aldosterone (Al), spironolactone (Sp), dexamethasone (De), 17 $\alpha$ -hydroxy progesterone (Hp), corticosterone (Co), deoxy-corticosterone (Do), and tri-iodo thyronine (T3)) on the expression of the Na,K-ATPase enzyme and its mRNA's was studied in MDCK cells. The glucocorticoids agonists De and Co both significantly increased the number of cells found in treated cultures compared to controls. This result was entirely consistent with those of Taub *et al.*, (1979) who reported an increase in cell density after treatment with the glucocorticoid hydrocortisone. No significant increases in cell density were found with the other agonists. Small increases in the number of Na,K-ATPase enzyme units per cell (Do, +19.0%; Al, + 16.5%; Co, +14.6%; De, +8.1%), compared to control cells, were found after hormone treatment. The order of magnitude of increases was similar to the order of potency of type IV steroid hormone receptors (Do > Al > Co > De; Ludens *et al.*, 1978), although only the increases in enzyme units found after treatment with Al or Co were statistically significant. No increases in Na,K-ATPase were found after the treatment of cells with Hp or T3. The abundance of total RNA per cell after treatment with the various hormones was mostly decreased, only Al treatment produced an increase. The significance of the changes in total RNA per cell was not assessed. The effect of the various hormone agonists and antagonists on the expression of Na,K-ATPase mRNA's was then studied by the hybridisation of isoform-specific DNA probes to a Northern blot of total RNA, extracted from hormone treated MDCK cells. None of the hormone treatments were found to induce the expression of detectable quantities of  $\alpha 2$  or  $\alpha 3$  mRNA's, and only De was found to increase the relative abundance of  $\alpha 1$  and  $\beta 1$  mRNA's (70% and 65% respectively), when this was expressed per  $\mu$ g of total RNA.

When the relative abundance of  $\alpha 1$  and  $\beta 1$  mRNA's was expressed per cell, the increases in the abundance of  $\alpha 1$  and  $\beta 1$  mRNA's found after De treatment were reduced to around 10% above that of control cells. However, the abundance of  $\beta 1$  mRNA in A1 treated cells, when expressed relatively per cell, was increased by 44% over control cells, although no change in  $\alpha 1$  mRNA abundance was found. The general effect of the other hormone agonists and antagonists was to decrease the relative abundance of the  $\alpha 1$  and  $\beta 1$  mRNA's to varying degrees. The statistical significance of the changes in mRNA abundances were not assessed. No correlation was found between increases in the Na,K-ATPase enzyme abundance and changes in mRNA abundance. The lack of a correlation between these parameters suggests that the hormone agonists used, probably have no effect on the abundance of Na,K-ATPase mRNA in MDCK cells, and that the cell surface expression of this enzyme is largely controlled post-transcriptionally by other factors including cell-cell and cell-substrate interactions. However, the lack of a response by these hormone agonists and antagonists may be due to a number of other reasons, such as use of wrong concentration of agonist, measurement of parameters at an incorrect time after the onset of treatment, or treatment of cell cultures at an unresponsive phase of the growth or culturing cycle. Other experiments would be needed to determine if this was the case.

APPENDICES

APPENDIX 1.

Calculation of specific ouabain binding to cells (see section 2.III.i.b.).

$$\text{Ouabain binding} = \frac{X \times [\emptyset] \times A}{Y \times C}$$

where:-

Ouabain binding = ouabain molecules bound per cell.

X = cell bound radioactive counts per plate.

= total radioactive counts per plate - radioactive counts on blank plates

[ $\emptyset$ ] = [Ouabain]

= concentration of ouabain used.

A = Avagadro's constant

=  $6.022 \times 10^{23}$  molecules per mole.

Y = radioactive counts per litre of radioactive ouabain solution.

= radioactive counts in standard solutions ( $100 \mu\text{l}$ )  $\times 10^4$  - radioactive counts on blank plates.

C = cell number per plate.

The formula calculates the amount of ouabain binding to cells incubated in both OK (potassium free) Krebs and 15K (15 mM potassium containing) Krebs solutions. Within an experiment, the mean value of ouabain binding to cells in 15K Krebs (non-specific) was calculated, and subtracted from each individual value calculated for ouabain binding in OK Krebs. The values obtained represent the specific ouabain binding. Plates are tissue culture plates containing cells being assayed, and blank plates are plates without cells assayed under the same conditions.



