A STUDY OF SODIUM TRANSPORT IN KIDNEY AND INTESTINE AND ITS SENSITIVITY TO ANGIOTENSIN

by

Douglas James Smith

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Department of Physiology and Biochemistry,
The University,
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ABSTRACT

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The extrusion of sodium and the uptake of potassium by sodium loaded, potassium depleted rat kidney cortex slices was studied, and the results interpreted in terms of two modes of sodium transport; Mode I being chloride independent but potassium dependent, and Mode II being potassium independent but having a specific requirement for chloride. From the results of experiments with guinea-pig and rabbit kidney cortex slices, similar mechanisms are proposed in these tissues.

In an attempt to further characterize the two modes of sodium transport, a comparative study of the action of ethacrynic acid and ouabain on sodium and potassium transport was carried out. From the results obtained, it is suggested that ethacrynic acid has species dependent effects. In rabbit, and to a lesser extent guinea-pig kidney cortex, the drug appears to have some specificity for sodium transport by Mode II. In the rat, however, this inhibitor appears to exert non-specific effects. In all species, metabolic actions are apparent following extended incubations with this diuretic.

An attempt was made to study cation fluxes in intestinal rings. The tissues tested, however, were found unsuited to sodium loading, potassium depleting procedures.

An investigation into the action of angiotensin confirmed that this hormone, at low concentrations, stimulates the rate of sodium extrusion from sodium loaded, potassium depleted rat kidney cortex slices. This stimulation was found to be potassium independent, but required the presence of chloride ions in the incubation media. The actions of angiotensin on guinea-pig kidney cortex slices were found to be comparable to those observed in the rat.

The stimulation of sodium extrusion by sodium loaded, potassium depleted rat kidney cortex slices and the stimulation of fluid transport by everted colon sacs prepared from nephrectomized, adrenalectomized rats was found to be calcium, but not magnesium, dependent. Evidence is presented suggesting that angiotensin action does not induce changes in calcium flux, and it is proposed that calcium is required for the interaction of angiotensin with its receptor.

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Abbreviations used

ACTH	adrenocorticotrophic hormone
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ATP-ase	adenosine 5'triphosphate phosphohydrolase
°C	degree centegrade
cAMP	cyclic 3'5' adenosine monophosphate
Ci	curies
cm.	centimeter
cpm.	counts per minute
DNA	desoxyribose nucleic acid
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol tetraacetic acid
eq	equivalents
ether	diethylether
ETHA	ethacrynic acid
fig.	figure
g.	acceleration due to gravity
g	gram
h	hour
kg	kilogram
LH	leutinizing hormone
M	molar
meq	milliequivalent
min	minute
ml	millilitre
mM	millimolar
mm	millimetre
mv	millivolt
NAD	nicotinamide adenine dinucleotide
osc.	oscillations
Osm	osmoles
pd	potential difference
RNA	ribose nucleic acid
scc	short circuit current
sec	second
Tris	tris hydroxymethyl aminomethane
TSH	thyroid stimulating hormone
V/V	volume per volume
wt	weight

Chapter 1

Review of the literature

INTRODUCTION

In terrestrial vertebrates, the rigid control of body salt and water balance is essential to the normal functioning of biochemical and metabolic processes. The organs which have evolved to tackle the major proportion of this control are the kidneys. In amphibia, in addition to renal control, salt and water excretion is also regulated by the skin and urinary bladder. In birds, this secondary control is exerted by the salt gland, whilst in fish, the gills are of importance. In mammals the kidney is highly developed, and is responsible for the major control of fluid balance. However, the large and small intestines, the skin, and the gall bladder also contribute to the maintenance of body salt and water content.

It is generally accepted that the excretion of sodium, chloride and water, the major components of extracellular fluid, is ultimately controlled by changes in the rate of net sodium transport across epithelial membranes. The mechanism of these movements is not, however, fully resolved. Koefoed-Johnsen & Ussing (1958) formulated a model for sodium transport in which sodium ions are pumped across the serosal membranes of the epithelial cells in exchange for potassium. This, they propose, is not a 1 : 1 exchange and leads to a net movement of chloride down its electrochemical gradient, thus generating an osmotic gradient which leads to water flow in the same direction. Several workers, however, have proposed a second route for sodium, this mechanism being independent of potassium ions, and transporting sodium and chloride as an isotonic solution (Diamond, 1964, 1969; Kleinzeller & Knotkova, 1964; Whittembury, 1968; Whittembury & Proverbio, 1970; Munday, Parsons & Poat, 1971). Whether chloride movements are induced by an inbalanced sodium potassium exchange, tightly coupled to sodium movements as proposed by Diamond (1964, 1969), or secondary to the unilateral movement of sodium

as proposed by Whittembury and associates, could possibly be resolved by observing renal sodium pump activity in a variety of ionic media, or in the presence of inhibitors. Ouabain and ethacrynic acid are two compounds which have been proposed to have a high specificity for potassium dependent and potassium independent sodium pump activity respectively (Proverbio, Robinson & Whittembury, 1970; Whittam & Willis, 1963; Whittembury, 1968). However, the specificity of ethacrynic acid is in dispute, this drug having been demonstrated to cause secondary and possibly species dependent effects. Investigations carried out with these two inhibitors on sodium and potassium movements in kidney slices might shed some light on their specificity, the degree of species variation, and the relative importance of the two proposed modes of sodium transport.

The mechanism of salt, water, sugar and amino acid transport by the kidney tubule and the intestinal epithelium have many similarities (Schultz & Curran,1970; Kleinzeller, 1971) including similar sensitivities to the salt retaining hormones aldosterone and angiotensin (Castles & Williamson, 1965; Crocker & Munday, 1970; Barraclough, Jones & Marsden, 1967; Crocker & Munday, 1967). An investigation of angiotensin action in guinea-pig and rat kidney, and rat colon, and the observation of some of the ionic requirements of this action might lead to a greater understanding of the action of this hormone in transporting epithelia.

Structure of transporting epithelia

The structure of the intestine is typical of transporting epithelia. It is a three-layered structure, the innermost layer facing the lumen is the mucosa, the middle layer, the sub-mucosa, and the outer layer, the serosa. In contrast, the kidney tubule is more simple, being composed of a monolayer of epithelial cells, whilst amphibian

skin is intermediate in complexity.

Whatever the structure of the epithelium, it is generally assumed that the transport properties of the tissue reside in the specialised cells of the mucosal epithelium. Most epithelial cells have a large luminal surface area, due to the presence of microvilli, forming a brush border layer.

Adjacent epithelial cells are fused together at their apical margins by a terminal bar, obliterating the space between the two unit membranes. This is the zonula occludens of Farquar & Palade (1963), otherwise known as the tight junction. The presence of tight junctions and microvilli are characteristic of transporting epithelia, and form the basis for the concept that transepithelial transport is transcellular rather than intercellular. However, the assumption that the tight junctions are impermeable barriers is probably erroneous, and will be discussed in more detail later.

It is well known that sodium transport is dependent upon oxidative metabolism in a wide range of epithelial tissues including toad bladder (Leaf, Page & Anderson, 1959), frog skin (Zerahn, 1956), small intestine (Barry, Dickstein, Matthews, Smyth & Wright, 1964), rat colon (Edmonds & Marriott, 1968) and kidney (Whittam & Willis, 1963). Furthermore, in many tissues, including toad bladder (Leaf, 1965) and kidney (Lassen, Munck & Thaysen, 1961; Kiil, Auckland & Refsum, 1961) there is a tight relationship between active ion transport and oxygen consumption. These observations are supported morphologically by the presence of large numbers of mitochondria in the epithelial cells of the transporting epithelia.

Evidence that transepithelial sodium transport is active

It has been shown in amphibian (Geibisch, 1968) and mammalian proximal tubule (Geibisch, Klose, Malnic, Sullivan & Windhager, 1964)

in small intestine (Hendley & Smyth, 1957; Rummel & Stupp, 1960), in frog skin (Koefoed-Johnsen & Ussing, 1958) and in toad bladder (Leaf, 1965), that the transport of sodium from the mucosal to the serosal solution may take place against a chemical gradient, although, normally in the intestine and proximal tubule, a sodium concentration gradient does not develop. The existence of a chemical gradient does not preclude the possibility of passive transport if a downhill electrical gradient exists across the epithelium. However, it has been shown, in kidney tubules (Geibisch, 1961; Burg & Orloff, 1971; Malnic & Aires, 1970), rat jejunum (Barry, Smyth & Wright, 1965) and colon (Edmonds & Marriott, 1967), frog skin (Koefoed-Johnsen & Ussing, 1958) and toad bladder (Leaf, 1965) that these epithelia generate an electrical potential, the lumen being negative with respect to the blood, against which sodium transport can take place.

Using the short-circuit current method of Ussing & Zerahn (1951), Leaf, Anderson & Page (1958) set up toad bladders with identical Ringer on each side, and found that when they reduced the transepithelial potential difference to zero, current flowed in the external circuit. Repeating this experiment in the presence of ^{22}Na and ^{24}Na, they were able to show asymmetric sodium movements, the net sodium flux being equal to the short-circuit current, confirming the similar observations of Ussing & Zerahn (1951) in frog skin. Using mammalian intestine, Barry, Smyth & Wright (1965) and Edmonds & Marriott (1968) have shown that in this tissue also, net sodium transport occurs in the absence of a chemical or electrical gradient.

Further evidence that sodium transport is active is provided by the work carried out with metabolic inhibitors, which reduce or abolish the flow of sodium from the lumen to the blood, without altering the passive blood to lumen sodium flux (Curran, 1960; Schultz & Zalusky, 1964). From the results quoted, we may propose that while serosal to

mucosal movements of sodium are passive, mucosal to serosal movements require an active step.

The classical model of active sodium transport

Koefoed-Johnsen & Ussing (1958) proposed the following model for transepithelial ion transport in frog skin, a model which is generally accepted as being applicable to all transporting epithelia.

The mucosal membrane is permeable to sodium but less permeable to potassium, thus behaving as a sodium electrode. Sodium enters the cell passively through the mucosal membrane down its electrochemical gradient, as shown by the dotted arrow in Fig 1., passes across the cell, and is extruded at the basal and lateral membranes into the extracellular spaces. The basal and lateral membranes behave as potassium electrodes, that is, they are permeable to potassium but less permeable to sodium. The extrusion of sodium is active and involves an exchange of sodium ions for potassium ions, as shown by the solid arrows in Fig 1. The enzyme system causing this sodium extrusion and potassium uptake is proposed to be the sodium potassium ATPase, first described by Skou (1957). The ratio of potassium influx to sodium efflux, at least in toad bladder, is less than one (Essig & Leaf, 1963) leading to an inbalance of charge across the epithelium making the lumen negative relative to the extracellular fluid, which, they propose, causes the movement of chloride from the lumen across the epithelium and into the extracellular fluid. This results in an osmotic gradient which is the driving force for osmotic water flow across the epithelium.

Mucosal entry of sodium

It has been shown in many tissues that the potential across the mucosal membrane is in favour of passive entry (Cereijido & Rotunno, 1967; Leaf, 1965; Rose & Schultz, 1971). It has also been shown in

actively transporting frog skins (Koefoed-Johnsen & Ussing, 1958), intestine (Lindeman & Solomon, 1962; Schultz & Curran 1968) and proximal kidney tubules (Geibisch, 1960, 1968; Windhager & Geibisch 1965) that the cell sodium concentration is considerably lower than that in the mucosal solution. However, Cereijido, Reisin & Rotunno (1968) and Biber, Chez & Curran (1966) have been able to demonstrate sodium uptake, by frog skins, from a mucosal solution containing a considerably lower concentration of sodium than is found in the epithelial cells. This observation could be explained in terms of passive uptake if the activity, as opposed to concentration, of sodium ions within the cell is low as has beensuggested by Lee & Armstrong (1972).

Although the entry of sodium into the epithelial cells of most transporting tissues appears to be a passive process, this entry step is probably not simple diffusion. In toad bladder, Frazier has shown that this entry exhibits saturation kinetics (Frazier & Leaf, 1964) and is competetively inhibited by other alkali metal ions, guanidine and aminoguanidine (Frazier, 1964). This evidence is in favour of a specific interaction at the brush border, and indicates that sodium entry may by carrier mediated, a conclusion reached by Frizzell & Schultz (1972) in a more recent study.

Intracellular sodium pool

Little evidence is available concerning the state of sodium inside the cell. After entry, it is assumed that sodium moves across the cell to be extruded at the lateral and basal membranes. Many workers have suggested that the intracellular concentration of sodium is low, (Lee & Armstrong, 1972; Rotunno, Kowalewski & Cereijido, 1967) and Cereijido & Rotunno (1967) propose that only a small proportion of cell sodium is involved in active transport. These results suggest a small sodium pool. The size and number of transport pools is not easily determined,

and a range of values have been reported. Frazier, Dempsey & Leaf (1962) and Finn & Rockoff (1971), suggest that in toad bladder, as much as 50% of tissue sodium may be in the transport pool, whereas, Nagel & Adolf (1971) and Rotunno & Cereijido (Quoted by Rotunno et al., 1966) believe that in frog skin, this value may be as little as 10%. These differences may reflect a tissue and species variation, dependent on the sodium gradient normally existing across the epithelium. The total number of pools in epithelial cells is not known, although Cereijido, Reisin & Rotunno (1968) propose the existence of three, and this has been suggested as the number in frog skin (Finn & Rockoff, 1971), although, Vanatta & Bryant (1970) suggest as many as seven in toad bladder.

At this time, techniques are not available to accurately measure pool size or number, and we may only assume that not all cell sodium is readily exchangeable and that only a small proportion of this cell sodium is in the transport pool. Other interpretations are that only a small fraction of the epithelial cells are involved in sodium transport, or that sodium transport is extracellular.

Serosal exit of sodium

It has been well documented that sodium extrusion from the epithelial cells into the blood is against a chemical and electrical gradient, in the following tissues; mammalian intestine (Barry et al., 1964, 1965; Edmonds & Marriott, 1967, 1968, 1970), kidney proximal tubule cells (Geibisch, 1960, 1968; Windhager & Geibisch, 1965), frog skin (Koefoed-Johnsen & Ussing, 1958) and in toad bladder (Leaf, 1965). Thus the active extrusion of sodium from the cell to the extracellular fluid must be an active process. The mechanisms of this active transport are described in the following sections.

The sodium potassium ATPase

Skou, in 1957 discovered a membrane bound enzyme in crab nerve which required the presence of sodium, potassium and magnesium for full activity, and which was inhibited by cardiac glycosides such as ouabain. This enzyme has since been demonstrated to occur in all animal cells studied. Whether it is mainly present on the serosal or mucosal membranes is not clear, as in rat intestine, Berg & Chapman (1965) showed that most activity was associated with the brush border, whereas, Quigley & Gotterer (1969) found little on this membrane and propose that most is situated on the serosal membrane. The latter suggestion is supported by work on amphibian epithelia (Farquar & Palade, 1966; Keller, 1963; Bartoszewicz & Barrnett, 1964).

A correlation between Na-K ATPase activity and sodium transport has been observed in many tissues by many workers. Schatzman (1953) first showed that ouabain inhibited cation movements in red blood cells, and Post, Merritt, Kingsolving & Albright (1960) demonstrated a comparable reduction in Na-K ATPase activity. Post et al. (1960) also observed the following correlations between sodium transport and Na-K ATPase activity:-

Sodium transport and the Na-K ATPase both have very specific requirements for ATP as an energy source.

Both mechanisms require the presence of sodium and potassium together in the incubation buffer, the omission of one or other of these cations reducing the activity of sodium transport and enzyme activity to a low level.

The Km's of the two mechanisms are similar.

Ouabain causes a marked reduction in both parameters, the Ki's of this inhibition being identical in the two cases.

The ouabain inhibition of transport and enzyme activity can be competitively antagonised by elevated buffer potassium.

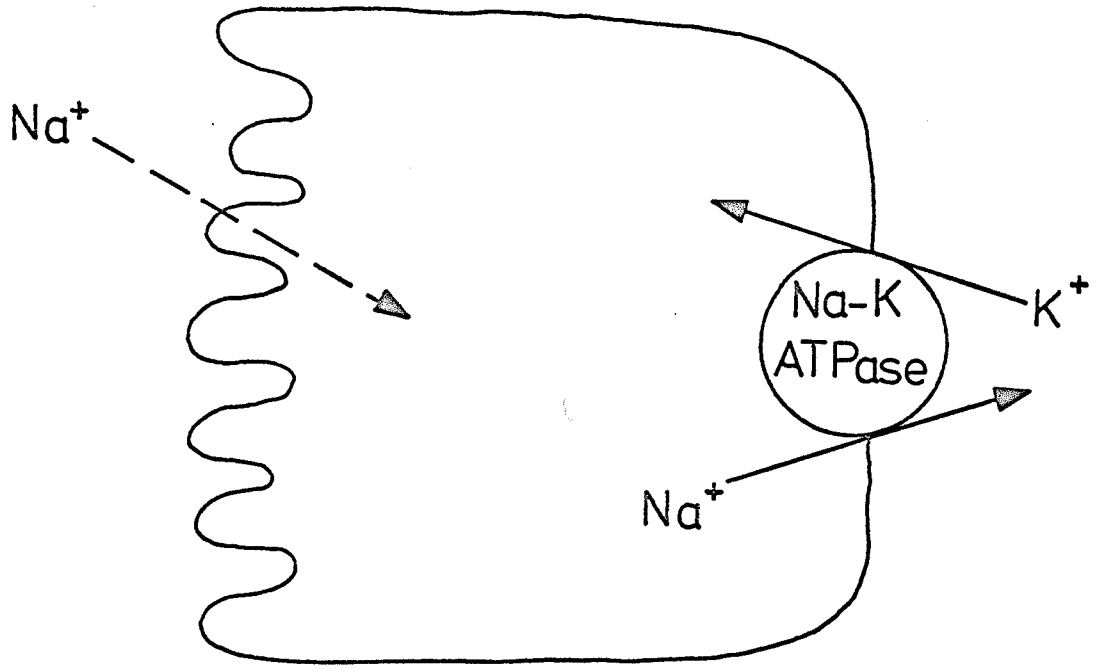
Similar observations have been made in kidney cortex (Whittam & Willis, 1963; Proverbio, Robinson & Whittembury), and in rabbit ileum, the ouabain inhibition of ATPase activity parallels an equivalent inhibition of short circuit current (Schultz & Zalusky, 1964).

It has been shown that ouabain is only active when added to the solution bathing the serosal membranes of the intestine, and is inactive when added mucosally (Schultz & Zalusky, 1964; Edmonds & Marriott, 1968; Newey, Sanford & Smyth, 1968). This is good evidence for the Na-K ATPase being the active serosal sodium pump, as ouabain cannot cross cell membranes (Landon, Jazeb & Forte, 1966; Csaky & Hara, 1965). Again, ouabain inhibited sodium transport and Na-K ATPase activity to a similar degree in frog skin (Koefoed-Johnsen, 1957), toad bladder (Bonting & Canady, 1964) and chicken kidney (Orloff & Burg, 1960).

A change in the rate of sodium transport is often associated with similar changes in Na-K ATPase activity. For example, colons from toads on a high sodium diet (Ferreira & Smith, 1968) and kidneys from adrenalectomised rats (Landon et al., 1966) show reduced sodium transport capacity and Na-K ATPase activity. Katz & Epstein (1967, 1968) have studied both Na-K ATPase activity and tubular sodium reabsorption under a variety of conditions, and in all cases such as unilateral nephrectomy, administration of methyl prednisolone and a high protein diet, the increase in tubular sodium reabsorption was paralleled by an increase in Na-K ATPase activity. The activity of other renal enzymes was also measured, and no change in their activity was observed.

The exact mechanism by which the transport of sodium and potassium is coupled to the hydrolysis of ATP has not been fully resolved. Post, Kume, Tobin, Orcutt & Sen (1969) postulated that a cyclic sodium dependent phosphorylation and potassium dependent dephosphorylation of a membrane carrier occurs, and suggested that sodium may bind to the phosphorylated carrier, whilst potassium has a higher affinity for the

Fig 1. The Classical Model of Active Sodium Transport



Sodium enters the cell through the mucosal membranes down an electrochemical gradient. This ion is then actively pumped out of the cell via the serosal membranes in exchange for potassium.

carrier in its nonphosphorylated form. They have shown the existence of a membrane fraction which can be phosphorylated by ATP and hydrolysed in the presence of potassium ions, this hydrolysis being inhibited by ouabain.

Second sodium pump

An indication that sodium could be actively transported independently of the sodium potassium exchange pump was observed by Hoffmann (1966) and Hoffmann & Kregenow (1966), who showed that, in red blood cells, removal of potassium or the addition of a maximally inhibiting concentration of ouabain, although abolishing potassium uptake, only reduced sodium transport by about 50%. This residual sodium movement was found to be totally inhibited by the diuretic ethacrynic acid. Measuring pump activities during substrate depletion, and after the addition of ATP analogues, indicated that these two mechanisms had different energy sources.

Kleinzeller (1961) and Kleinzeller & Knotkova (1964) observed that sodium loaded, potassium depleted kidney cortex slices extrude sodium, chloride and water as an isotonic solution in the absence of potassium or in the presence of ouabain. The sodium potassium exchange pump is inhibited under these conditions, so that the sodium, chloride and water extrusion must occur by a different mechanism. These authors proposed that the cells of the cortex had a contractile mechanism, actively squeezing fluid out of the cells. Whittembury and his associates have carried out series of experiments to further investigate these findings, and from their work on guinea-pig kidney cortex slices (Whittembury, 1966, 1968; Whittembury & Proverbio, 1970) and perfused toad kidney (Whittembury & Fishman, 1969) and from the work carried out by other investigators (Macknight, 1968; Willis, 1966, 1968; Burg & Orloff, 1964), the existence of two separate pumps in the kidney is

fairly well established. A potassium independent pump transporting sodium, chloride and water, regulating cell volume and being mainly concerned with the net transport of fluid, this pump being insensitive to ouabain, but inhibited by ethacrynic acid. The potassium dependent, ouabain sensitive sodium potassium exchange pump, they suggest, is only involved in the maintenance of cell sodium and potassium content, and perhaps the net movement of potassium. Support for this theory is provided by the work of Munday, Parsons & Poat (1971), Maude (1969, 1970) and Robinson (1971). The existence of a similar chloride linked sodium pump in the intestine has been suggested (Robinson, 1970; Barry et al. 1965), and Barry proposes that this mechanism is electrically neutral and stimulated by metabolisable hexose sugars such as glucose, mannose and fructose. The actively transported sugars such as glucose and galactose on the other hand may stimulate the sodium potassium exchange pump, which appears to be, at least in the intestine, electrogenic. This suggestion is supported by work carried out by Taylor, Wright, Schultz & Curran (1968). There is also some evidence that amino acids stimulate the sodium potassium exchange pump (Robinson, 1970).