## APPENDIX 2.

Calculation of the specific activity of radiolabelled DNA.

The radiolabelling of DNA is performed by the use of the Amersham International "Multiprime" labelling kit. This kit incorporates the use of the Klenow fragment of E. coli. DNA polymerase I for the purposes of radiolabelling. "Multiprime" labelling leads to net DNA synthesis, and so the total amount (ng) of DNA at the end of the reaction must be calculated.

**Total DNA (ng).** =  $\mu\text{Ci}$  of  $[\alpha^{32}\text{P}]$ -dNTP's added x proportion of label incorporated x (4 x mean M. Wt. of nucleotides) / specific activity of radiolabelled nucleotide (Ci / mmol) + input DNA (ng).

**Radioactivity (dpm).** =  $\mu\text{Ci}$  of  $[\alpha^{32}\text{P}]$ -dNTP's added x  $2.22 \times 10^6$  (dpm /  $\mu\text{Ci}$ ) x proportion incorporated of label incorporated.

where:-

Radioactivity added = 50-125  $\mu\text{Ci}$ .

Proportion of label incorporated. = i.e. 50% incorporation of total radiolabel  
= a proportion of 0.5.

Mean M. Wt. of nucleotides = mean molecular weight of nucleotides (deoxy-nucleotide monophosphates)  
= 330 g/mole.

Specific activity of nucleotide. = normally 3000 Ci/mmol ( $[\alpha^{32}\text{P}]$  dATP).

Input DNA = normally 25-50 ng.

Dpm = radioactive disintegrations per minute.

**Specific Activity of DNA (dpm/ug)** =  $\frac{\text{Radioactivity incorporated (dpm)} \times 10^3}{\text{DNA (ng)}}$

### APPENDIX 3.

#### Preparation and composition of solutions.

- Potassium free :- 11.1 mM glucose, 136.9 mM NaCl, 1.2 mM (OK) Krebs buffer. MgSO<sub>4</sub>, 2.8 mM CaCl<sub>2</sub>, 0.6 mM Na<sub>2</sub>HPO<sub>4</sub>, 12 mM HCl, 13.7 mM tris-base, and 1% dialyzed newborn calf serum. Krebs solutions were adjusted to give a pH of 7.4 at either 0 or 37°C, as required.
- Bacterial lysis buffer. :- 50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA, and 5 mg / ml lysozyme.

#### Gel electrophoresis solutions.

- 10 x Gel loading buffer. :- 50% glycerol, 0.4% bromophenol blue, and 0.1 M Na phosphate buffer pH 7.0.
- 10 x Tris-Borate-EDTA (TBE) buffer. :- 89 mM tris base, 89 mM boric acid, and 2 mM EDTA pH 8.0.
- 10 x MOPS buffer :- 0.4 M morpholinopropanesulphonic acid, 100 mM Na acetate and 10 mM EDTA pH 7.0.
- Deionised formamide :- deionised by stirring in the presence of 0.1 g / ml of Amberlite mixed bed ion exchange resin, for 30 minutes.

#### Total RNA extraction solutions.

- Cell lysis buffer :- 5 M guanidinium isothiocyanate, 50 mM tris pH 7.5 and 10 mM EDTA.
- TNESDS buffer :- 150 mM NaCl, 10 mM Tris, 1 mM EDTA and 0.1% SDS, pH 7.5.
- Chloroform :- contained chloroform and iso-amyl alcohol in a ratio of 24:1 (v/v).
- Phenol :- was composed of a mixture of re-distilled phenol, 0.1% 8-hydroxy-quinoline and 0.2% β-mercaptoethanol, with the addition of saturating quantities of 100 mM Tris pH 7.5.

DEP-H<sub>2</sub>O :- double distilled, "Milli-Q" water, treated with 0.1% di-ethyl pyro-carbonate (DEP), stirred for 12 hours, and autoclaved (121 °C for 20 minutes).

mRNA isolation solutions.

Loading buffer :- 0.5 M NaCl, 1 mM EDTA, 0.5% SDS and 10 mM Tris-HCl pH 7.5.

Clearance buffer :- 0.1 M NaCl, 1 mM EDTA and 10 mM Tris pH 7.5.

Elution buffer :- 1 mM EDTA and 10 mM Tris-HCl pH 7.5.

Wash buffer :- 0.1 M NaOH and 5 mM EDTA.

2 x Loading buffer :- 1 M NaCl, 1% SDS, 2 mM EDTA and 20 mM Tris pH 7.5

Hybridisation solutions.

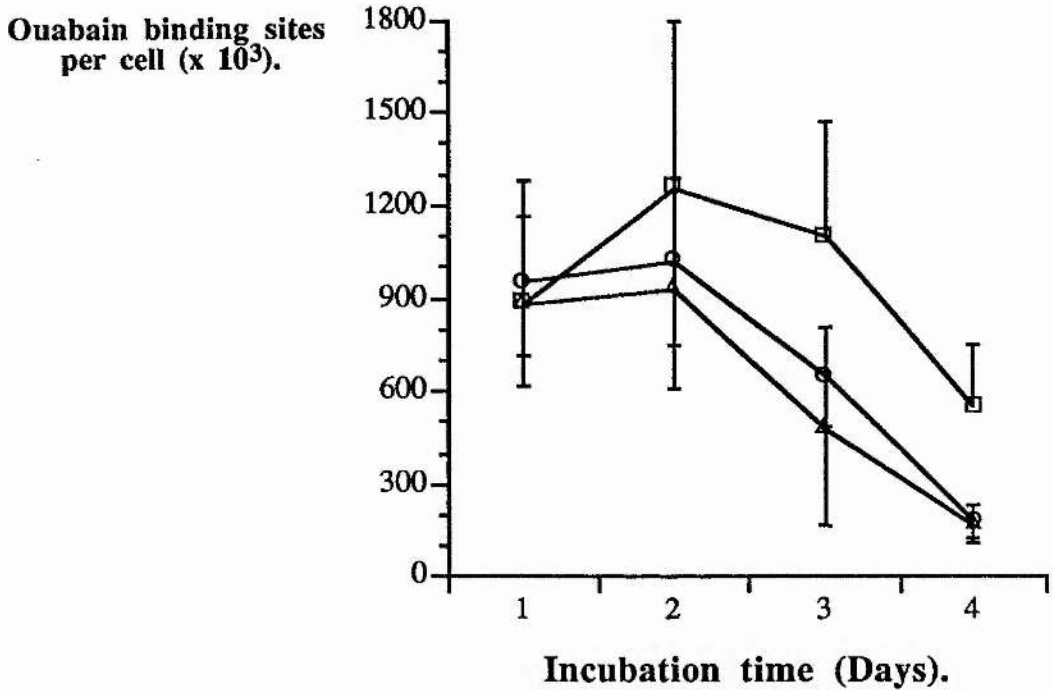
20 x Standard saline citrate (SSC) :- 3 M NaCl, 0.3 M tri-Na citrate pH 7.0

100 x Transfer buffer. :- 2 M Tris, 1 M Na acetate 50 mM EDTA pH to 7.4 with glacial acetic acid.

100 x Denhardt's solution. :- 2% ficoll, 2% polyvinyl pyrolidone and 2% bovine serum albumin (Pentax fraction V).

## Appendix 4.

Cell surface expression of Na,K-ATPase (ouabain binding) during the growth of cells at different initial seeding densities.



The figure illustrates the level of specific [<sup>3</sup>H]-ouabain binding to MDCK strain I cells grown on plastic substrates at different initial seeding densities. The cells were seeded at high ( $\Delta$ ;  $8.0 \times 10^4$  cells per  $\text{cm}^2$ ), medium ( $\circ$ ;  $2.6 \times 10^4$  cells per  $\text{cm}^2$ ), and low ( $\square$ ;  $9.5 \times 10^3$  cells per  $\text{cm}^2$ ) density. The data represent the mean values from three experiments performed in this laboratory in 1978. The experiments were performed and the data supplied by Mrs P.H. Ogden. Error bars represent standard deviations where  $n = 9$ , except in medium and high density seeded cells on day 4 where  $n = 6$ .

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