The presence of a neutral sodium chloride pump in the gall bladder has been described (Diamond 1964, 1969; Martin & Diamond, 1966). This epithelium does not generate a potential, yet transports large quantities of isotonic sodium chloride. According to the Ussing model, this transport cannot be coupled to the sodium potassium exchange pump. Diamond and his coworkers also showed that replacing buffer chloride by sulphate causes a marked reduction in both sodium transport and oxygen consumption. They propose that this neutral sodium chloride extrusion causes the development of local osmotic gradients in the intercellular spaces which drag water in the same direction, a proposal which they have formulated into the standing gradient theory.

The existence of two sodium pumps in a range of tissues seems

highly likely. The existence of a neutral sodium chloride pump has been proposed in muscle cells from work carried out with frog skeletal muscle (Leblanc & Erlij, 1969).

Extracellular transport

In addition to transcellular sodium transport, Ussing & Windhager (1964) propose a second route for sodium transport. In this model sodium, chloride and water are transported through the tight junctions between the cells into the extracellular fluid. Although these tight junctions appear anatomically to be completely fused (Farquar & Palade, 1963), Geibisch and coworkers (Windhager, Boulpaep & Geibisch, 1967; Geibisch, Boulpaep & Whittembury, 1971) have demonstrated that in kidney proximal tubules, the electrical resistance across this region is low compared to the resistance across the cell membranes. In addition Whittembury & Rawlins (1971) have demonstrated the movement of sulphate, a relatively large molecule, through the tight junctions into the peritubular spaces.

An extracellular transport route was proposed in frog skin to account for inconsistencies in the classical Ussing model of transepithelial transport. Although the concentration of free sodium ions in epithelial cells appears to be low, the approximate concentration in frog skin appears to be in the order of 30mM (Rotunno et al., 1967). Yet Cereijido, Reisin & Rotunno (1968) were able to show uptake from a solution containing 1mM sodium. This observation could either be interpreted on the basis of extracellular transport or by postulating a mechanism of active mucosal uptake. That active mucosal uptake could occur via the Na-K ATPase seems unlikely, as Farquar & Palade (1966) have shown that the mucosal membranes of frog skin are devoid of significant quantities of this enzyme.

Edmonds & Pilcher (1972) and Barnaby & Edmonds (1969) proposed

that an extracellular route for potassium transport exists in rat colon, since a large proportion of transported potassium does not exchange for cell potassium. They also showed that the epithelial resistance to potassium is very low, and were able to demonstrate two components of potassium transport, one of which had a fast turnover, and was most easily explained by postulating an extracellular route. Edmonds & Nielsen (1968) suggest a similar route for sodium transport across the colon, as sodium and chloride movements across the mucosal barrier were not consistent with passive diffusion. Although the mucosal barrier has some ATPase activity in this tissue (Berg & Chapman, 1965), active mucosal uptake would result in an increased cell sodium concentration with increased transport rates, and blocking transepithelial transport would abolish the regulation of cell ion content, which is not consistent with the available evidence. The preceeding observations lead Cereijido & Rotunno (1968) to propose the following model for extracellular transport. This is shown in Fig 2.

The outer leaflets of the mucosal membrane are lipid, and have fixed polar groups with a high selectivity for sodium and lithium. This proposal is supported by the observations of Morel & Leblanc (1973) that sodium and lithium are accumulated by the stratum granulosum of frog skin, from the bathing solution, against a concentration gradient, and independent of serosal extrusion. The sodium ions reaching the membrane, they suggest, have a greater tendency to move tangentially than to enter the cell, jumping from fixed charge to fixed charge until they reach the zonula occludens. The outer non-lipid layer covering the innerfacing cell membranes have fixed charges selective for potassium, and prevent sodium from crossing. Pumps are located on this membrane, and sodium is actively transported across this membrane into the extracellular fluid. Additional support for this theory exists:-

a) Passing current inwards across the epithelium will cause

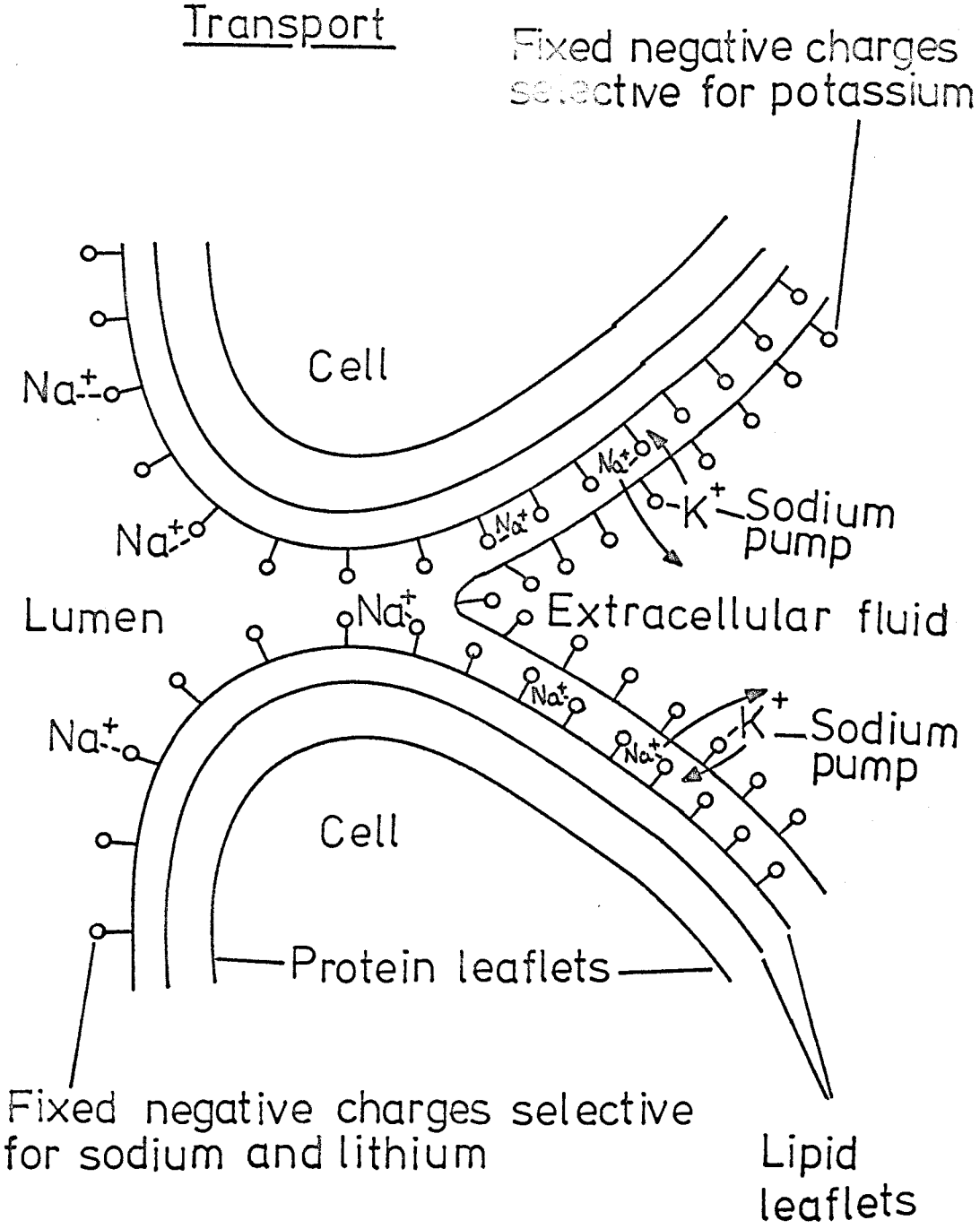
sodium ions to move from the mucosal to the serosal solution. Passing current in the reverse direction should cause potassium ions to move towards the lumen, thus blocking the pores. This should increase the transepithelial resistance, and indeed, such observations have been recorded both in frog skin (Candia, 1967) and in Necturus proximal tubules (Spring & Paganelli, 1972). If the outer mucosal membrane has a high preference for sodium over potassium, high mucosal potassium concentrations should increase the epithelial resistance. This was demonstrated and shown to be reversible (Ussing, 1969; Vôute & Ussing, 1970). Ussing also observed that shrinkage decreased, and swelling increased the short circuit current, which is in agreement with the model.

b) If all transport is transcellular rather than extracellular, a change in buffer sodium or a change in short circuit current would be likely to have a large effect on the electrical potential of the cell. However, Cereijido & Curran (1965) showed that a 40 fold change in buffer sodium, or a five fold change in short circuit current is without effect on the potential difference of the cell.

c) Studies carried out in rat (Solomon, 1957) and Necturus (Spring & Paganelli, 1972) proximal tubules demonstrated that electrical changes in this preparation are consistent with the existence of an extracellular transport channel, and the role of this described pathway is the subject of much current work.

Thus there is considerable evidence to suggest that some transepithelial sodium movements are intercellular. However, whether this transport is through or between cells, the active step appears to involve an active sodium pump.

Fig 2. Model of Extracellular Sodium Transport



Potassium transport

A widely, but by no means universally accepted view, is that potassium transport is linked to the active transport of sodium in the opposite direction. The main evidence for this is the potassium requirement for transport by the Na-K ATPase. It has been shown by many workers that removal of potassium ions from the incubation medium of transporting epithelia markedly reduces sodium transport, indicating that potassium transport is probably not secondary to sodium transport but tightly linked to it. A similar effect is observed with ouabain, a known inhibitor of the transport ATPase. Whether potassium transport is active, depending on the sodium for potassium exchange pump to provide the energy (Edmonds, 1967), or passive and secondary to the development of an electrical gradient produced by the active transport of sodium (Turnberg, 1971; Koefoed-Johnsen & Ussing, 1958; Leaf, 1965) the rate and direction of potassium movements will depend on the relative permeabilities of the mucosal epithelial cell membranes to this ion.

Chloride transport

According to the classical model of transport by frog skin *in vitro*, chloride movements are passive and secondary to the development of a potential across the epithelium (Ussing & Zerahn, 1951). However, Jørgensen, Levi & Zerahn (1954) suggest active movements *in vivo*, as do Zadunaisky & DeFisch (1964) and Martin (1964) in this tissue. In the small intestine, where a potential exists, *in vitro* transport of chloride appears to be passive. Clarkson & Toole (1964) were unable to demonstrate net chloride movement in the absence of an electrochemical gradient, and measurements by Schultz, Zalusky & Gass (1964) also suggested passive movements of chloride. *In vivo*, chloride transport has been suggested to be active (Curran & Solomon, 1957; Kinney & Code, 1964; Turnberg, Bieberdorf & Fordtran, 1969), although Edmonds &

Marriott (1967) have shown that, in rat colon *in vivo*, chloride movement is passive.

Frömter & Hegel (1966) and Maude (1970) have been unable to demonstrate the existence of a potential across the wall of the proximal tubule of the rat, whereas, Burg & Orloff (1970) have reported the existence of a small potential across the proximal tubule of the rabbit. Burg & Orloff believe this potential to be sufficient to cause passive chloride transport. Malnic, Aires & Vierra (1970) repeating the earlier work of Kashgarian, Stoechle, Gottschalk & Ullrich (1963) showed that, in rats, the equilibrium concentration of chloride in the tubule was the same as that in the plasma, indicating net chloride secretion. Using acidotic conditions and acetazolamide, they concluded that these chloride movements are dependent upon the acidification of the proximal urine, and propose an HCl secreting pump. Rector & Clapp (1962) suggest that these movements obey the Nernst equation, and are therefore passive. Recent evidence provided by the work of Rocha & Kokko (1973) indicates that, in the thick ascending loop of Henle, chloride transport is active and provides the driving force for sodium and water movements.

Water transport

Although Parsons & Wingate (1961) and Hakim, Lester & Lifson (1963) suggest the existence of active water movements in the intestine of the rat and dog respectively, most investigations into the mechanism of water transport support the generally accepted view that water follows solute and is, therefore, passive. Curran (1960) and Clarkson & Rothstein (1960) were unable to demonstrate net water transport in the absence of an osmotic gradient across different regions of the intestine. In frog skin (Kirschner, Maxwell & Fleming, 1960) and toad bladder (Hays & Leaf, 1962) fluid transport has been shown to be passive, although it may not be tightly linked to sodium (Bentley, 1958).

Similarly, Whittembury, Oken, Windhager & Solomon (1959) have demonstrated that water movements in the kidney are passive and secondary to solute movements, and Whittembury and his associates have good evidence to propose that these water movements are secondary to sodium transport by the second sodium pump (Whittembury, 1968; Whittembury & Proverbio, 1970).

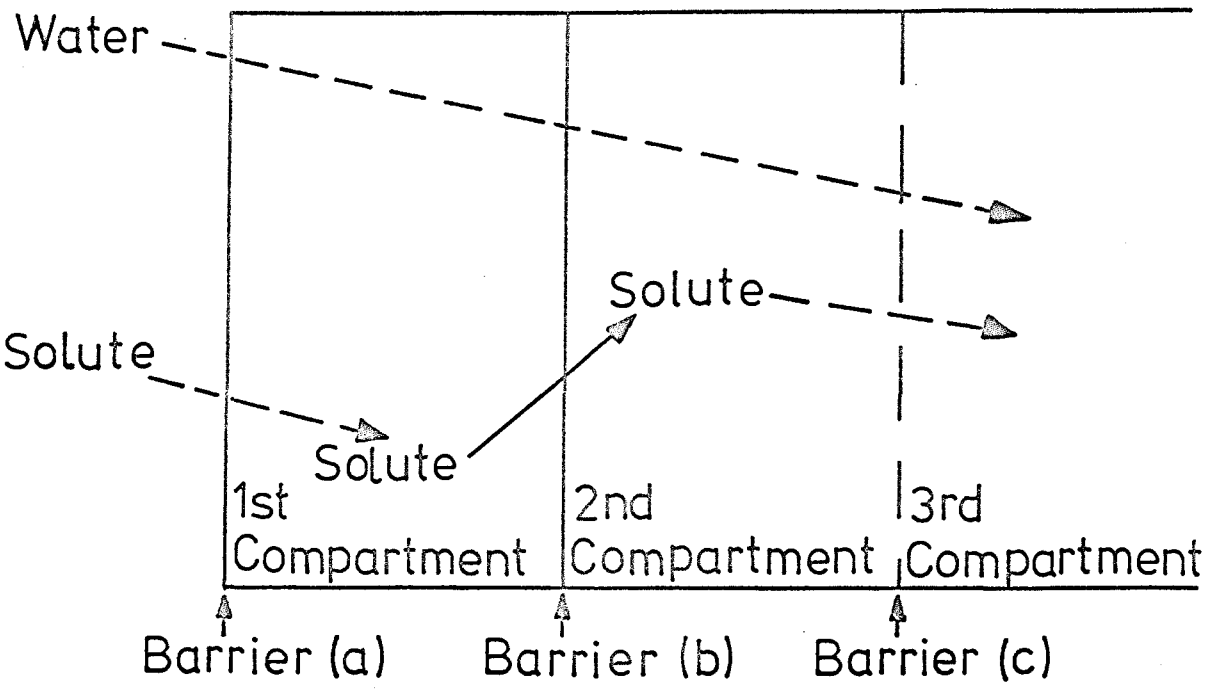
There are three main models for water flow, all requir ing the existence of three compartments. They propose that solute is actively transported across the barrier between compartment one and two. This causes osmotic water flow into compartment two. The increased hydrostatic pressure generated in compartment two causes water to flow from compartment two into compartment three.

The Curran Model

This model is shown in Fig 3.

Curran (1960) proposed the following model for water transport across epithelia. Solute enters compartment one through barrier (a). It is then actively transported into compartment two through barrier (b). This causes osmotic water flow from the mucosal solution via compartment one, the cell, through barrier (b), the serosal and lateral membranes, into compartment two, the extracellular fluid, causing an increased hydrostatic pressure in this compartment. This causes fluid movement from compartment two through barrier (c), possibly the basal membranes, into compartment three, possibly the blood. This model requires that the reflection coefficient of barrier (b) is higher than that of barrier (c), so that water movements from compartment three to compartment two are limited, as Staverman (1951) has shown that the osmotic pressure across a membrane is directly related to its reflection coefficient. Curran & MacIntosh (1962) were able to demonstrate water transport across a model epithelium using dialysis membrane and sintered glass as barriers (b) and (c) under conditions where the

Fig 3. The Curran Model of Water Transport



Solute enters compartment 1 through barrier (a). It is then pumped into compartment 2 through barrier (b), generating an osmotic gradient. Water then flows through barriers (a) and (b) into compartment 2 down its osmotic gradient. An increased hydrostatic pressure in compartment 2 causes fluid flow into compartment 3.

solute concentration in compartment two was higher than in compartment one.

The Diamond Model

This model is shown in Fig 4.

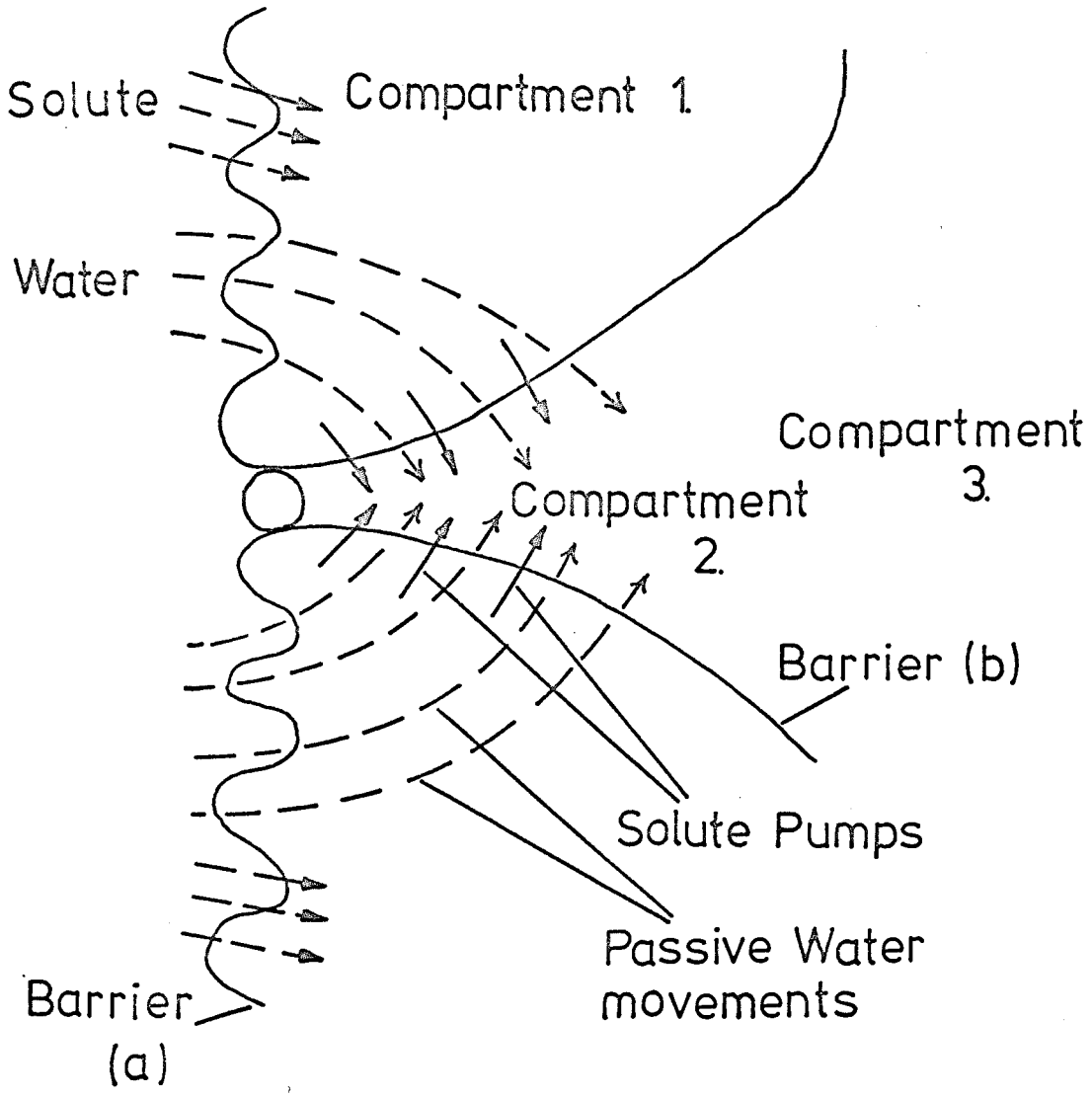
Diamond (1964, 1969; Diamond & Bossert, 1967) modified the Curran model in the following way. He proposed that solute enters the cell, compartment one, through the brush border membrane, barrier (a), and is actively transported into the intercellular channels, compartment two, via the lateral cell membranes, barrier (b), giving rise to high concentrations of solute in these channels. Solute then diffuses down its concentration gradient towards the open ends of the channels, and simultaneously, water flows into this compartment down its osmotic gradient, so that osmotic equilibrium is achieved at the end of the channel. As the process is continuous, the result will be a standing osmotic gradient, from the tight junction to the open end of the channel. The fluid of constant osmolarity, distal to the intercellular channels constitutes compartment three. This model does not propose the existence of any barriers other than the brush border and the lateral cell membranes.

The Fordtran Model

This model is shown in Fig 5.

This model proposed for human jejunum by Fordtran, Rector & Caster (1968) is similar to the Diamond model, but involves the intercellular movements of water and sodium chloride in response to an osmotic gradient set up by the active transcellular transport of glucose and bicarbonate. They propose that the tight junctions are permeable to sodium, chloride and water, but impermeable to glucose and bicarbonate. It is suggested that sodium bicarbonate and glucose are transported into

Fig 4. The Diamond Model of Water Transport

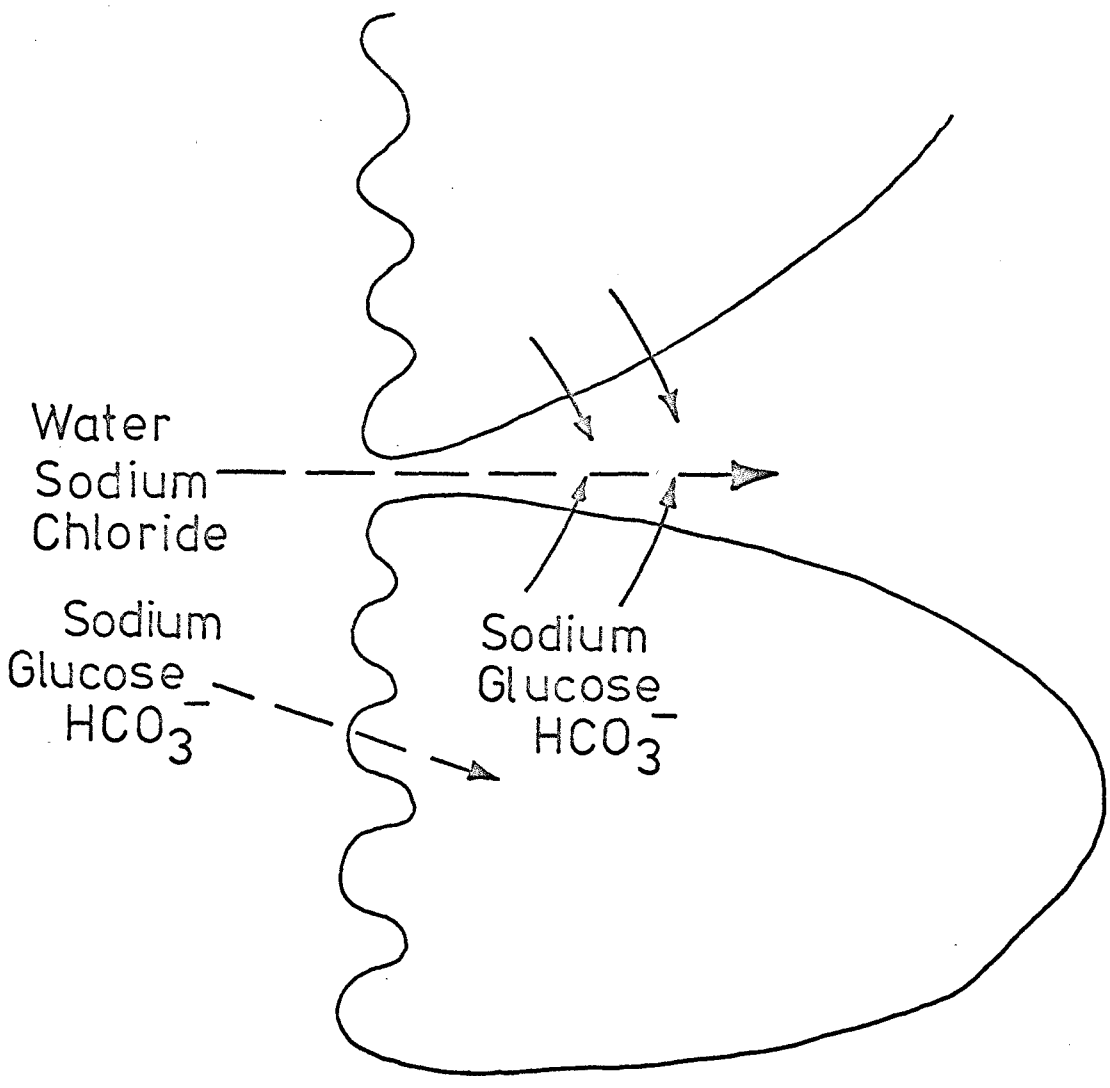


Solute enters the cell (compartment 1) via the brush border membrane (barrier (a)) and is pumped through the lateral cell membranes (barrier (b)) into the intercellular spaces (compartment 2). Water then flows into this compartment down an osmotic gradient, so that osmotic equilibrium is achieved at the end of the channel (compartment 3).

the intercellular channels generating a local osmotic gradient. Sodium chloride and water flow through the tight junctions down this osmotic gradient.

In all three models, it is assumed that water transport is passive and is in response to osmotic gradients, generated by the active transport of sodium.

Fig 5. The Fordtran Model of Water Transport



Sodium, bicarbonate and glucose enter the cell and are pumped into the intercellular spaces generating a local osmotic gradient. Water diffuses down this gradient through the tight junctions carrying sodium and chloride with it.

HORMONES

Angiotensin

Structure

Angiotensin II is an octapeptide having the structure indicated below, and first elucidated by Elliott & Peart (1957).

NH_2-Asp-Arg-Val-Tyr-Val-His-Pro-Phe-COOH

This is the form of angiotensin found in most mammals, although in man and the pig, valine 5 is replaced by isoleucine.

Formation and breakdown

The hormone renin, a protein with a molecular weight of 40,000 approx is secreted from the juxtaglomerular cells of the kidney. It acts on its substrate, angiotensinogen, in the plasma, cleaving the molecule at the leucyl-leucyl bond, to produce the decapeptide, angiotensin I. Renin substrate is an alpha globulin of molecular weight 57,000 approx produced by the liver. Angiotensin I has limited physiological activity, although it will bind to the adrenal medulla more strongly than angiotensin II (Lin & Goodfriend, 1970) and has been demonstrated to have a direct dipsogenic action (Bryant & Falk, 1973). This compound is converted to angiotensin II by the removal of the dipeptide histidyl-leucine from the C terminal end of the peptide, in the presence of converting enzyme found mainly in the lungs, but also in the plasma. Angiotensin II is broken down by angiotensinases present in most tissues including the kidney and intestine, and non-specifically by peptidases.

Control of formation and activity

The amount of angiotensin in the circulation is controlled mainly by alterations in the rate of renin secretion, and in changes in renin activity. The levels of circulating renin are controlled by various factors. Haemorrhage, or a reduction in blood flow to the kidney are powerful stimuli for renin release. Changes in body sodium affect the levels as does feeding a low sodium diet, the latter leading to increased renin secretion, whereas, sodium loading depresses renin release (Ganong & Boryczka, 1967). Although the detailed mechanism by which renin release is induced is not well understood (see review by Lee, 1969), two theories have been proposed for the control of renin secretion.

The Baroceptor theory

This theory proposes that the juxtaglomerular cells of the kidney respond to changes in vascular volume and pressure by altering the rate at which renin is released. This theory was first proposed by Tobian (1960). The juxtaglomerular cells may respond to either changes in intravascular afferent arterial pressure, a change in the transmural pressure gradient (this would also take into account the renal interstitial pressure), or changes in wall tension (this would also take into account any changes in arterial diameter).

The Macula Densa theory

Vander (1967) proposed that the macula densa is sensitive to a decreased sodium load, an increased load at the macula densa reducing renin release. Thurau and his associates (Thurau, Valtin & Schnerman, 1968) believe, however, that it is an increase in sodium load at this point that is the stimulus for renin release.

Nervous control

There is considerable evidence for an involvement of the renal nerves in controlling renin secretion, stimulation of these nerves causing renin release (Vander, 1967). Kidney denervation has also been shown to block the increase in plasma renin activity in response to haemorrhage (Hodge, Lowe & Vane, 1966).

Control of renin activity

Besides alterations in the plasma renin concentration, renin activity may also vary (Harris, Munday, Noble & Winch, 1973). A renin inhibitor has been demonstrated (Sen, Smeby & Bumpus, 1967), and a renin activator may exist. However, little is known about the mechanism by which these compounds act.

The actions of angiotensin

The cardiovascular system

Angiotensin is a powerful constrictor of vascular smooth muscle, causing a marked increase in blood pressure. There is not, however, an increase in cardiac output which may in fact decrease. See review by Regoli (Regoli, Park & Rioux, 1974)

The nervous system

It has been suggested that angiotensin has a direct effect on the hypothalamus, intracranial injections of renin or pure angiotensin causing a rapid drinking response in the rat (Fitzsimons, 1970; Epstein, Fitzsimons & Rolls, 1970) and the monkey (Sharpe & Swanson, 1974). ADH release has also been proposed to occur in response to low doses of angiotensin (Mouw, Bonjour, Malvin & Vander, 1971). In addition, increases in the rate of noradrenaline secretion and synthesis, and a

reduction in its breakdown have been proposed (Zimmerman, Gomer & Liao, 1972; Roth, 1972; Khairallah, 1972).

The control of aldosterone secretion

It has been demonstrated in dogs (Davis, 1959; Ganong, Mulrow, Boryczka & Cera, 1962) and sheep (Blair-West et al., 1965) that angiotensin will stimulate the secretion of aldosterone. This appears to be via a mechanism separate from that utilized by ACTH, as basic differences exist between the activity of these two hormones, angiotensin not acting through adenyl cyclase, having a different time course of action, and different ionic requirements. Further, angiotensin has a relatively high specificity for aldosterone secretion, whereas, ACTH has little effect on aldosterone secretion, but a high specificity for cortisol release (Ganong, Biglieri & Mulrow, 1966). The rat, however, appears insensitive to this effect of angiotensin. Eilers & Peterson (1964) failed to find a decrease in aldosterone secretion following nephrectomy, and Marieb & Mulrow (1964) could not demonstrate an increase in aldosterone secretion following angiotensin infusion. Increases in aldosterone secretion by the rat have been observed, but only with concentrations of angiotensin well outside physiological limits (Dufau & Kliman, 1968).

Actions on the kidney

The physiological effects of angiotensin are mainly on salt and water handling by the kidney. These actions have been reviewed by Thurau et al. (1968). Angiotensin is known to affect the renal handling of sodium in a biphasic manner (Barraclough, Jones & Marsden, 1967), low doses causing antinatriuresis and antidiuresis, high doses causing natriuresis and diuresis.

The diuretic actions of the hormone are generally accepted as

being due to a direct effect on transtubular transport (Malvin & Vander, 1967). However, whether the antinatriuretic and antidiuretic actions are also due to a direct effect on the tubules, or are secondary to renal vasoconstriction is still in dispute. Barraclough et al. (1967) believe that this response is a result of a direct tubular action, whereas, Malvin & Vander (1967) and Bonjour & Malvin (1969) suggest that antinatriuresis and antidiuresis are secondary to renal vasoconstriction which in turn leads to a reduction in glomerular filtration rate. In support of this view, Carrière & Friborg (1969) observed a redistribution of blood flow in the cortex following the infusion of low doses of angiotensin. However, Lindheimer, Lalone & Levinsky (1966) and others have shown that is possible to change GFR considerably without affecting sodium excretion. In vitro studies by Munday et al. (1971) on rat kidney cortex slices have shown that low doses of angiotensin (10^{-12}M) will stimulate the extrusion of sodium from these slices, indicating that angiotensin has some direct actions on tubular sodium reabsorption processes, as this preparation is devoid of an intact blood supply.

The stop-flow experiments carried out by Vander (1963) indicate that angiotensin exerts an inhibitory effect on fluid transport by the distal tubules and collecting ducts. Leyssac (1964) and Munday et al. (1971), however, suggest that an action at the proximal tubule is likely.

Actions on the intestine

The existence of an extra-adrenal factor controlling the transport of sodium in the intestine was first proposed by Spencer (Ross & Spencer, 1954), who noted that sodium loading resulted in a further reduction in sodium transport in adrenalectomized rats. Using spironolactone in place of adrenalectomy, Clarke, Miller & Shields (1967) showed a similar reduction in transport following sodium loading in the dog. The first

direct observations of an involvement of angiotensin in the control of intestinal sodium transport were made by Crocker & Munday (1967, 1969, 1970). They showed that fluid transport by jejunal sacs prepared from sodium loaded or adrenalectomised, nephrectomized rats is significantly increased in the presence of low doses of angiotensin. Davies, Munday & Parsons (1970) extended these studies, and showed that the ileum and colon as well as the jejunum are sensitive to the hormone, and furthermore, that low doses (10^{-11} - 10^{-12}M) of angiotensin stimulate transport, whereas, high doses (10^{-8} - 10^{-9}M) are inhibitory. These experiments were carried out on stripped sacs where the muscle and nerve plexi are removed, suggesting that these effects are due to the direct action on a transport mechanism. Observations in vivo have also demonstrated a biphasic action of angiotensin on jejunal fluid transport (Bolton, Munday, Parsons & Poat, 1974).

Actions on other epithelia

No effect of angiotensin on the short circuit current (scc) generated by toad bladder (Coviello & Crabbe, 1965) or toad skin (Barbour, Bartter & Gill, 1964) have been observed. However, later studies of the action of the hormone on sodium transport by frog skin (McAfee & Locke, 1967) and water transport by toad kidney (Coviello, 1969) and toad bladder (Coviello, 1970) have demonstrated significant increases in these movements. A possible explanation of this apparent discrepancy is that angiotensin, at low concentrations, stimulates a neutral sodium pump which would not involve an increase in scc. An inhibition of fluid transport by the gall bladder with moderate doses of angiotensin has also been observed (Frederiksen & Leyssac, 1969), suggesting the inhibition of a neutral sodium pump.

The mechanism of action of angiotensin

Most polypeptide hormones exert their effects through an action on protein synthesis or through a change in cellular cAMP levels.

Little evidence is available to indicate that angiotensin works via an action on adenyl cyclase. Davies, Munday & Parsons (1972) working on rat descending colon, and Munday, Parsons & Poat (1972) working on rat kidney cortex slices were unable to mimic, inhibit or simulate the effects of angiotensin using cAMP, dibutyryl cAMP or theophyline. Furthermore, no change in kidney cortex cAMP levels was observed following the addition of angiotensin to cortex slices (Munday, Parsons & Poat, 1974). Again Kaplan (1965) concluded that cAMP could not be implicated in the action of angiotensin on the secretion of aldosterone from the adrenal cortex in vivo. In contrast, Coviello (1971) has demonstrated that in isolated toad bladder, both angiotensin and cAMP induced a hydrosmotic water flow, furthermore, that the angiotensin effect was potentiated by theophylline. In addition, Hornych, Meyer & Milliez (1972) demonstrated that both angiotensin and dibutryl cAMP stimulate sodium transport by the ascending colon of the rat.

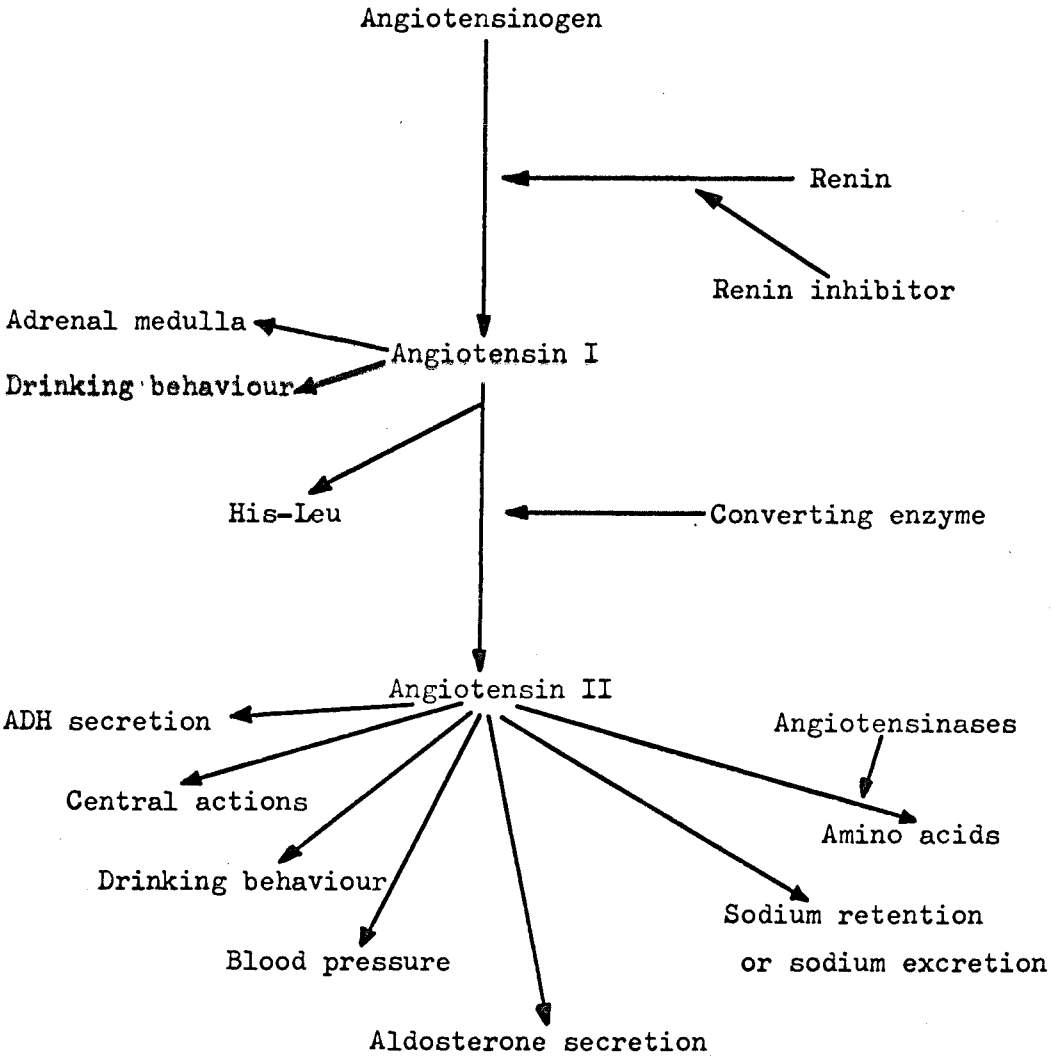
This apparent discrepancy between angiotensin action in kidney cortex slices, descending colon and adrenal cortex on one hand, and toad bladder, and ascending colon on the other may represent a true tissue difference. However, whereas ascending colon (Hornich et al., 1972) and toad bladder epithelia (Ussing & Zerahn, 1951) are sensitive to ADH, no definitive evidence is available for an effect of this hormone on proximal kidney tubules, descending colon, or adrenal cortex. A more likely explanation, therefore, is that the responses of ascending colon and toad bladder to theophylline and cAMP are due to effects on vasopressin sensitive mechanisms rather than angiotensin stimulated processes. Recently a study undertaken in two laboratories has failed to show a stimulation of adenyl cyclase activity or tissue cAMP levels

following angiotensin additions (personal communication Meyer, Paris; Munday, Southampton).

The work of Davies, Munday & Parsons (1969) demonstrated that the angiotensin stimulation of fluid transport by rat colon is inhibited by the protein synthesis blockers puromycin and cyclohexamide, but is unaffected by the transcription inhibitor, actinomycin D. They suggest from these results that angiotensin works via an action on protein synthesis at the stage of translation. A similar study has been carried out using rat kidney cortex slices and comparable results have been obtained (Munday et al., 1972).

Munday et al. (1971) have shown that angiotensin will stimulate sodium extrusion from sodium loaded, potassium depleted rat kidney cortex slices in the absence of potassium or in the presence of maximally inhibiting concentrations of ouabain. This sodium extrusion, they propose, cannot be via the sodium potassium exchange pump, as this is blocked under the conditions used, and they suggest that this stimulation of sodium extrusion is brought about by an increase in activity of a potassium independent pump of the type described by Whittembury (1968) and Whittembury & Proverbio (1970). They also were able to show that the effect of the hormone was only evident after a lag period of three minutes.

The renin angiotensin system:- Summary



Antidiuretic hormone (ADH)

Formation

This hormone is synthesised by the hypothalamus, and released from the neurohypophysis. It is generally accepted that the secretion of ADH is controlled through two main systems, osmoreceptors situated near the supra-optic nucleus in the hypothalamus (Verney, 1947,1948) and volume receptors associated with the atria of the heart and the great vessels (Share, 1968). Consequently, the main stimuli for ADH release are dehydration and haemorrhage, although nervous stimuli such as anxiety may also alter the rate at which the hormone is released.

Actions of ADH

The primary target organ of this hormone is the kidney, where the permeability of the distal tubules and collecting ducts to water is increased, allowing more water to be passively reabsorbed in this region. There is also some evidence that ADH increases sodium transport in the ascending loop of Henle, and decreases medullary and papillary blood flow (Cross, Trace & Vattuone, 1974), both effects resulting in the development of a greater solute gradient across the medulla, which in turn increases the capacity of the kidney to produce concentrated urine.

There is considerable controversy regarding the effects of ADH on the intestine. Auslebrook (1961) reported an increase in sodium transport with high concentrations, whereas, Crocker & Munday (1970) using rat jejunum, Davies *et al.* (personal communication) and Edmonds & Marriott (1968) working on descending rat colon, were all unable to show a response to this hormone. However, Hornych, Meyer & Milliez (1972) have demonstrated an increase in fluid transport by ascending rat colon following the addition of ADH.

Mechanism of action

Much of the work carried out with ADH has utilized amphibian epithelia as a target tissue, and most of the theory of the mechanism of action of this hormone has developed from results obtained using the isolated frog skin and toad bladder. ADH has been shown to increase both passive water and active sodium movements across frog skin (Ussing & Zerahn, 1951) and toad bladder (Crabbé & DeWeer, 1965; Leaf, Anderson & Page, 1958). The increase in passive water movements are proposed to occur by an increase in the permeability of the mucosal membranes (Peachy & Rasmussen, 1961), possibly by an increase in luminal pore diameter as suggested by Whittembury, Sugino & Solomon (1960) in kidney. Thus the increase in active sodium transport is proposed to occur by an increase in the luminal permeability to sodium ions, allowing more of this ion to reach the serosally orientated sodium pumps (Cofré & Crabbé, 1967; Leaf, 1965).

These changes in mucosal membrane permeability are probably due to an increased cAMP level in the epithelial cells, induced as a result of the interaction of ADH with an adenyl cyclase (Leaf, 1965: Orloff & Handler, 1965). It is likely that this model of ADH action is also applicable to the kidney.

Aldosterone

Formation

Aldosterone is a steroid hormone synthesised in the zona glomerulosa of the adrenal cortex. The major control of aldosterone secretion, in species other than the rat, is generally accepted to be via the renin angiotensin system (Ganong et al., 1966). Other important controlling influences are sodium depletion, dehydration and elevated serum potassium (Davis, 1963), while ACTH and ADH appear to have a smaller and less important role.

Actions of aldosterone

In rat kidney in vivo, aldosterone causes a kaliuresis followed by an antinatriuresis (Fimognari, Fanestil & Edelman, 1967; Castles & Williamson, 1965) by an action at the distal tubule. In the intestine, aldosterone has been shown to stimulate sodium reabsorption by human colon (Levitan & Inglefinger, 1965), rat jejunum and ileum (Hill & Clarke, 1969) and rat jejunum (Crocker & Munday, 1970), and to increase potassium secretion by rat colon (Shields, Mulholland & Elmslie, 1966). Aldosterone has also been shown to stimulate sodium transport and scc across amphibian bladder and skin (Sharp & Leaf, 1966; Edelman, 1968; Crabbé & DeWeer, 1964) and toad colon (Cofré & Crabbé, 1967).

Mechanism of action

Aldosterone appears to exert its effect in a way typical of steroid hormones. In brief, the proposed events are: 1) Migration of aldosterone into the cytoplasm of the target cell; 2) Binding of the steroid to its aporeceptor; 3) Transformation of this complex into an active steroid-receptor complex; 4) Migration of the active complex

into the nucleus; 5) Binding to specific genomic sites; 6) Activation or derepression of transcription which results in induction of the synthesis of specific proteins; and 7) Augmentation of sodium transport by the action of aldosterone induced proteins. Evidence has been presented suggesting that this general pattern applies to the action of most steroid hormones (Raspé, 1970).

A series of experiments recently reported by Edelman (Lahav, Dietz & Edelman, 1973) provide further information on the validity of this sequence. From their work with inhibitors on toad bladder, they demonstrated that whereas the early addition of the transcription inhibitor, actinomycin D, blocked the response to aldosterone, addition of this compound towards the end of the latent period did not alter the time of onset or the initial magnitude of the response. Furthermore, Herman (Quoted by Edelman, 1969) demonstrated that the addition of cyclohexamide, an inhibitor of translation, at a time when protein synthesis would be expected to occur blocked the increase in sodium transport. However, if cyclohexamide and aldosterone are added to bladders at the same time, and the cyclohexamide subsequently washed out 75minutes later, the sodium transport response was neither delayed nor reduced in magnitude.

There is evidence from the work of Majumdar & Trachewsky (1971) that aldosterone may enhance both ribosomal RNA synthesis as well as a shift in or augmentation of messenger RNA synthesis. Newer information lends credence to the dual role hypothesis in the regulation of gene expression. Two specific nuclear polymerases have been identified, and Liew, Liu & Gornall (1972) have demonstrated that prior injections of aldosterone enhanced the activities of both these enzymes.

The nature of the synthesised protein, or proteins is unknown, although three suggestions have been made. It may be the Na-K ATPase, although no correlation between aldosterone administration and ATPase

activity has been observed either in toad bladder (Bonting & Canady 1964) or rat kidney (Landon et al., 1966). However, Jørgensen (1968) showed later that aldosterone causes an increase in rat kidney Na-K ATPase activity only after treatment of microsome fractions with deoxycholate. The second proposal is that the protein synthesised is the brush border carrier protein. This is supported by Sharp & Leaf (1966) and Crabbé & DeWeer (1965) who have shown that an increase in the sodium content of toad bladders is induced following aldosterone administration. Thirdly, Fanestil, Porter & Edelman (1967) observed that ADH has a more marked effect in the presence of aldosterone and they propose that aldosterone acts by increasing the energy supply to the sodium pump. However, more recent evidence suggests that the permissive effect of aldosterone is mediated by a steroid dependent decrease in the rate of degradation of cAMP (Orloff, Handler & Staff, 1973).

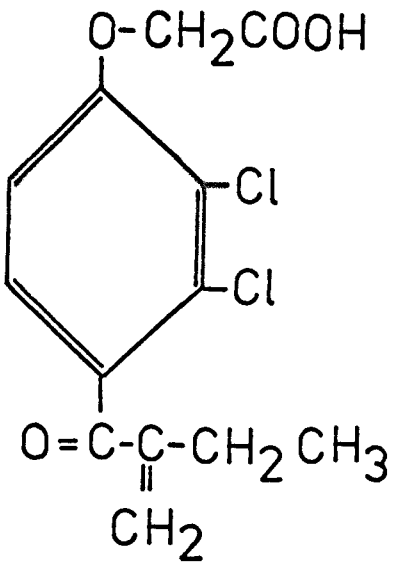
Other hormones

Although several other hormones affect sugar transport (Levin, 1969), little evidence is available for a direct action of these hormones on transepithelial ion transport. Edmonds, Thomson & Marriott (1970) have shown that aldosterone action requires the presence of thyroid hormone for action, and Simone & Solomon (1970) observed that injection of growth hormone into hypophysectomized rats on a high sodium diet caused kaliuresis and antinatriuresis. Insulin has also been implicated in the control of sodium transport; Auslebrook (1965) has observed that the high level of transport in alloxan diabetic rats is reduced on treatment with insulin, and Crabbé (1967) noted an increase in scc and pd after administration of insulin to toad colon sacs.

Inhibitors

Ethacrynic acid

This compound was first synthesised by Schultz et al. (1962) and has the structure shown below.



It was designed to react with the sulphahydryl groups of the transport ATPase, hence the introduction of a conjugated double bond system, a system which has a high affinity for S-H groups. The addition of an oxyacetic acid residue ensures that the compound is excreted into the kidney tubule, a very important criterion in diuretic drug design.

Beyer, Baer, Michaelson & Russo (1965) observed that the intravenous infusion or oral administration of this drug causes a marked saliuresis and diuresis in the dog, the rabbit and to a lesser extent the guinea-pig, whereas it was without effect in the rat. Using rabbit kidney cortex slices, these workers obtained results to suggest that ethacrynic acid is secreted into the proximal tubule and absorbed more distally. They also showed that this compound abolishes the solute gradient from the cortex to the inner medulla, and it has been suggested that the increased sodium load presented to the distal tubule increa ses

the activity of the sodium potassium exchange pump, leading to an increased loss of potassium in the urine (Baer & Beyer, 1966). Stopped flow studies by Beyer et al. (1965) indicate that this diuretic may also act at the level of the proximal and distal tubules.

Mechanism of action

The mechanism of action of ethacrynic acid appears to be highly complex. The molecule was specifically designed to react with the S-H groups of the transport ATPase, and Kraimer & Kaiser (1970) have shown an interaction with protein sulphahydryl groups. Futhermore, Duggan & Noll (1965), Duggan, Baer & Noll (1965), Davis (1970), Bannerjee, Khanna & Sen (1970) have demonstrated an inhibition of the Na-K ATPase in vitro, although Robinson (1972) was unable to obtain such an effect using dog kidney cortex slices. It is doubtful that the effects of ethacrynic acid on the transport ATPase can explain the actions of the inhibitor in vivo, as the findings of Nechay et al. (1967) and Hook & Williamson (1965) demonstrate that it is possible to obtain an inhibition of ATPase activity after in vivo administration of ethacrynic acid to rats without a concommitant natriuretic and diuretic response.

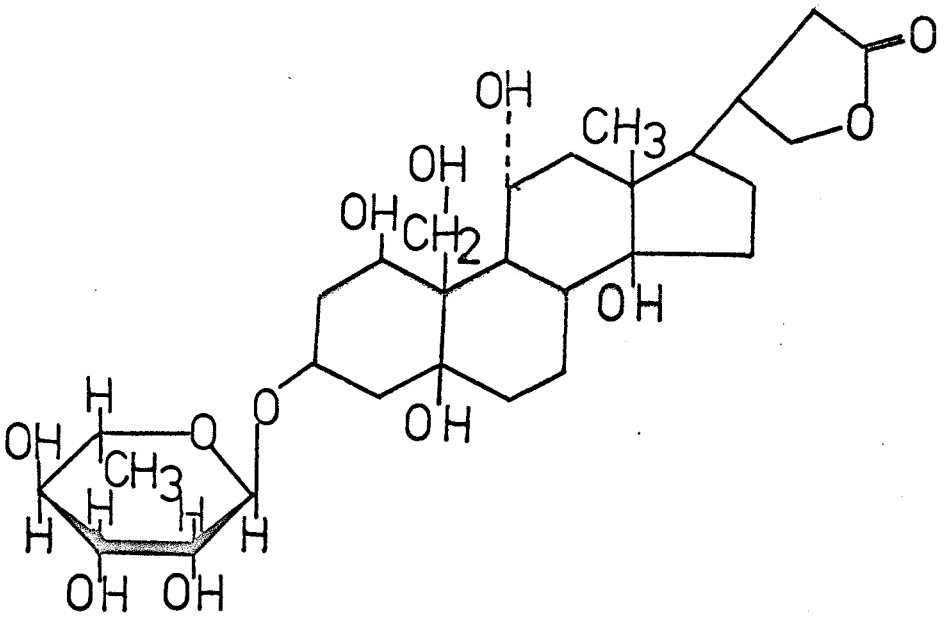
Whittembury & Fishman (1969) using perfused toad kidney showed that ethacrynic acid causes an increase in cell sodium and chloride without any change in cell potassium. These results and the results of experiments performed by Whittembury (1968) and Whittembury & Proverbio (1970) on kidney slices, Hoffman & Kregenow (1966) on red blood cells and Leblanc & Erlij (1969) on frog skeletal muscle, led to the proposition that ethacrynic acid is a specific inhibitor of a second sodium pump independent of the sodium potassium exchange pump. However, there are indications that the effect of ethacrynic acid may be more complicated than was first suspected. Jones & Landon (1967) have shown that the drug inhibits respiration in kidney cortex slices with a

relatively slight effect on glycolysis, suggesting a direct mitochondrial action. A primary action on metabolism was also suggested by the experiments of MacKnight (1969) and Poat, Poat & Munday (1970) using kidney cortex slices. Work carried out by Daniel Kidwai, Robinson, Freemen & Fair (1971) on rat uterus adds support to these observations, these workers having demonstrated a direct inhibition of oxidative phosphorylation with, in addition, a slight inhibition of glycolysis, these actions leading to a marked decrease in cellular ATP levels. Daniel et al. (1971) also demonstrated that rat uterus takes up and concentrates ethacrynic acid, and on fractionation, found the highest concentration of this drug in the mitochondrial fraction.

Thus ethacrynic acid may act via an inhibition of the transport ATPase, inhibition of oxidative phosphorylation, or inhibition of glycolysis. However, much of the work concerning the effects of this drug on respiration have been carried out in the rat, a species in which ethacrynic acid is inactive as a diuretic. Thus in vitro metabolic effects may not be related to the in vivo saliuretic and diuretic effects on those species sensitive to the drug.

Ouabain

Ouabain is a cardiac glycoside obtained from Strophanthus gratus and first isolated by Schwartz et al. (1929). It has the structure shown below.



This drug is widely used in the treatment of congestive heart failure, as a consequence of its ability to increase heart muscle efficiency. Apart from an action on the heart, ouabain and other cardiac glycosides have an important action on the kidney and other transporting epithelia. It has been shown that this compound has a biphasic action on salt excretion. In the chicken, low doses of ouabain stimulate sodium, chloride and water reabsorption and increase the activity of the Na-K ATPase, while high doses have the opposite effect (Palmer & Nechay, 1964). Similarly, the transport of sodium by toad bladders (McClane, 1965) and the activity of mammalian kidney ATPase (Palmer, Lasseter & Melvin, 1966) show a similar concentration dependent biphasic response to cardiac glycosides. Besides the effects of this compound on the heart and kidneys, a loss of potassium and a gain of sodium by the skeletal muscle is observed, this resulting in an

increase in plasma potassium and a drop in plasma sodium (Williams et al. 1971).

Mechanism of action

Schatzman (1953) showed that cardiac glycosides prevent the uptake of potassium and the loss of sodium from cold stored red blood cells when these are incubated at 37°C in the presence of glucose. He found that ouabain had no effect on metabolic activity of the cells, suggesting that the drug was either disconnecting energy from the sodium pump, or directly inhibiting this pump. Glynn (1957) suggested that the effect of cardiac glycosides was exerted by competition with potassium for its site on the transport ATPase. Post et al. (1969) propose that the ATPase has two forms, E_1 and E_2, these are phosphorylated or dephosphorylated in the presence of sodium and potassium respectively. E_1 conversion to E_2 is catalysed by magnesium ions. Ouabain, they propose, reacts preferentially with E_2 phosphate, and have shown that when this form of the enzyme is incubated with ouabain, a marked resistance to splitting was observed, indicating a more stable intermediate. This is supported by the work of Hoffman (1966) who proposes that phosphorylation of the Na-K ATPase causes a conformational change, such that the outside of the membrane is susceptible to attack by ouabain. He also demonstrated that ouabain is only active when added to the outside of the membrane, the side from which potassium is actively transported. It has also been shown that infusion of potassium with ouabain tends to prevent the inhibitory actions of the latter on sodium transport in the dog kidney (Heidenreich, Laaff & Fülgraff, 1966). These observations suggest a highly specific effect of this drug on sodium potassium exchange pump activity.

Chapter 2

Materials and methods

METHODS

Tissue Preparations

a) Everted colon sacs

Colon sacs were prepared by the method of Wilson and Wiseman (1954) as described for the small intestine.

Male albino Wistar rats weighing between 240 and 260g were anaesthetized by intraperitoneal injection of "Nembutal" at a dose of 0.1ml/100g body weight. After reflexes had disappeared, the animal was opened along the ventral mid-line and the colon exposed. A canulla was tied into the proximal end of the colon through which ice-cold oxygenated saline (0.9% sodium chloride) was perfused to wash out the lumen contents. The entire colon was removed from the animal by freeing it from the attached mesentery, and cutting close to the anus. The tissue was transferred to a beaker of ice-cold oxygenated saline, still attached to the canulla and washed. It was then everted on a glass rod, rinsed, blotted on damp filter paper (Whatman No. 50) and weighed. One end of the colon was then closed with a ligature, and a hypodermic needle introduced into the open end, through which about 1ml of Krebs' bicarbonate buffer containing glucose (500mg/100ml) was injected (Krebs & Henseleit, 1932). The sac was closed by tying a second ligature whilst removing the needle. The sac was reweighed and transferred to a 50ml Erlenmeyer flask containing 10ml of the same buffer. The flask was gassed with a mixture of 5% carbon dioxide in oxygen, placed in a water bath at 37°C and shaken at 80 oscillations per minute for one hour. At the end of the incubation period, the sac was removed, blotted carefully on damp filter paper and reweighed. If dry weights or serosal volumes were required, the sac was opened, the serosal fluid removed, the volume measured, and the tissue dried to constant weight at 105°C.

In some experiments, sacs stripped of muscle were prepared as

follows. The washed colon was placed on a glass rod (7mm diameter) so that the muscle was under slight tension. The muscle was cut with a blunt scalpel and teased off with the fingers. The mucosa was then everted and converted into a sac as described above.

Expression of results

Mucosal fluid transport = increase in sac weight/hr

Serosal fluid transport = increase in serosal fluid volume/hr

Mucosal ion transport = $(C_i \times MV_i) - (MC_f \times (MV_i - MFT))$

Serosal ion transport = $(SC_f \times SV_f) - (C_i \times SV_i)$

Where,

C_i = initial buffer ion concentration

MV_i = initial mucosal volume

SV_i = initial serosal volume

SV_f = final serosal volume

MC_f = final mucosal buffer ion concentration

SC_f = final serosal buffer ion concentration

MFT = mucosal fluid transport

Results were expressed either per g dry weight or per g wet weight of tissue.

b) Intestine rings

The intestine was everted as described above, and cut transversly into thin rings with a razor blade. The rings were loaded with sodium and depleted of potassium by incubation in potassium-free, glucose-free Krebs' bicarbonate buffer. The loading procedure was carried out either at $0^{\circ}C$ for 1-2hrs in flasks open to the air, or at $37^{\circ}C$ for 20mins in flasks from which the oxygen had been removed by gassing with a mixture of 5% carbon dioxide in nitrogen. A sample of the slices was assayed for sodium and potassium as described below. The remainder were

transferred to flasks (3 rings/flask) containing fresh buffer gassed with a mixture of 5% carbon dioxide in oxygen. This buffer contained glucose (500mg/100ml) and potassium (5.5mM). The slices were then incubated, either at 37°C or 25°C for times varying between 4mins and 30mins, after which, they were removed, rinsed in distilled water, blotted gently on damp tissue, placed in preweighed flasks and dried at 105°C to constant weight. The tissue was digested in concentrated nitric acid, made up to 10ml with distilled water and the concentrations of sodium and potassium determined by flame photometry, using internal standards to corresct for interference. Ion concentrations were expressed as μEq/g dry weight of tissue.

Extracellular space

Extracellular space was measured by the method of Rosenberg, Blair & Segal (1961) using either C^{14} inulin or H^{3} inulin. Ion changes were then expressed as μEq/g of intracellular fluid.

c) Kidney cortex slices

Male albino Wistar rats weighing between 240 and 260g were stunned by a blow on the head, and killed by cervical fracture. The kidneys were removed immediately, and placed in ice-cold saline. Keeping the kidney cold throughout, thin cortex slices were prepared by cutting with a razor blade on an ice-cold glass plate. The outermost slices were rejected as these have been shown to contain atypical amounts of sodium and potassium (Whittam & Davies, 1953). The slices were then placed in a 50ml Erlenmeyer flask, gassed with nitrogen and incubated at 37°C for 12mins in a modified Krebs' phosphate buffer in the absence of glucose and potassium. Under these conditions, pump activity is abolished and the cells of the tissue load with sodium and deplete of potassium. Some of these slices were taken for analysis of sodium

and potassium content, while the remainder were placed in a series of 10ml Erlenmeyer flasks (2 slices/flask) containing fresh buffer of the same composition as before. These flasks were gassed with oxygen and allowed to equilibrate to 25°C in a water bath set at this temperature while shaking at 120 oscillations per minute. The final composition of this modified Krebs' buffer (Poat & Munday, 1971) was 143mM sodium chloride, 3mM calcium chloride, 1.5mM magnesium sulphate, 3.5mM sodium phosphate buffer pH 7.4, 10mM glucose and 5.5mM potassium phosphate buffer pH 7.4. The final volume of this buffer was 5ml. The flasks were incubated, usually for 10mins, after which, the slices were removed, rapidly rinsed in distilled water, blotted lightly on damp tissue, placed in preweighed 5ml flasks, dried to constant weight at 105°C, and the sodium and potassium contents estimated as described.

In some experiments, sodium chloride was replaced by the sodium salt of replacement anions and calcium chloride replaced by calcium sulphate. The osmolarity of the solution was maintained by the addition of mannitol where necessary, and the loading time was extended to 20mins to allow cell chloride to fully exchange with the replacement anion.

Estimation of tissue sodium and potassium

The dry tissue was ashed by heating in a small volume of concentrated nitric acid until the slices had dissolved and until all liquid had been driven off. The residue was then made up to a final volume of 10ml or 25ml with hot distilled water and assayed for sodium and potassium using an EEL flame photometer. Over the range of sodium and potassium present in the extracts, the relation between flame intensity and ion concentration was linear. Distilled water was sprayed between each reading. Sodium and potassium content of slices is expressed as μEq of ion/g dry weight of tissue.

Operative procedures

All instruments were sterilized by immersion in 'Hibitane' methanol (0.05%) for at least half an hour before the operation. The solution was also used to sterilize ligatures (No. 60 linen thread) and the skin around the incisions. Post-operative animals were allowed to recover in a quiet room maintained at 25°C.

Combined Nephrectomy and Adrenalectomy

Rats were anaesthetized with an oxygen/ether mixture, and placed in the prone position on the operating table. The skin was shaved below the ribs on both sides of the spine. An incision was made on one side, and the skin retracted to expose the body wall musculature. A small cut was made through the muscle layer, this cut being extended by blunt dissection. At this point, the kidney is visible and, by gently grasping with forceps, may be pulled through the opening together with the peri-renal fat. The adrenal was removed by tearing the gland and the adrenal fat away from the kidney. Care was taken to remove peri-adrenal fat. The adrenal vessels are small and do not require ligation. To remove the kidney, a ligature, previously soaked in "Hibitane" was tied around the ureter, renal artery and renal vein, and the organ cut away above the ligature. The incision was then closed. The same procedure was then repeated on the other side of the body to remove the second kidney and adrenal gland.

Diet

Rats were allowed food (Rank's 41B diet cubes) and water ad libitum.

Measurement of oxygen consumption by homogenates and slices of kidney cortex

Oxygen consumption was measured using a Gilson respirometer with 0.2ml of a 10% (w/v) solution of potassium hydroxide in the centre well of the manometer flasks to absorb respired carbon dioxide.

a) Kidney cortex homogenates

A 10% (w/v) homogenate was prepared in the modified Krebs' phosphate buffer described earlier and 0.2ml added to the side arm of the manometer flasks. The main compartment contained 2.6ml of the same buffer and 0.2ml of a 0.2M solution of sodium succinate as an energy source. Any inhibitors were dissolved in the buffer in the main compartment. After incubation, the protein content of the homogenate was estimated, and the oxygen consumption expressed as µl of oxygen consumed/mg of protein.

b) Kidney cortex slices

The substrate (0.2ml of a 0.2M sodium succinate solution) was added from the side arm to the main compartment which contained two slices in 2.8ml of buffer containing added inhibitors when required. Oxygen consumption was recorded every 5mins. After 1hr, the slices were removed from the flasks, rinsed in distilled water, gently blotted on damp tissue and dried at 105°C to constant weight. Oxygen consumption was expressed as µl of oxygen consumed/mg dry weight of tissue.

In both experiments, respiration was initiated by tipping the contents of the side arm into the main compartment.

Protein estimation

Protein was estimated by the Biuret method (Gornall, Bardawill & David, 1949). 0.2ml of homogenate was added to 5ml of 6% trichloracetic acid (TGA), the precipitated protein centrifuged, washed with more TCA and recentrifuged. Lipid was extracted by incubating in ethanol/water (50/50 v/v) for two hours, centrifuging, incubating twice in ethanol for half an hour, followed by centrifugation (Cleland & Slater, 1953). The pellet was then dissolved in 4ml of Biuret reagent and made up to 5ml with distilled water. After allowing the colour to develop, absorption was measured at 540mµ, using bovine serum albumin as the standard.

Thin layer chromatography

Thin layer chromatography was carried out using F.254 T.L.C. plates in either an acid or a neutral solvent.

The acid solvent had the composition:

Butanol 120vols, Acetic acid 30vols, Water 50vols.

The neutral solvent had the composition:

Ethanol 100vols, Butanol 100vols, Water 50vols.

Estimation of carbonic anhydrase activity

Carbonic anhydrase activity was estimated by the colourimetric method of Roughton & Booth (1946), which is based on the time taken to reduce the pH of a buffer from 7.95 to 6.3 as a result of the formation of carbonic acid from carbon dioxide. Two drops of bromothymol blue, 2ml of water and 0.3ml of carbonic anhydrase extract were added to 3ml of 0.022M veronal buffer. This mixture was then gassed with nitrogen at 0°C and to it was added 5ml of ice-cold water saturated with carbon dioxide. Enzyme activity was then estimated by the reduction in time taken for the indicator to change colour.

Enzyme activity was then calculated from the following formula:

$$E.U. = \frac{t_o - t}{t - 1}$$

where t and t_o are the reaction times in the presence and absence of catalyst respectively, and E.U. is the enzyme unit. This formula allows one second for mixing. Carbonic anhydrase activity of kidney cortex slices was measured by homogenizing 10mg of tissue in 1ml of isotonic saline, diluting with more saline to 10ml, and then proceeding as above. When added acetazolamide was at a concentration of 5mM. Enzymic activity is expressed as E.U./mg protein.

Chemicals

Angiotensin (Hypertensin Ciba) Angiotensin II amide.

Angiotensin was dissolved in sterile saline (0.9% sodium chloride) to a concentration of 10^{-5}g/ml and stored in sealed, siliconized, sterile ampoules. Dilution to the required concentration was carried out immediately prior to use. When added to intestinal sacs, angiotensin was in the serosal solution only.

Ethacrynic acid (2,3-dichloro-4-(2 methylenebutyryl) phenoxyacetic acid)

Ethacrynic acid was a gift from Dr. M. MacPhail, Merck, Sharp & Dohme Ltd. It was made up fresh every day in Krebs' phosphate buffer, the pH being adjusted to 7.4 with dilute sodium hydroxide.

Ouabain (Strophanthin G)

Ouabain was obtained from Sigma chemical Co. Ltd. and was dissolved in Krebs' phosphate buffer immediately before use.

EGTA (Ethyleneglycol-bis(β-amino-ethyl ether)n,n'-tetra-acetic acid)

EGTA was obtained from Sigma chemical Co. Ltd. and was made up in Krebs' phosphate or bicarbonate buffer immediately prior to use, adjusting to pH 7.4 with sodium hydroxide.

Siliconization

Ampoules for storing angiotensin were siliconized by immersion in a 2% solution of dichloro-dimethyl silane in carbon tetrachloride for 15mins. These were then emptied, washed twice with acetone, once with acetone/water (50/50 v/v) and then at least three times with distilled water.

Cleaning of glassware

All glassware was cleaned before use by soaking in Pyroneg (Diversay Ltd.) for at least twelve hours, followed by washing in tap water and rinsing in at least three changes of distilled water.

Statistical evaluation of results

The standard error of the mean for each set of results was calculated from the following formula.

$$S.D. = \sqrt{\frac{d^2}{n - 1}}$$

$$S.E. = \frac{S.D.}{\sqrt{n}}$$

where,

S.D. is the standard deviation,

d is the deviation of an indevidual value from the mean,

S.E. is the standard error,

n is the number of observations.

The value for students 't' can them be calculated:

$$t = \frac{M_1 - M_2}{\sqrt{(S.E._1)^2 + (S.E._2)^2}}$$

where, M_1 and M_2 are the means of the two sets of results to be compared. The value of P, the probability, was obtained from tables. Any differences with a P value greater than 0.05 were taken as not significant and referred to in the tables as N.S.

Chapter 3

Results

Section 1

The dependence of sodium transport by rat kidney cortex slices on the presence of other ions

SECTION 1. The dependence of sodium transport by rat kidney cortex slices on the presence of other ions

The requirement for cations

POTASSIUM

It has been suggested by several workers, particularly Whittembury (1968) and Whittembury & Proverbio (1970), that sodium transport by the kidney takes place through two separate mechanisms; one requiring the presence of potassium, the other being independent of this ion.

From the results presented in Table 1, it can be seen that rat kidney cortex slices incubated anaerobically at 37°C for 12mins in the absence of potassium and glucose become depleted of potassium and loaded with sodium. When these sodium loaded, potassium depleted slices are reincubated aerobically at 25°C for 10mins in the presence of glucose and potassium, they extrude sodium and take up potassium. In the absence of potassium in the second incubation buffer, sodium extrusion from the slices is significantly reduced ($P < 0.01$), but by no means abolished, confirming the existence of a potassium independent sodium transport mechanism in addition to the sodium-potassium exchange pump.

To determine whether the tissue is fully loaded with sodium after the first incubation, wet weights and dry weights of sodium loaded, potassium depleted slices were compared, and the tissue fluid content calculated. This was found to be 5.15mls of fluid/g dry weight of tissue. Taking the loaded sodium value of 729 ± 18 µEq/g dry weight of tissue, this gives a tissue sodium concentration of 142 ± 3 µEq/ml of tissue fluid. As the buffer sodium concentration is 143µEq/ml, the tissue may be assumed to be fully loaded with sodium.

Table 1. The effect on tissue sodium and potassium content of incubating rat kidney cortex slices anaerobically at 37°C for 12mins in the absence of potassium and glucose, and reincubating aerobically at 25°C for 10mins in the presence of glucose and in the presence or absence of potassium

	Tissue Na	Na loss	Tissue K	K gain	
Fresh slices	293±17		282±12		(6)
After first incubation	729±18		107±5		(6)
After second incubation					
+ potassium	501±16	228±16	203±7	96±7	(7)
- potassium	608±13	121±13	108±3	1±3	(11)

All values expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

CALCIUM

It has been observed that the removal of calcium from the incubation fluids of isolated frog skin results in a reduction in sodium transport rates (Curran & Gill, 1962), and Kleinzeller (1961) has indicated that incubating kidney cortex slices in the absence of calcium alters the near steady state tissue cation level. The possible involvement of calcium in the activity of a potassium independent sodium transport mechanism in kidney cortex slices was studied by incubating sodium loaded, potassium depleted slices in a potassium-free buffer in the presence of EGTA at a concentration of 5mM, to remove free calcium ions. A potassium-free buffer was used, so that any effects on sodium transport linked to potassium transport would not be observed. It can be seen that EGTA does not inhibit sodium extrusion from these slices (Table 2), indicating that calcium ions are not required for potassium independent sodium pump activity.

The requirement for anions

Sodium extrusion from loaded kidney cortex slices independent of potassium is also independent of calcium so that it is improbable that this movement of sodium is in exchange for another cation. It has been shown (Barry, Smyth & Wright, 1965) that in the intestine, a non-electrogenic sodium transport mechanism exists, and Shaikh (1972) has provided evidence to suggest that this mechanism involves the neutral movement of sodium with an anion, and is concerned with the bulk movement of fluid. If a similar mechanism exists in the kidney, this would require the movement of an anion with, and in the same direction as sodium. This would also be the case if the pump proposed by Whittembury (1968) was non-electrogenic.

In view of these observations, an investigation was carried out to study the effect of anions on the fraction of sodium transport which is not linked to potassium transport.

Table 2. Sodium extrusion and potassium uptake by sodium loaded, potassium depleted rat kidney cortex slices during a 10min reincubation in the absence of potassium and in the presence or absence of 5mM EGTA

	Na loss		K gain		
Control	104±10	N.S.	-6±2	P<0.02	(13)
+ EGTA	120±10		-15±2		(8)

All values expressed as µEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

BICARBONATE

The first series of experiments was designed to determine the possible involvement of bicarbonate ions in the potassium independent sodium transport process.

Rat kidney cortex slices were loaded with sodium and depleted of potassium in the usual way, and reincubated in either a bicarbonate rich or a bicarbonate free buffer. A comparison between sodium loss and potassium gain by these slices is given in Table 3, and indicates that exogenous bicarbonate is unnecessary for sodium pump activity since the rates of sodium extrusion and potassium uptake are both unaffected by the presence or absence of bicarbonate ions in the incubation medium.

Alternatively, the endogenous production of bicarbonate ions may be necessary to support active sodium extrusion, and so acetazolamide, an inhibitor of carbonic anhydrase activity, was added to the buffer during the second incubation period (Table 4). Krebs' phosphate buffer was used as the incubation fluid for these experiments in order to exclude bicarbonate ions from the solution. Again, no reduction in sodium extrusion or potassium uptake rates was observed, neither is there a significant change in tissue sodium or potassium content after incubating fresh slices with acetazolamide for 30mins, as is shown by the results presented in Table 5.

In order to check that the inhibitor did indeed block carbonic anhydrase activity, an homogenate of kidney cortex was prepared, and assayed for enzyme activity. It was found that at the concentration used for the earlier experiments (5mM), acetazolamide completely abolished carbonic anhydrase activity when added to an homogenate preparation (Fig 6).

These results, taken together, suggest very strongly that bicarbonate ions are not involved in sodium transport by the kidney cortex.

Table 3. Sodium extrusion and potassium uptake by rat kidney cortex slices loaded with sodium and depleted of potassium by a 12min anaerobic incubation in Krebs' phosphate buffer in the absence of glucose and potassium during a 10min aerobic incubation in either Krebs' phosphate or Krebs' bicarbonate buffer in the presence of glucose and potassium

	Na loss		K gain		
Reincubate + bicarbonate	263±6		128±4		(9)
		N.S.		P<0.05	
Reincubate - bicarbonate	246±8		115±4		(9)

All values expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Table 4. The effect of acetazolamide on sodium extrusion and potassium uptake, by sodium loaded, potassium depleted rat kidney cortex slices. Acetazolamide was added to the second incubation buffer at a range of concentrations.

Acetazolamide (mM)	Na loss	K gain	
0	246±8	115±4	(8)
0.1	258±13	116±8	(3)
1.0	255±17	120±5	(3)
5.0	270±4	128±3	(3)

All values expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Table 5. The effect of incubating fresh rat kidney cortex slices for 30mins in the presence or absence of 5mM acetazolamide, on tissue sodium and potassium concentrations

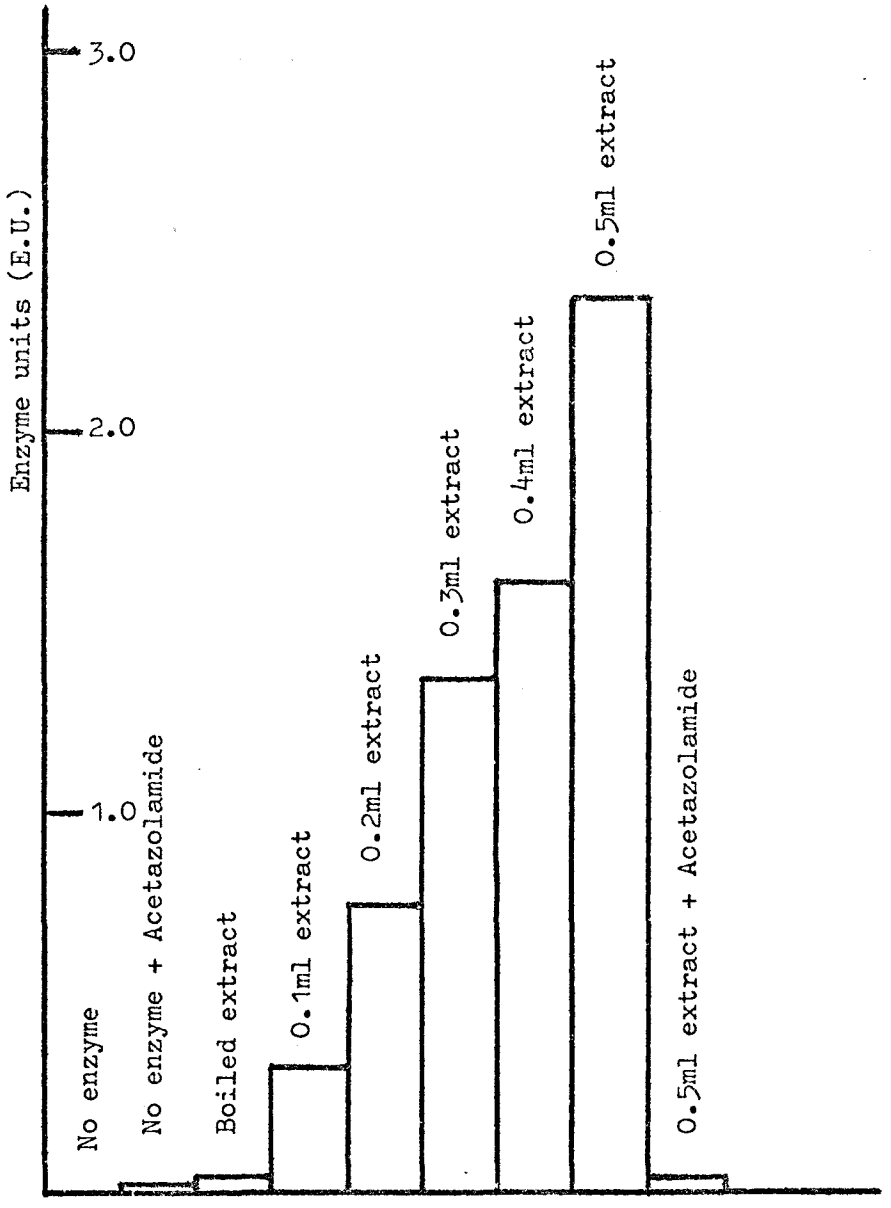
	Tissue Na		Tissue K		
Control	348±12	N.S.	315±9	N.S.	(6)
Acetazolamide	339±4		326±8		(6)

All values expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Fig. 6 The activity of carbonic anhydrase in the presence and absence of 5mM acetazolamide



All results are means of two estimations

CHLORIDE

Chloride, like bicarbonate, is a major constituent of extracellular fluid and is therefore a likely candidate for the anion in any anion linked sodium pump.

In order to investigate this possibility, slices were loaded with sodium and depleted of potassium and subsequently reincubated in a series of buffers in which chloride had been replaced with one of a number of replacement anions. A wide range of anions was used to negate any effects peculiar to any one particular anion.

The optimum loading and incubation periods were determined by loading kidney cortex slices for 12 to 30mins and then reincubating for 10mins in phosphate buffer containing sulphate in place of chloride. Sodium extrusion and potassium uptake by these slices was compared with those movements occuring in a chloride containing buffer after loading for the same time periods. It can be seen from the results presented in Tables 6 and 7 that, in the absence of chloride ions, sodium extrusion from loaded slices is markedly reduced without a significant change in potassium uptake, the reduction in sodium loss being near maximal after 20mins loading. Loading slices for 20 or 30mins in a buffer containing chloride causes a significant reduction in the subsequent pumping ability (Table 7). This appears to be a consequence of generalized damage, as the sodium loss: potassium gain ratio remains constant. However, loading for shorter periods of time does not allow complete exchange of tissue chloride with the replacement anion, as is shown by the high sodium extrusion rate after loading slices for 12mins in the absence of chloride. Consequently, 20mins was chosen as the optimum loading period, followed by a 10min incubation period, this latter time was chosen so that, as in the previous experiments, the rate of sodium and potassium transport could be studied, as opposed to the final equilibrium, these times being used for all other replacement experiments.

Table 6. The effect of sodium loading and potassium depleting rat kidney cortex slices for different times in buffer containing sulphate in place of chloride on the subsequent sodium extrusion and potassium uptake during a 10min reincubation in a similar buffer

Loading time	Na loss		K gain		$\frac{\text{Na loss}}{\text{K gain}}$	
12"	206±8	P< 0.001	96±4	N.S.	2.15	(8)
20"	132±9	N.S.	99±2	N.S.	1.33	(8)
30"	118±6		92±3		1.27	(8)

All values expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Table 7. The effect of sodium loading and potassium depleting rat kidney cortex slices for different times in a normal buffer containing chloride, on the subsequent sodium extrusion and potassium uptake during a 10min reincubation in a similar buffer

Loading time	Na loss		K gain		$\frac{\text{Na loss}}{\text{K gain}}$	
12"	232±14	N.S.	105±5	P<0.01	2.21	(8)
20"	195±16	P<0.01	88±2		2.22	(16)
		N.S.		P<0.01		
30"	178±9		78±2		2.28	(16)

All values expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Pump activity in the presence of chloride

Following an anaerobic incubation in a potassium-free, glucose free medium, kidney cortex slices contain a high level of sodium, while tissue potassium is reduced to a low level. Reincubation of these slices causes sodium to be extruded and potassium to be taken up in a ratio of over 2:1 which may be interpreted as indicating a high level of potassium independent sodium transport (Table 8).

Pump activity following sulphate replacement of chloride

Loading slices for 20mins in a chloride-free, sulphate replacement buffer causes a near normal loss of potassium, while the uptake of sodium is significantly lower than in the presence of chloride ($P < 0.001$). Table 9 shows that on reincubation in the same buffer, sodium extrusion is significantly lower than in the presence of chloride ($P < 0.01$), with no significant change in potassium uptake. The consequent reduction in the sodium loss:potassium gain ratio indicates a reduction in potassium independent sodium extrusion, with no change in sodium-potassium exchange pump activity.

Pump activity following isethionate replacement of chloride

As in the previous experiment, the ability of slices to take up sodium from buffer containing isethionate in place of chloride during the first incubation is seriously impaired. This may be due to the low degree of ionisation of sodium isethionate, leading to a reduced sodium ion activity. Potassium depletion is again normal. Sodium loss on reincubation is markedly reduced and potassium uptake slightly increased, with a consequent reduction in the sodium loss:potassium gain ratio to near unity, again indicating almost complete inhibition of a potassium independent sodium pump mechanism (Table 10), together with an apparent increase in sodium-potassium exchange activity.

Table 8. Sodium and potassium movements in rat kidney cortex slices after loading for 20mins and reincubating for 10mins in buffer containing chloride

	Tissue Na	Na loss	Tissue K	K gain	Na loss / K gain	
Load 20"	706±10		104±2			(10)
Incubate 10"	512±15	195±15	192±2	88±2	2.22	(16)

All values expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthsis

Table 9. The effect on sodium and potassium movements in rat kidney cortex slices of loading for 20mins and reincubating for 10mins in buffer containing sulphate in place of chloride

	Tissue Na	Na loss	Tissue K	K gain	Na loss / K gain	
Load 20"	605±5		128±5			(5)
Incubate 10"	470±4	135±4	218±2	90±3	1.50	(8)

All values expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Table 10. The effects on sodium and potassium movements in rat kidney cortex slices of loading for 20mins and reincubating for 10mins in buffer containing isethionate in place of chloride

	Tissue Na	Na loss	Tissue K	K gain	Na loss / K gain	
Load 20"	546±12		100±6			(7)
Incubate 10"	416±7	141±7	219±4	120±7	1.18	(15)

All values expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Pump activity following citrate replacement of chloride

When slices are loaded in a buffer containing citrate in place of chloride, the uptake of sodium is near normal, but the loss of potassium is excessive, possibly due to the calcium chelating actions of citrate ions. On reincubation, sodium extrusion is near normal, whereas potassium uptake is markedly increased, leading to only a slight reduction in the sodium loss:potassium gain ratio, once more indicating a reduction in potassium independent sodium extrusion together with an increased sodium-potassium exchange activity (Table 11).

Pump activity following gluconate replacement of chloride

The pattern of sodium pump activity in a buffer containing sodium gluconate in place of sodium chloride is indicated by the results shown in Table 12 and is similar to that observed when chloride is replaced by sulphate, that is, a low tissue sodium content after loading, a reduced sodium extrusion rate but a normal potassium uptake rate on reincubation, leading to a reduction in the sodium loss:potassium gain ratio, again indicating reduction in potassium independent sodium extrusion.

Pump activity following acetate replacement of chloride

The findings presented in Table 13 show that when slices are loaded in sodium acetate, the tissue takes up excessive quantities of sodium, either by a change in extracellular space, or an accumulation of this compound brought about by a change in the degree of ionisation of sodium acetate inside and outside the cells. On reincubation, sodium loss is slightly increased, whereas potassium uptake is markedly increased, indicating a reduction in potassium independent sodium extrusion overshadowed by a marked increase in potassium dependent sodium extrusion.

Table 11. The effect on sodium and potassium movements in rat kidney cortex slices of loading for 20mins and reincubating for 10mins in buffer containing citrate in place of chloride

	Tissue Na	Na loss	Tissue K	K gain	Na loss / K gain	
Load 20"	680±15		43±3			(10)
Incubate 10"	490±5	190±5	167±3	124±3	1.53	(16)

All values expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Table 12. The effect on sodium and potassium movements in rat kidney cortex slices of loading for 20mins and reincubating for 10mins in buffer containing gluconate in place of chloride

	Tissue Na	Na loss	Tissue K	K gain	Na loss / K gain	
Load 20"	511±16		76±2			(5)
Incubate 10"	383±8	128±8	166±3	90±3	1.42	(8)

All values expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Table 13. The effect on sodium and potassium movements in rat kidney cortex slices of loading for 20mins and reincubating for 10mins in buffer containing acetate in place of chloride

	Tissue Na	Na loss	Tissue K	K gain	Na loss / K gain	
Load 20"	922±16		121±2			(10)
Incubate 10"	702±18	219±18	267±2	145±2	1.51	(16)

All values expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Pump activity following bicarbonate replacement of chloride

On loading with this anion, the accumulation of sodium by the tissue is similar to that observed with acetate, and again a normal potassium depleted value is obtained. Table 14 shows that on reincubation, however, sodium extrusion is so reduced, that the sodium loss:potassium gain ratio is not significantly different from unity, which can be interpreted as complete inhibition of sodium extrusion not linked to potassium uptake.

Pump activity following pyruvate replacement of chloride

With this anion, tissue sodium again does not reach its normal high level after loading, again indicating a low ionisation of sodium pyruvate, or a low permeability of the tissue to pyruvate. On reincubation, both sodium loss and potassium gain are reduced resulting in a reduced sodium loss:potassium gain ratio, however, this reduction is slight suggesting a low level of chloride-pyruvate exchange during loading. Table 15.

Pump activity following succinate replacement of chloride

On replacing chloride with succinate, no change is observed in the loaded sodium or potassium value, neither is there any reduction or increase in the rate of potassium uptake. The only parameter which varies with this replacement anion is sodium loss during the second incubation, this being significantly reduced by more than 50% ($P < 0.001$), leading to a sodium loss:potassium gain ratio close to unity (Table 16). This indicates that this ion enters the tissue with reasonable ease, probably due to the high degree of dissociation and high permeability of sodium succinate, this anion exchanging well with chloride

Table 14. The effect on sodium and potassium movements in rat kidney cortex slices of loading for 20mins and reincubating for 10mins in buffer containing bicarbonate in place of chloride

	Tissue Na	Na loss	Tissue K	K gain	Na loss / K gain	
Load 20"	1027±39		110±17			(10)
Incubate 10"	946±44	71±9	198±10	87±6	0.82	(13)

All values expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Table 15. The effects on sodium and potassium movements in rat kidney cortex slices of loading for 20mins and reincubating for 10mins in buffer containing pyruvate in place of chloride

	Tissue Na	Na loss	Tissue K	K gain	Na loss / K gain	
Load 20"	651±4		80±1			(5)
Incubate 10"	502±6	149±6	158±3	78±3	1.91	(8)

All values expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Table 16. The effects on sodium and potassium movements in rat kidney cortex slices of loading for 20mins and reincubating for 10mins in buffer containing succinate in place of chloride

	Tissue Na	Na loss	Tissue K	K gain	Na loss / K gain	
Load 20"	734±15		86±2			(10)
Incubate 10"	640±12	93±11	170±6	83±5	1.12	(15)

All values expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

To carry out further experiments, a replacement anion was required which is similar in its properties to chloride. Although most of the replacement anions used in this study cause a reduction in the rate of sodium extrusion, this need not reflect an inhibition of the potassium independent sodium pump. Certain anions, in particular isethionate, gluconate and sulphate are not easily taken up by the tissue, suggesting that they enter the cells of the kidney with difficulty. This could be due to the low degree of ionisation of their sodium salts, however, if this low entry rate is due to the low permeability of the tissue to these larger anions, on reincubation, the initial rate of sodium extrusion could well exceed the extrusion rate of the replacement anion, leading to the development of a large potential across the serosal cell membranes, thereby limiting further sodium movements.

As the tissue exhibited a normal rate of sodium uptake during a loading procedure in buffer containing succinate in place of chloride, this indicates that the tissue permeability barriers to succinate and chloride are similar, and that the degree of ionisation of sodium succinate is comparable to the degree of ionisation of sodium chloride. However, this ion is divalent, making it less desirable as a replacement anion. Unfortunately, a monovalent anion which would replace chloride as well as does succinate was not found, the inorganic monovalent halides not tested, probably being too toxic in the concentrations required, and so succinate was used as the preferred anion in the following series of experiments.

Pump activity in buffer containing chloride, or succinate, in the presence or absence of potassium in the reincubation buffer

Table 17 shows the effect of omitting potassium and chloride, both in turn, and together on sodium and potassium transport. Slices

Table 17. The effect of potassium in the second incubation buffer on sodium extrusion and potassium uptake by rat kidney cortex slices loaded for 20mins and reincubated for 10mins in buffer containing chloride, or buffer containing succinate in place of chloride

Buffer	Na loss	K gain	
Chloride + K^+	195±16	88±2	(16)
Chloride - K^+	107±10	-6±2	(8)
Succinate + K^+	93±11	83±5	(15)
Succinate - K^+	9±8	-13±1	(8)

All values expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

loaded in buffer containing chloride and reincubated in buffer containing both chloride and potassium, extrude sodium and take up potassium in a ratio of about 2:1. Omission of potassium from the second incubation buffer causes a reduction in sodium extrusion by about 50%, together with the abolition of potassium uptake, indicating the inhibition of a 1:1 sodium-potassium exchange. Omitting chloride from both incubations and adding succinate as a replacement anion causes a reduction in sodium extrusion, again by about 50%, with no effect on potassium uptake, indicating the abolition of a sodium extrusion mechanism linked to chloride movement, and leaving a residual 1:1 sodium-potassium exchange. After loading in the absence of chloride, omission of potassium and chloride from the second incubation buffer reduces both sodium loss and potassium gain to zero, indicating the total inhibition of both sodium transport mechanisms.

These results add weight to the hypothesis that sodium transport is carried out via two mechanisms, one requiring the presence of potassium and being an exchange pump, the other being independent of potassium but requiring the movement of chloride in the same direction.

The chloride dependence of sodium transport by kidney cortex slices of the guinea-pig and the rabbit

To determine whether the properties of the potassium independent sodium pump mechanism in other species are similar to those in the rat, kidney cortex slices were prepared from guinea-pigs, loaded with sodium and depleted of potassium during a 20min incubation, and reincubated for 10mins, in buffer containing chloride, or buffer in which chloride had been replaced with succinate. The results presented in Table 18 show that replacing chloride with succinate causes the tissue to take up more sodium and lose considerably more potassium, than in the presence of

Table 18. The effect on sodium and potassium movements in guinea-pig kidney cortex slices of loading for 20mins and reincubating for 10mins in buffer containing chloride, or buffer containing succinate in place of chloride

	Tissue Na	Na loss	Tissue K	K gain	Na loss / K gain	
Buffer + Cl^-						
Load	604±6		114±4			(24)
Incubate	350±6	250±6	221±3	107±3	2.34	(9)
Buffer - Cl^-						
Load	659±9		70±3			(9)
Incubate	446±13	213±13	254±4	184±4	1.16	(10)

All values expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

chloride, during the first incubation.

To test whether the slices are fully loaded after the first incubation in the presence of chloride, an experiment was carried out to determine the sodium and water content of slices after loading in the presence of chloride. The sodium content of the tissue after loading was found to be 621±18 μEq of sodium/g dry weight of tissue, and when the wet and dry weights of the tissue were compared, they were found to contain 4.40ml of fluid/g dry weight of tissue. Thus the sodium concentration of loaded guinea-pig kidney cortex slices is 141±4 μEq of sodium/ml of tissue fluid. This value is not significantly different from the concentration of sodium in the buffer, and the slices may, thus, be considered fully loaded with sodium after the first incubation.

During the second incubation, replacement of chloride by succinate causes a significant decrease in sodium loss ($P < 0.05$), and a marked and highly significant increase in potassium uptake ($P < 0.001$), resulting in a halving of the sodium loss:potassium gain ratio to near unity. These results are best interpreted as a marked inhibition of potassium independent sodium extrusion, and a considerable stimulation of potassium linked sodium extrusion.

When the same experiment was carried out using rabbit kidney cortex slices (Table 19), again, a high level of tissue sodium and a low level of tissue potassium is observed after loading in the absence of chloride. No reduction in sodium extrusion during the second incubation occurs, sodium movements apparently being maintained by the potassium dependent mechanism, as indicated by the 100% increase in potassium uptake, and the halving of the sodium loss:potassium gain ratio.

The increase in kidney cortex sodium-potassium exchange activity which is observed in the guinea-pig and the rabbit after replacing chloride by succinate could occur for two reasons. Firstly,

Table 19. The effect on sodium and potassium movements in rabbit kidney cortex slices of loading for 20mins and reincubating for 10mins in buffer containing chloride, or buffer containing succinate or sulphate in place of chloride

	Tissue Na	Na loss	Tissue K	K gain	Na loss / K gain	
Buffer + Cl^-						
Load	628±7		126±3			(24)
Incubate	445±18	183±18	196±11	70±11	2.61	(7)
Buffer - Cl^- + succinate						
Load	687±11		56±2			(4)
Incubate	499±13	188±13	199±6	143±6	1.31	(5)
Buffer - Cl^- + sulphate						
Load	567±21		86±2			(4)
Incubate	436±12	131±12	186±1	100±1	1.31	(5)

All results expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

that the energy normally utilized for potassium independent sodium pump activity can be made available to the potassium linked pump, this then working at a faster rate to raise the tissue potassium content from the very low level achieved after loading. This would suggest that in these two species, the activity of the potassium linked sodium pump may be influenced by the activity of the potassium independent sodium pump. Secondly, that succinate, present at high concentrations in the tissue after loading, can be metabolized, and the energy provided, utilized for potassium linked sodium pump activity.

To test whether the observed increase in sodium-potassium exchange activity is due to a stimulation of metabolism by the added succinate, or whether sodium normally moving on a chloride linked pump is being channelled to the potassium linked pump, rabbit kidney cortex slices were loaded and reincubated, for the same times as before, in buffer containing the non-metabolizable anion, sulphate, in place of chloride. It was found (Table 19), that a significant stimulation of potassium uptake by the tissue from the low value obtained after loading in this buffer, is again observed during the second incubation ($P<0.05$), the decrease in sodium extrusion being insignificant. In this buffer, the sodium loss: potassium gain ratio is the same as is observed in a buffer containing succinate in place of chloride, suggesting that the reduction in tissue potassium during the first incubation to a very low level, and the inhibition of potassium independent sodium transport, caused by the omission of chloride, leads to a stimulation of sodium-potassium exchange activity, this probably not resulting from a metabolic affect of succinate. This is quantitatively different to the responses obtained using rat kidney cortex slices, although, in all three species, chloride appears necessary for sodium transport by the potassium independent sodium pump.

Section 2

The mechanism of action of ethacrynic acid in the kidney

SECTION 2. The mechanism of action of ethacrynic acid in the kidney

Although it is generally accepted that ouabain has a high degree of specificity for a potassium linked sodium transport mechanism, the way in which ethacrynic acid exerts its diuretic effects is by no means understood.

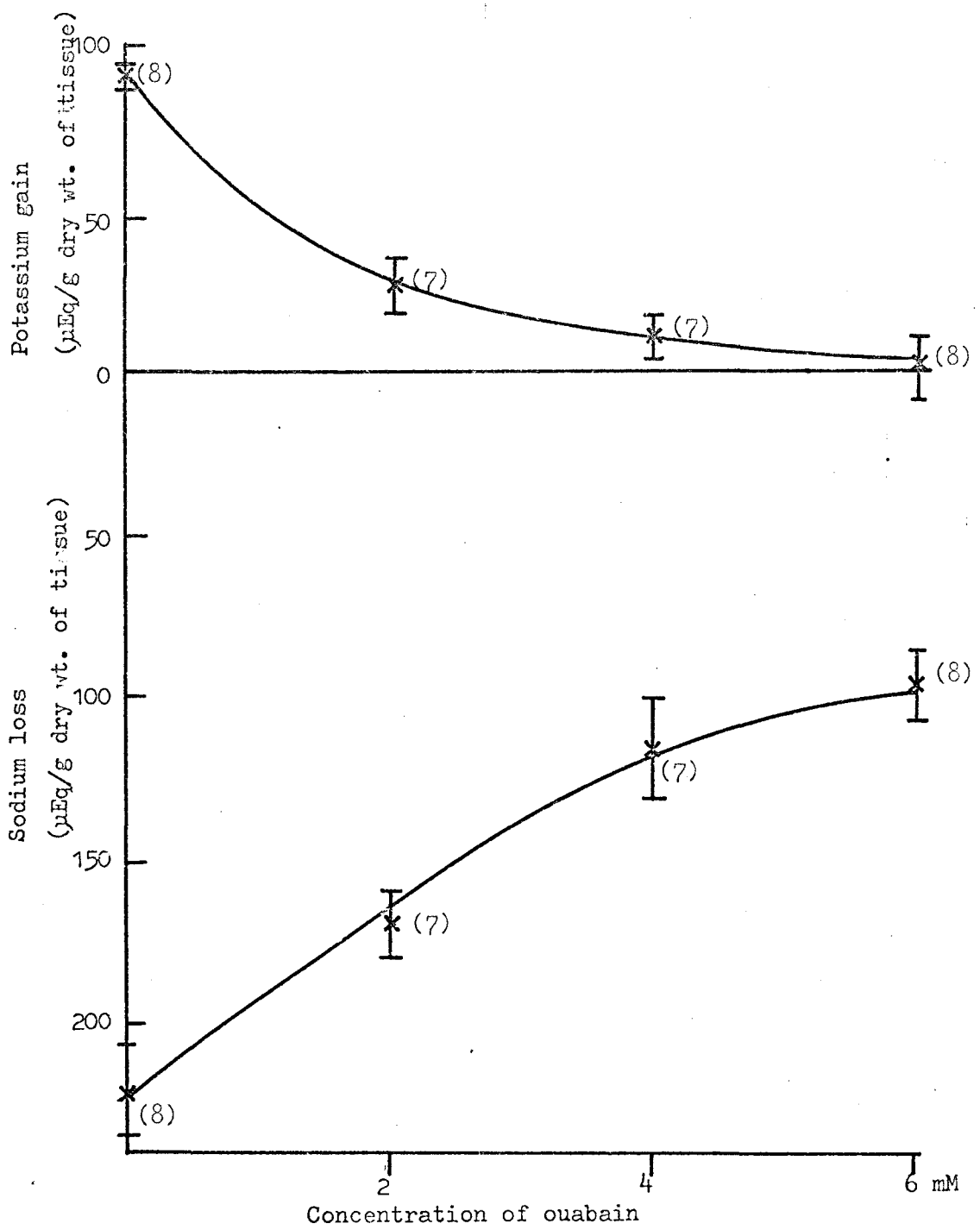
Results suggesting that ethacrynic acid acts by inhibiting respiration (Jones & Landon 1967; Poat, Poat & Munday 1970) have been obtained from experiments carried out in the rat, a species which does not show a diuretic response to this compound. On the other hand, administration of ethacrynic acid to the guinea-pig, rabbit and dog results in a marked diuresis and natriuresis, and it has been suggested, from work carried out on guinea-pig kidney cortex (Whittembury 1968), that the drug acts by a specific inhibition of a potassium independent sodium transport mechanism.

In an attempt to resolve these apparently conflicting results, an investigation was carried out into the effects of ethacrynic acid and ouabain on sodium movements, potassium movements and the consumption of oxygen by slices and homogenates of cortex taken from the kidneys of rats, guinea-pigs and rabbits.

The dose dependent effects of ethacrynic acid and ouabain on sodium and potassium movements in cortex slices prepared from rat and guinea-pig kidney

The effects of adding ouabain and ethacrynic acid to the buffer during the second 10min incubation period on sodium extrusion and potassium uptake by rat and guinea-pig kidney cortex slices which had been previously loaded by a 12min incubation are shown in Figs 7-10. From Figs 7 and 9, it can be seen that in both species, the addition of

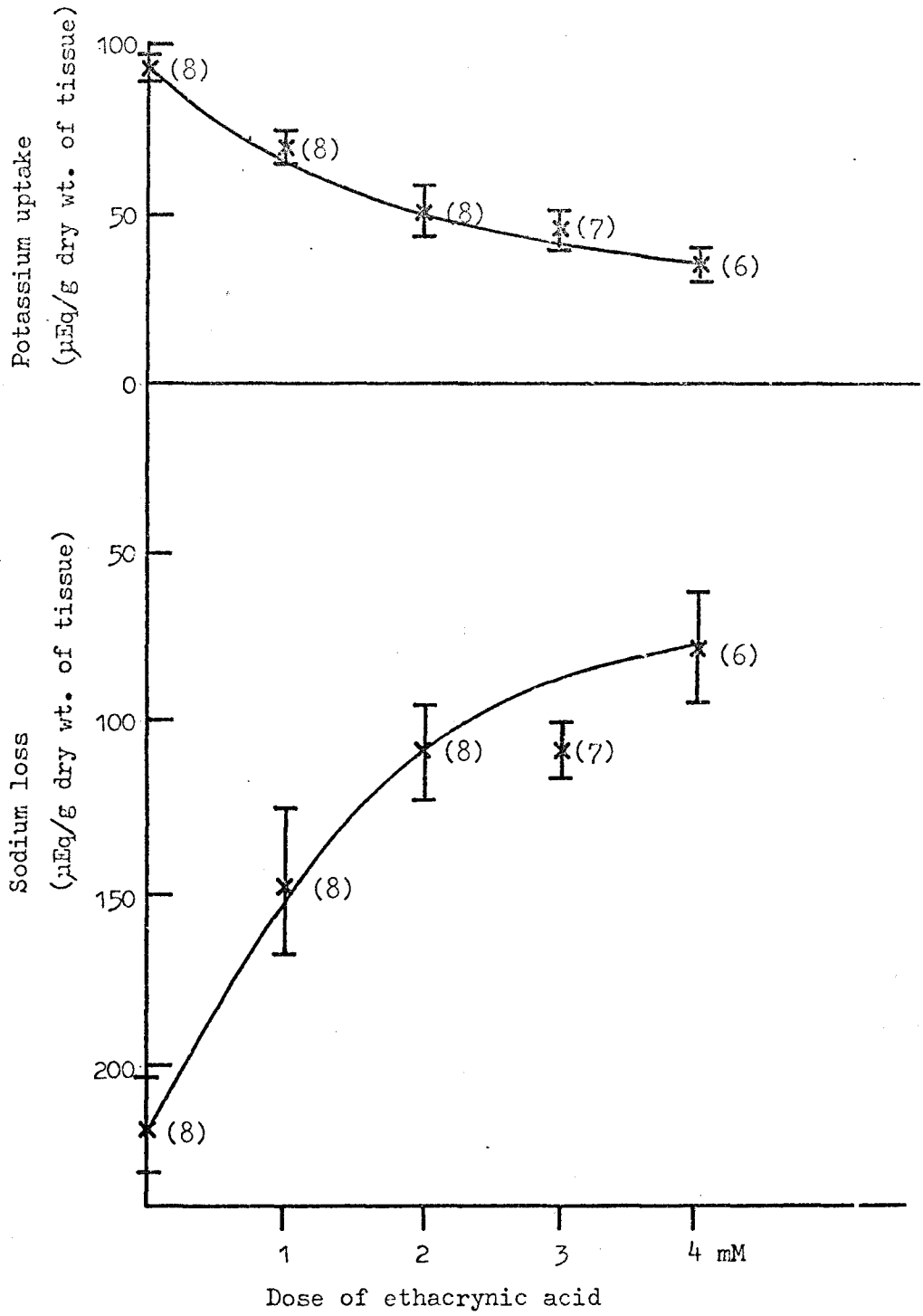
Fig. 7 The effect of ouabain on sodium extrusion and potassium uptake by sodium loaded, potassium depleted rat kidney cortex slices during a 10min incubation.



Results expressed as Means± S.E.M.

No. of observations in parenthesis

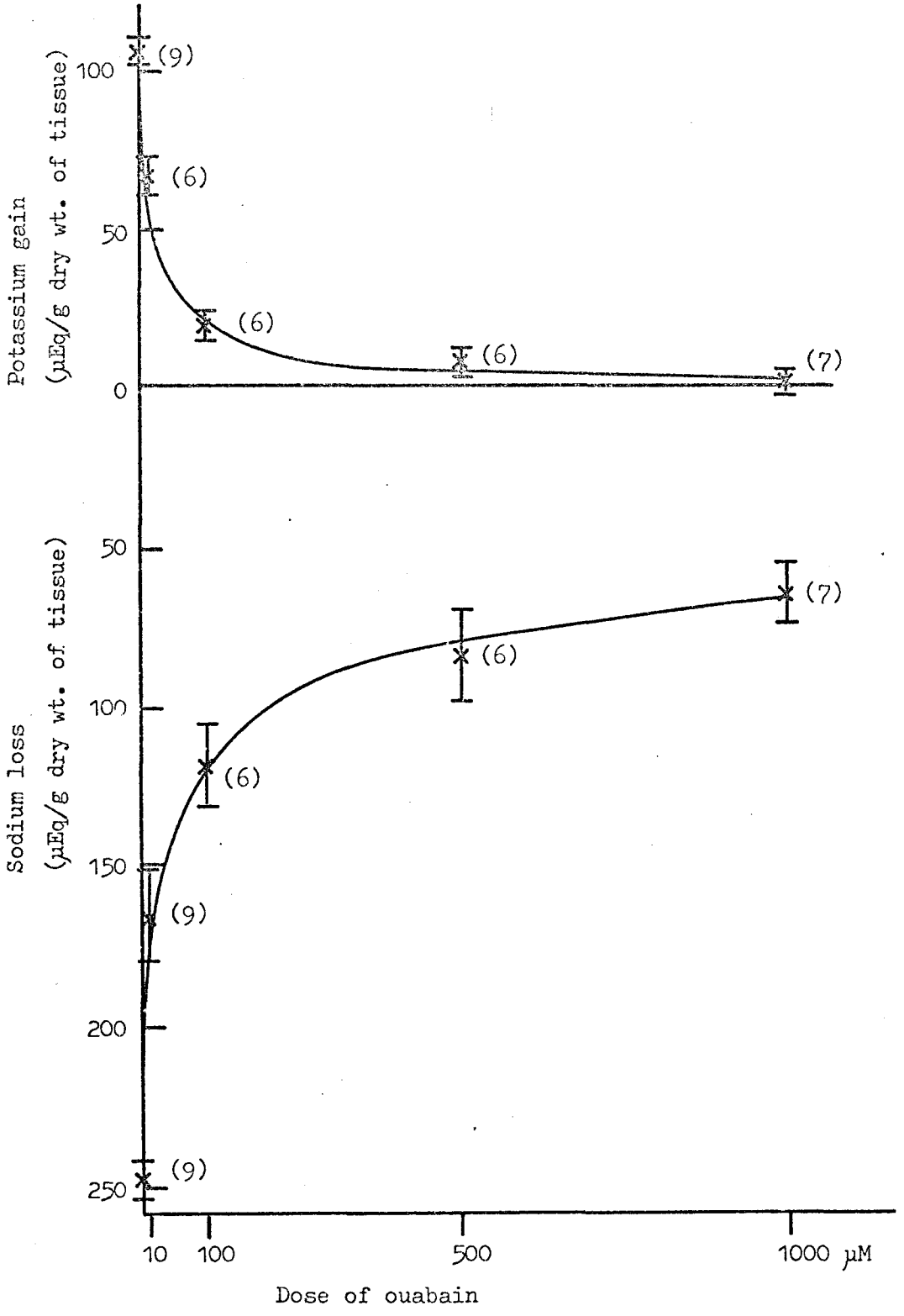
Fig. 8 The effect of ethacrynic acid on sodium extrusion and potassium uptake by sodium loaded rat kidney cortex slices during a second incubation



All results expressed as Means ± S.E.M.

No. of observations in parenthesis

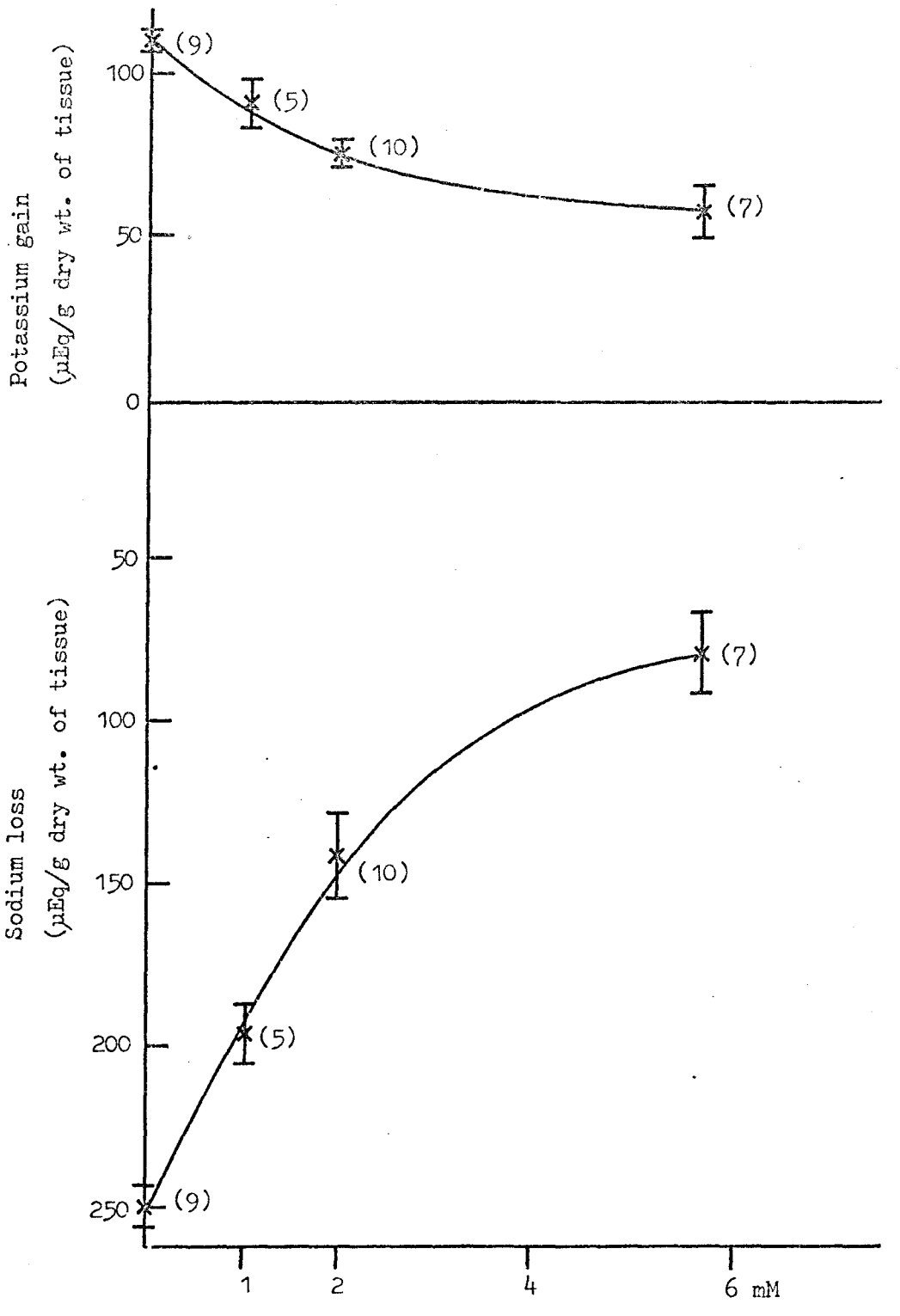
Fig. 9 The effect of ouabain on sodium extrusion and potassium uptake by sodium loaded guinea-pig cortex slices during a second incubation



Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Fig. 10 The effect of ethacrynic acid on sodium extrusion and potassium uptake by sodium loaded guinea-pig kidney cortex slices during a second incubation



Results expressed as Means ± S.E.M.

No. of observations in parenthesis

ouabain at high doses completely blocks potassium uptake whilst inhibiting sodium extrusion by between 60 and 70% of the control value. It may be noted that the guinea-pig kidney is considerably more sensitive to ouabain than is that of the rat, although the effects of this inhibitor are qualitatively similar. The addition of ethacrynic acid to the buffer during the second incubation (Figs 8 and 10) causes a similar reduction in sodium extrusion and potassium uptake in the rat kidney, whereas in the guinea-pig kidney, the effect on sodium transport is more marked, the addition of 6mM ethacrynic acid causing a 68% reduction in sodium loss with only a 40% reduction in potassium gain.

These results suggest that ethacrynic acid and ouabain are acting on different mechanisms, and that the degree of inhibition of a potassium linked pump is less in the guinea-pig than in the rat.

In order to compare the effects of ethacrynic acid and ouabain on sodium and potassium transport by the kidney cortex, slices were prepared from kidneys taken from the three species, and loaded in the normal way. These loaded slices were then reincubated for 10mins in the presence or absence of potassium, ethacrynic acid, ouabain, or in combinations of these conditions. Concentrations of the inhibitors were chosen which caused a similar degree of inhibition of sodium extrusion, so that the effects on potassium uptake could be compared. For this reason, the inhibitor concentrations were:-

	ETHA	Ouabain
Rat	3mM	4mM
Guinea-pig	2mM	10μM
Rabbit	2mM	15μM

As it has been previously shown (Whittembury & Proverbio, 1970; Munday, Parsons & Poat, 1971) that incubating loaded kidney cortex slices in the presence or absence of potassium, ouabain and ethacrynic acid does not result in a significant change in extracellular space, in this study, spaces were not measured, and it is proposed that the alterations in tissue ions are a reflection of the changes in cell ion content.

The results of these experiments are shown in numerical form in Tables 20-22 and in graphical form in Figs 11-13.

The effects of potassium and ouabain on sodium extrusion and potassium uptake by kidney cortex slices

In all three species, the omission of potassium from the second incubation buffer abolishes potassium uptake and causes a reduction in sodium extrusion of between 40 and 60%, indicating that of the total sodium extruded, about one half is potassium dependent, the remaining half being potassium independent. If this is so, the addition of ouabain would be expected to result in a much greater inhibition of potassium uptake than sodium loss, and this is what is observed in the rat. However, in the other two species, ouabain reduces the fluxes of both sodium and potassium to a similar degree, suggesting that, in the guinea-pig and rabbit, it is less specific as an inhibitor of potassium linked sodium transport.

The effect of ethacrynic acid on sodium extrusion and potassium uptake by kidney cortex slices

Ethacrynic acid causes a greater inhibition of sodium extrusion than potassium uptake in all three species. This is particularly marked in rabbit kidney cortex where sodium extrusion is inhibited by about 55% whereas potassium uptake is reduced by less than 20%. These results are consistent with the view that ethacrynic acid causes a

Fig. 11 The effect of omitting potassium, adding ouabain (4mM) or ethacrynic acid (3mM) on sodium extrusion (open columns) and potassium uptake (hatched columns) by rat kidney cortex slices during a 10min incubation. The slices were loaded with sodium and depleted of potassium by a 12min preincubation.

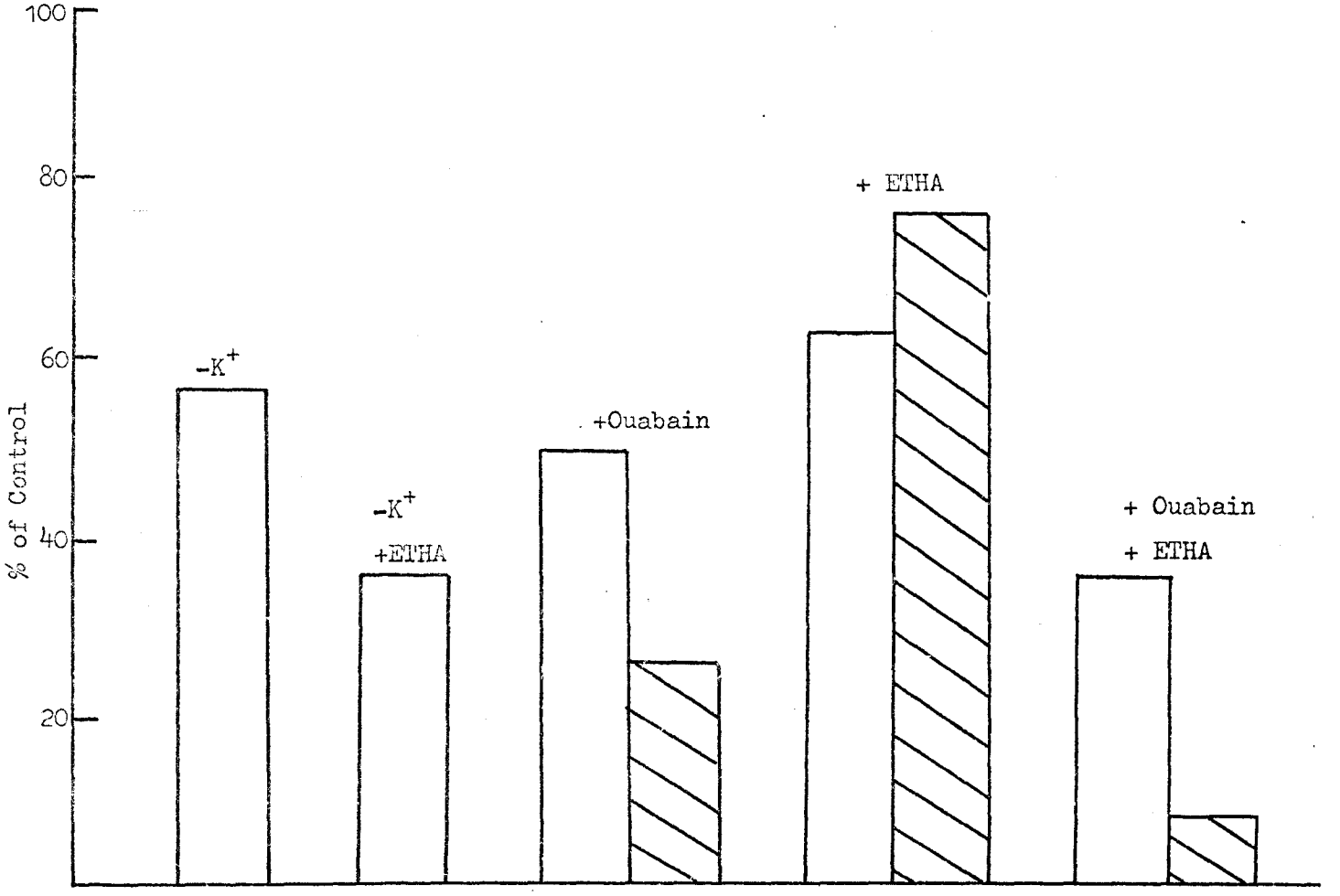


Fig. 12 The effect of omitting potassium, adding ouabain (10μM) or ethacrynic acid (2mM) on sodium extrusion (open columns) and potassium uptake (hatched columns) by guinea-pig kidney cortex slices during a 10min incubation. The slices were loaded with sodium and depleted of potassium by a 12min preincubation

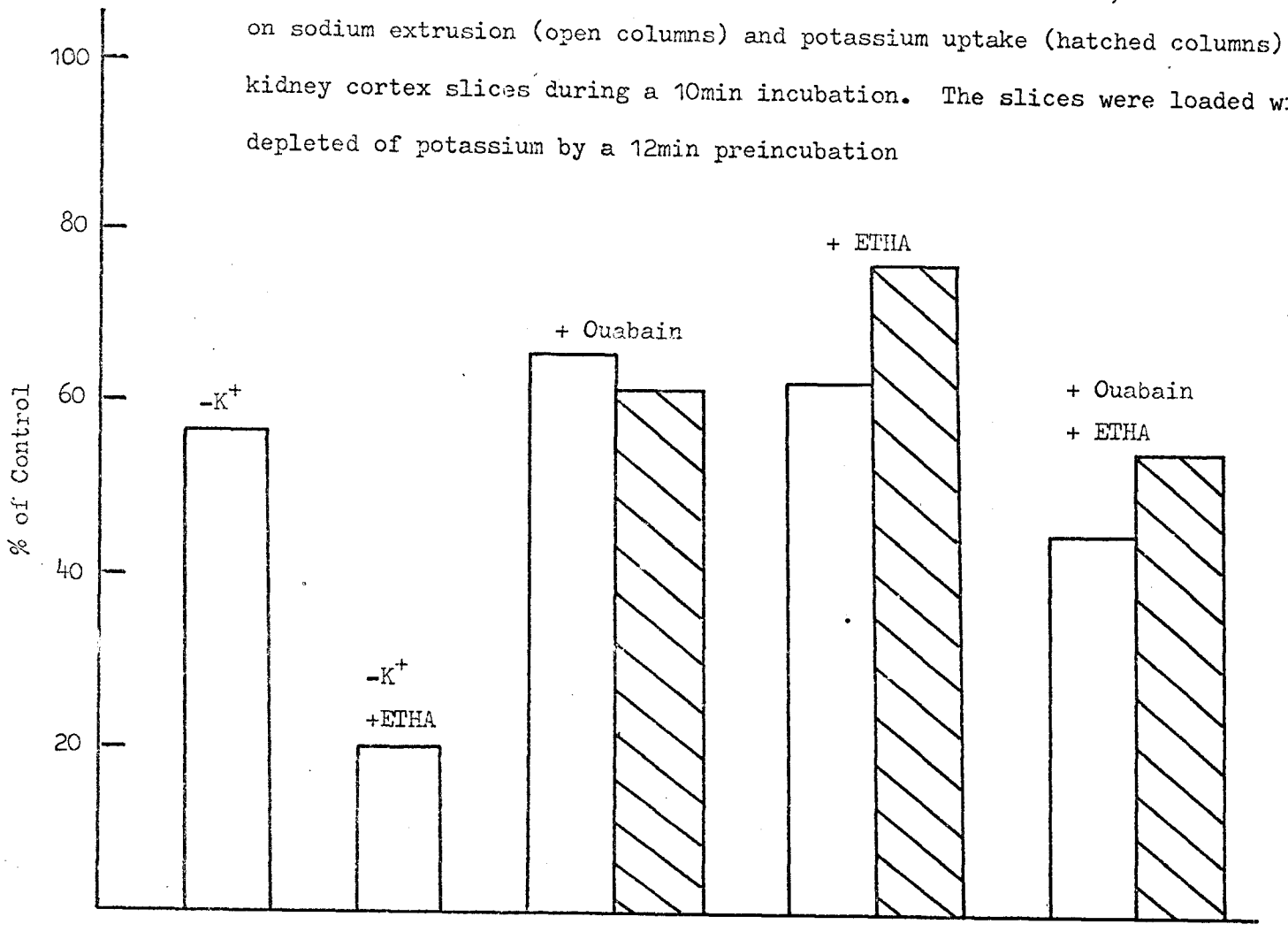


Fig 13. The effect of omitting potassium, adding ouabain (15μM) or ethacrynic acid (2mM) on sodium extrusion (open columns) and potassium uptake (hatched columns) by rabbit kidney cortex slices during a 10min incubation. The slices were loaded with sodium and depleted of potassium by a 12min preincubation.

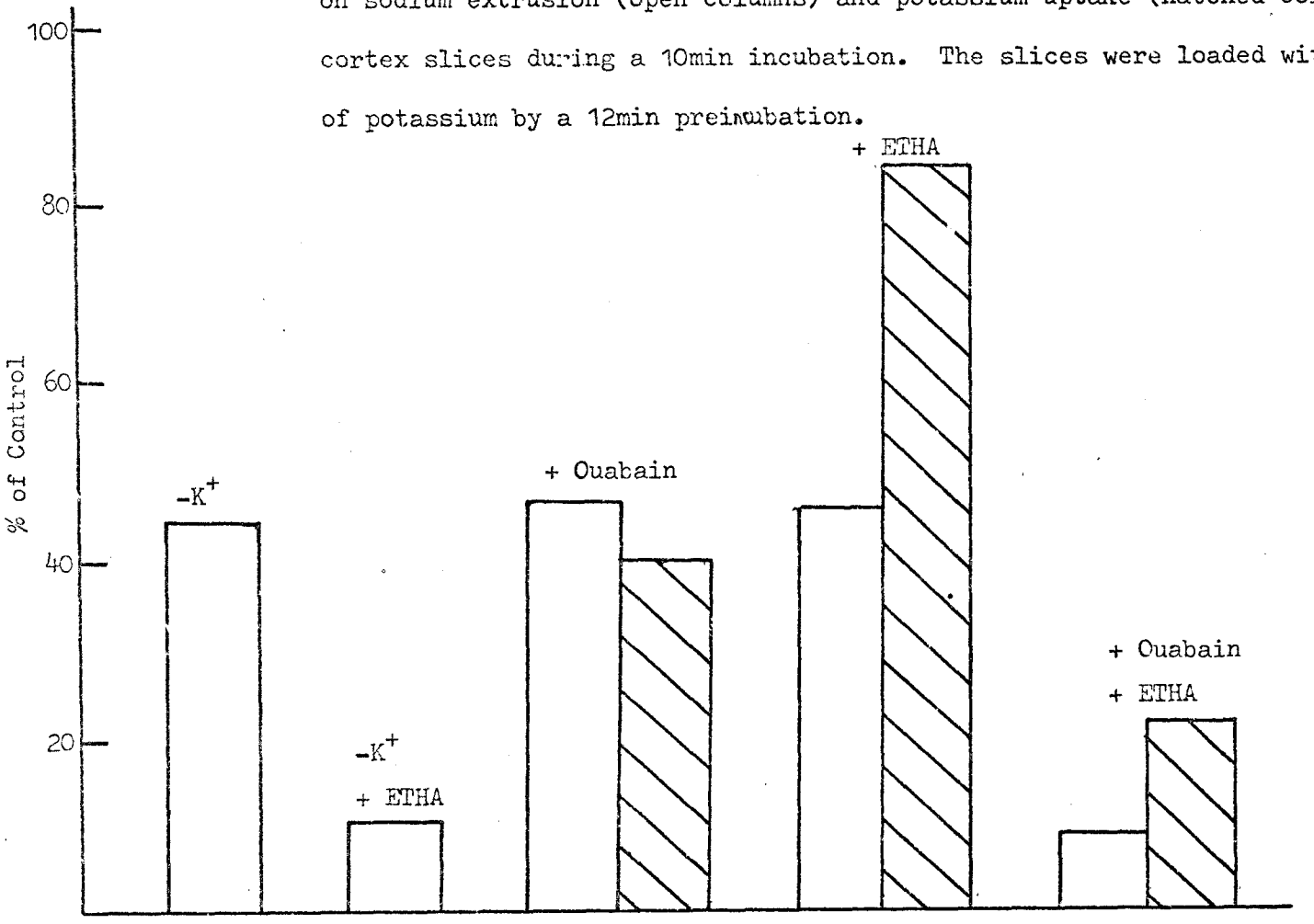


Table 20. The effects of adding ouabain (4mM) or ethacrynic acid (3mM) or omitting potassium from the incubation buffer, on sodium extrusion and potassium uptake by rat kidney cortex slices incubated for 10mins. The slices had been loaded with sodium and depleted of potassium by a 12min preincubation.

	Sodium loss	Potassium gain	
Control	228±16	96±7	(7)
Potassium-free	121±13	1±3	(11)
Ouabain	107±9	23±4	(5)
ETHA	113±15	60±7	(5)
Potassium-free + ETHA	76±12	-10±2	(12)
Ouabain + ETHA	76±12	7±3	(6)

After sodium loading, the slices contained 719±8μEq of sodium and 104±2μEq of potassium/g dry weight of tissue, these values being the means of 24 observations.

All values expressed as μEq of ion/g dry weight of tissue
Results expressed as Means ± S.E.M.
No. of observations in parenthesis

Table 21. The effect of adding ouabain (10μM) or ethacrynic acid (2mM) or omitting potassium from the incubation buffer, on sodium extrusion and potassium uptake by guinea-pig kidney cortex slices incubated for 10mins. The slices had been loaded with sodium and depleted of potassium by a 12min preincubation.

	Sodium loss	Potassium gain	
Control	250±6	107±3	(9)
Potassium-free	143±11	-11±3	(6)
Ouabain	163±14	65±4	(6)
ETHA	156±10	81±3	(16)
Potassium-free + ETHA	51±14	-5±4	(7)
Ouabain + ETHA	113±14	57±5	(6)

After sodium loading, the slices contained 604±8μEq of sodium and 114±4μEq of potassium/g dry weight of tissue, these values being the means of 24 observations.

All values expressed as μEq of ion/g dry weight of tissue
Results expressed as Means ± S.E.M.
No. of observations in parenthesis

Table 22. The effect of adding ouabain (15μM) or ethacrynic acid (2mM) or omitting potassium from the incubation buffer, on sodium extrusion and potassium uptake by rabbit kidney cortex slices incubated for 10mins. The slices had been loaded with sodium and depleted of potassium by a 12min preincubation.

	Sodium loss	Potassium gain	
Control	183±18	70±11	(7)
Potassium-free	81±10	-12±4	(8)
Ouabain	85±10	28±4	(12)
ETHA	82±8	58±6	(15)
Potassium-free + ETHA	21±5	-22±3	(8)
Ouabain + ETHA	17±9	15±7	(10)

After sodium loading, the slices contained 628±7μEq of sodium and 126±3μEq of potassium/g dry weight of tissue, these values being the means of 24 observations.

All values expressed as μEq of ion/g dry weight of tissue
Results expressed as Means ± S.E.M.
No. of observations in parenthesis

preferential inhibition of a potassium independant sodium transport mechanism.

The effect of adding ethacrynic acid in the absence of potassium or in the presence of ouabain on sodium extrusion and potassium uptake by kidney cortex slices

An indication of the specificity of ethacrynic acid can be obtained by incubating sodium loaded, potassium depleted cortex slices with ethacrynic acid either in the presence of ouabain or in the absence of potassium.

In the rat, ethacrynic acid causes only a slight reduction in sodium extrusion in the absence of potassium. Ouabain reduces sodium extrusion to a greater extent and, under these conditions, potassium uptake is reduced to a very low level, indicating a high degree of inhibition of the sodium potassium exchange pump, with a relatively slight effect on the potassium independent mechanism.

In the guinea-pig and rabbit, the addition of ethacrynic acid in the absence of potassium causes a marked reduction of sodium extrusion. In the guinea-pig, but not in the rabbit, the addition of ethacrynic acid in the presence of ouabain causes a slight reduction in both sodium extrusion and potassium uptake, in the latter species, a marked inhibition of sodium extrusion with only a moderate reduction in potassium uptake is observed. These findings are consistent with the view that, in this species, ethacrynic acid is inhibiting a potassium independent sodium extrusion mechanism.

The results presented above suggest that the action of ethacrynic acid in the rat is not a specific inhibition of a potassium independent pump of the type described by Whittembury (1968). In the guinea-pig, the degree of specificity is somewhat greater, whilst in the rabbit,

ethacrynic acid appears to have a high specificity for this mode of sodium transport.

The effect of ethacrynic acid and ouabain on sodium extrusion and potassium uptake by dog kidney cortex slices

The effects of adding ouabain and ethacrynic acid to the buffer during the second 10min incubation period on sodium extrusion and potassium uptake by dog kidney cortex slices which had been previously loaded by a 12min incubation are shown in Table 23. The addition of 100μM ouabain results in the complete inhibition of potassium uptake together with a reduction in sodium extrusion such as to suggest the specific inhibition of potassium linked sodium transport. Ethacrynic acid (2mM) causes a preferential reduction in sodium loss compared with potassium gain suggesting a relatively specific effect on potassium independent sodium extrusion. When the inhibitors are added together, sodium loss is further reduced.

The involvement of sulphahydryl groups in the action of ethacrynic acid

There is some indication that ethacrynic acid reacts with the sulphahydryl groups of the sodium dependent ATPase (Duggan & Noll, 1965, and others). Consequently, it is of interest to know whether the effects of this inhibitor on sodium pump mechanisms also require the interaction of the conjugated double bonds in the ethacrynic acid molecule with S-H groups at its site of action.

To test for this possibility, rat kidney cortex slices were sodium loaded and potassium depleted for 12mins in the usual way. These slices were then reincubated in the presence of 2mM ethacrynic acid or 5mM cysteine, together or separately. From the results shown in Table 24 it can be seen that the addition of cysteine is without effect on

Table 23. The effect of adding ouabain (100μM) or ethacrynic acid (2mM) to the incubation buffer, on sodium extrusion and potassium uptake by dog kidney cortex slices incubated for 10mins. The slices had been loaded with sodium and depleted of potassium by a 12min preincubation.

	Sodium loss	Potassium gain	
Control	229±16	99±5	(4)
Ouabain	99±16	-6±4	(4)
ETHA	71±24	53±17	(4)
Ouabain + ETHA	24±9	-24±8	(4)

After sodium loading, the slices contained 781±17μEq of sodium and 146±5μEq of potassium/g dry weight of tissue, these values being the means of 10 observations.

All values expressed as μEq of ion/g dry weight of tissue
Results expressed as Means ± S.E.M.
No. of observations in parenthesis

Table 24. The effect of adding ethacrynic acid (2mM) or cysteine (5mM) to the incubation buffer, on sodium extrusion and potassium uptake by rat kidney cortex slices incubated for 10mins. The slices had been loaded with sodium and depleted of potassium by a 12min preincubation.

	Sodium loss	Potassium gain	
Control	250±6	107±3	(6)
Cysteine	239±11	103±5	(6)
ETHA	141±13	75±3	(10)
Cysteine + ETHA	243±19	107±5	(6)

After sodium loading, the slices contained 719±8μEq of sodium and 104±2μEq of potassium/g dry weight of tissue, these values being the means of 24 observations.

All values expressed as μEq of ion/g dry weight of tissue
Results expressed as Means ± S.E.M.
No. of observations in parenthesis

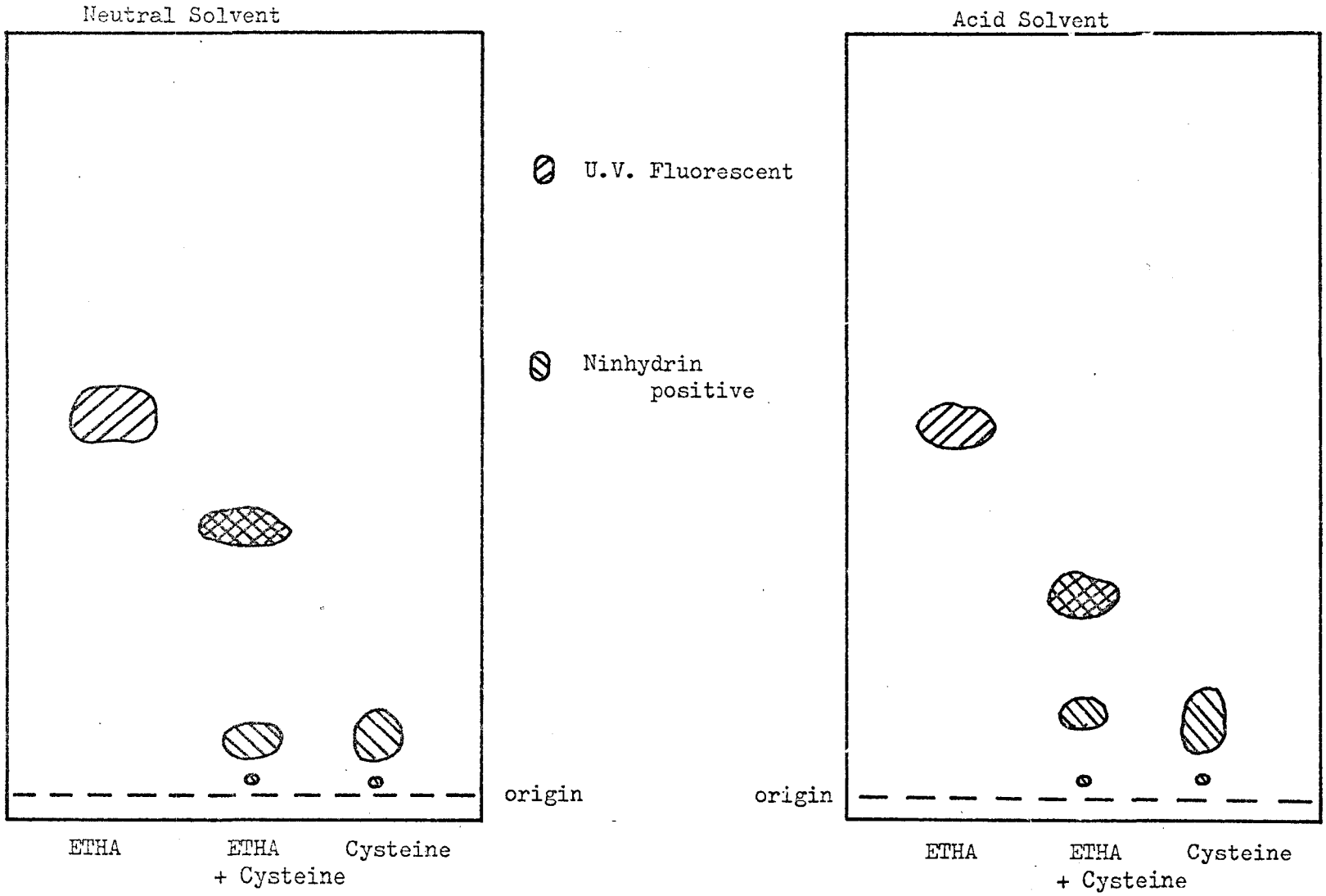
the movements of sodium and potassium, and that ethacrynic acid causes a reduction in both sodium extrusion and potassium uptake. However, ethacrynic acid is without effect when added in the presence of cysteine, indicating that the compound may exert its effect through an interaction with sulphahydryl groups, this interaction being prevented in the presence of cysteine.

In order to test whether the effect of cysteine is due to the protection of S-H groups at the site of action of ethacrynic acid, or due to an interaction of cysteine with ethacrynic acid, the two compounds were mixed in buffer at the same concentrations as used before and allowed to stand for 10mins at 25°C. A sample of this solution was diluted with 90% alcohol and spotted onto thin layer chromatography plates along with samples of cysteine and ethacrynic acid as markers. These plates were then run in either a neutral or acid solvent, allowed to dry, and the position of the compounds determined by examination of the plate under ultra-violet light. Finally, the plates were sprayed with ninhydrin to locate ninhydrin positive spots.

A record of these chromatograms is presented in Fig 14, and shows that in both solvents, ethacrynic acid gave a fast moving, U.V. fluorescent, ninhydrin negative spot, whereas, cysteine gave a slow moving, ninhydrin positive, non-fluorescent spot. The mixture of ethacrynic acid and cysteine gave two spots, one being slow moving, ninhydrin positive, non-fluorescent and corresponding to cysteine. The second spot was found to be both ninhydrin positive and U.V. fluorescent and located between the cysteine and ethacrynic acid spots. The most likely explanation of these findings is that this new compound is the cysteine conjugate of ethacrynic acid.

Consequently it appears that cysteine reacts with ethacrynic acid to cause the formation of a new compound which may be an inactive

Fig. 14 Chromatography of ethacrynic acid and cysteine, and a mixture of the two compounds



conjugate. The possibility that ethacrynic acid exerts its effect through an interaction of the molecule with tissue sulphahydryl groups cannot, therefore, be ruled out.

The effect of ethacrynic acid on potassium linked sodium pump activity

In order to compare the actions of ethacrynic acid on the potassium linked sodium pump, a series of experiments was carried out under conditions where the potassium independent sodium pump is blocked. Slices of kidney cortex were prepared from the three species used in the earlier study and sodium loaded for 20mins in buffer containing succinate in place of chloride. These slices were then reincubated for 10mins in the same buffer, in the presence of potassium and glucose and in the presence or absence of ethacrynic acid, again at the same concentrations used previously. The results presented in Table 25 show the effects of ethacrynic acid on potassium linked sodium transport in the three species. In the rat, sodium extrusion is abolished and potassium uptake reduced by 34%, indicating a marked inhibition of potassium dependent sodium transport, together with the loss of a sodium permiability barrier. Addition of ethacrynic acid to loaded guinea-pig kidney cortex slices in a chloride free buffer again causes an inhibition of the potassium linked sodium pump, the degree of inhibition being similar to that observed in the rat. In the rabbit, however, the reduction in this mode of transport due to ethacrynic acid is less than 20%, this being half that observed in the other two species.

These results suggest that although ethacrynic acid has a considerable effect on potassium linked sodium transport, in the rabbit, this inhibition is small relative to the effects on potassium independent sodium extrusion.

Table 25. The effect of adding ethacrynic acid to the incubation buffer, on sodium extrusion and potassium uptake by kidney cortex slices incubated for 10mins in buffer containing succinate in place of chloride. The slices had been loaded with sodium and depleted of potassium by a 20min incubation in a similar buffer.

	Na loss	Inhibition (%)	K gain	Inhibition (%)	
Rat					
Control	58±11		63±3		(10)
3mM ETHA	-4±13	100	41±3	34	(10)
Guinea-pig					
Control	213±13		184±4		(10)
2mM ETHA	125±10	41	118±5	30	(10)
Rabbit					
Control	188±4		143±6		(10)
2mM ETHA	153±13	18	121±5	15	(10)

All values expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

The effect of increasing the time during which ethacrynic acid is in contact with rat kidney cortex slices on sodium extrusion and potassium uptake

The suggestion that ethacrynic acid acts via a direct inhibition of cellular respiration requires that the compound enters the cells of the kidney. As the experiments reported so far have been carried out over a period of 10mins, the compound may not have had time to enter the kidney cells and exert its proposed intracellular effects, and a secondary, long term, metabolic effect, not related to a membrane action could have been overlooked.

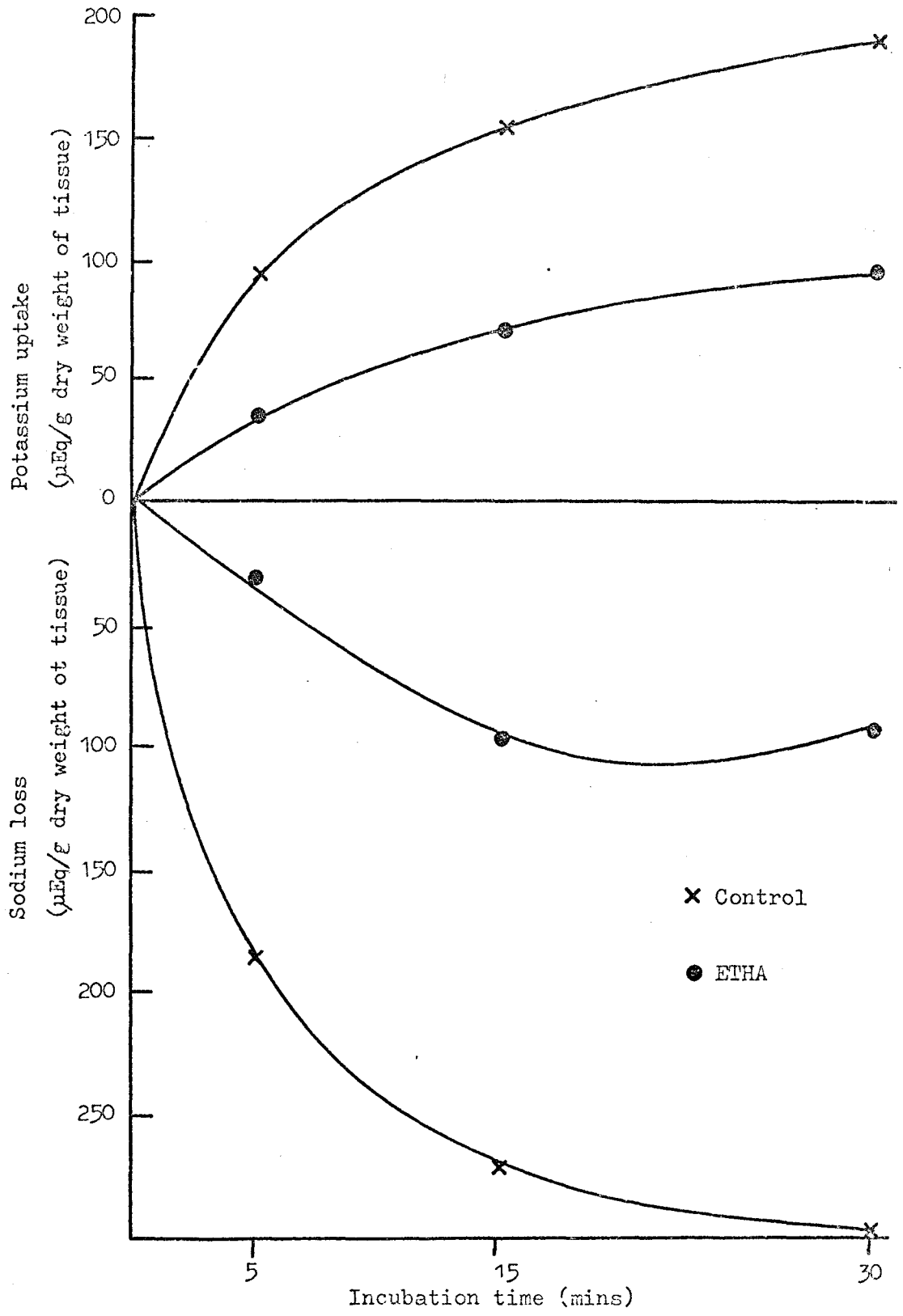
To investigate whether the inhibition of sodium transport caused by ethacrynic acid is increased by extending the time during which the tissue is in contact with the drug, two series of experiments were carried out.

In the first series, sodium loaded, potassium depleted rat kidney cortex slices were incubated in the presence or absence of 3mM ethacrynic acid for 5, 15 and 30mins. An examination of the results presented in Fig 15 show that after 30mins in the presence of ethacrynic acid, the slices start to take up sodium whereas potassium uptake is maintained at a linear rate.

In the second series, slices were loaded with sodium and depleted of potassium as before, and placed in buffer at 25°C in the presence or absence of 3mM ethacrynic acid, and allowed to remain at this temperature for 2, 15 or 30mins. After this time, pump activity was re-established by the addition of glucose and potassium, and the slices incubated for a further 10mins. The results presented in Fig 16 clearly show that preincubation of slices with ethacrynic acid markedly increases the degree of inhibition of both sodium extrusion and potassium uptake.

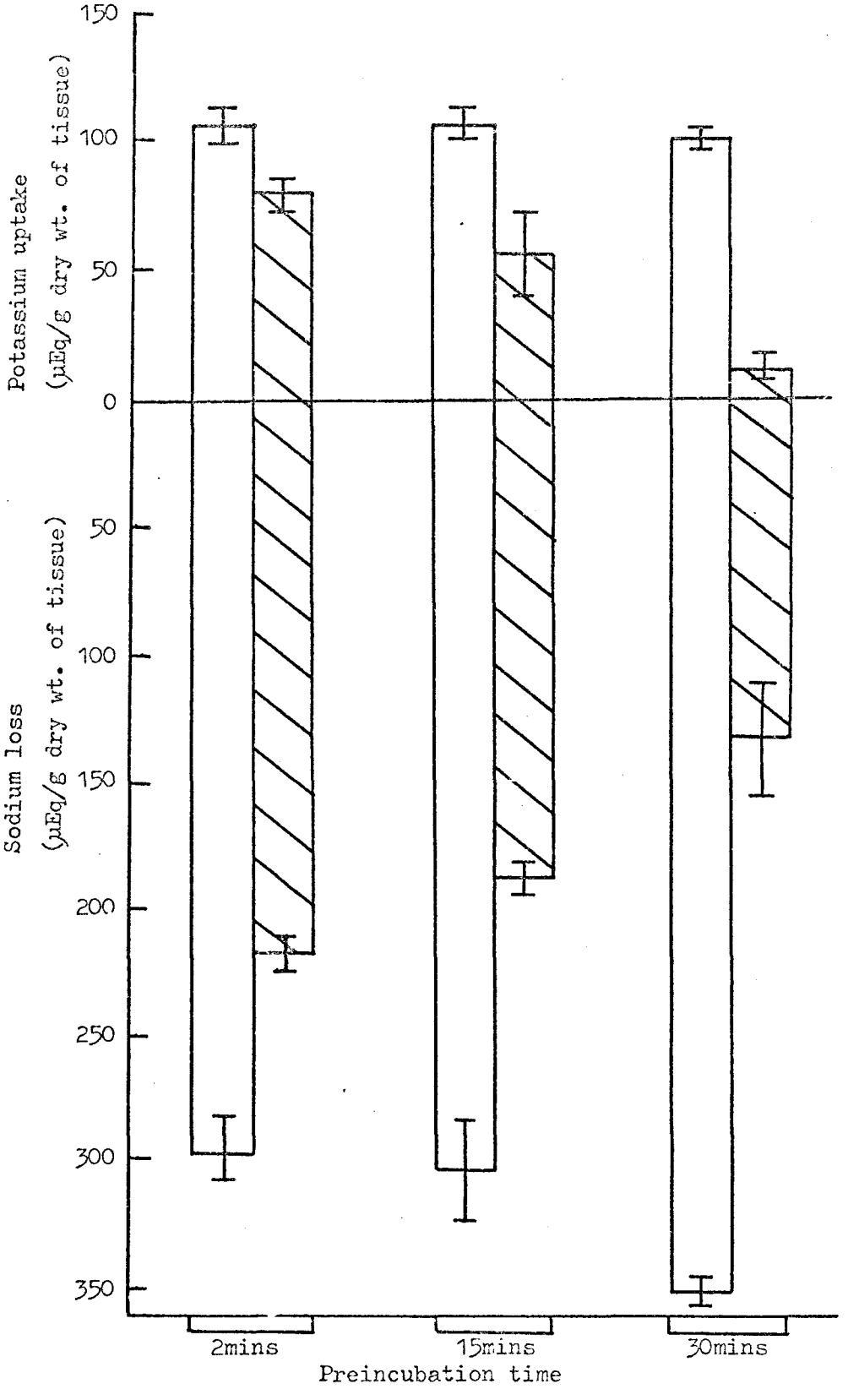
These experiments clearly show that ethacrynic acid has a long term effect which is probably not linked to any initial effect at the cell membrane.

Fig. 15 The time course of action of ethacrynic acid (3mM) on sodium extrusion and potassium uptake by rat kidney cortex slices. The slices were loaded with sodium and depleted of potassium by a 12min preincubation.



Each value is the mean of two observations

Fig. 16 The effect of preincubating sodium loaded, potassium depleted rat kidney cortex slices in the presence (hatched columns) or absence of 3mM ethacrynic acid on the subsequent sodium and potassium movements during a 10min incubation



Results expressed as Means of 4 observations ± S.E.M.

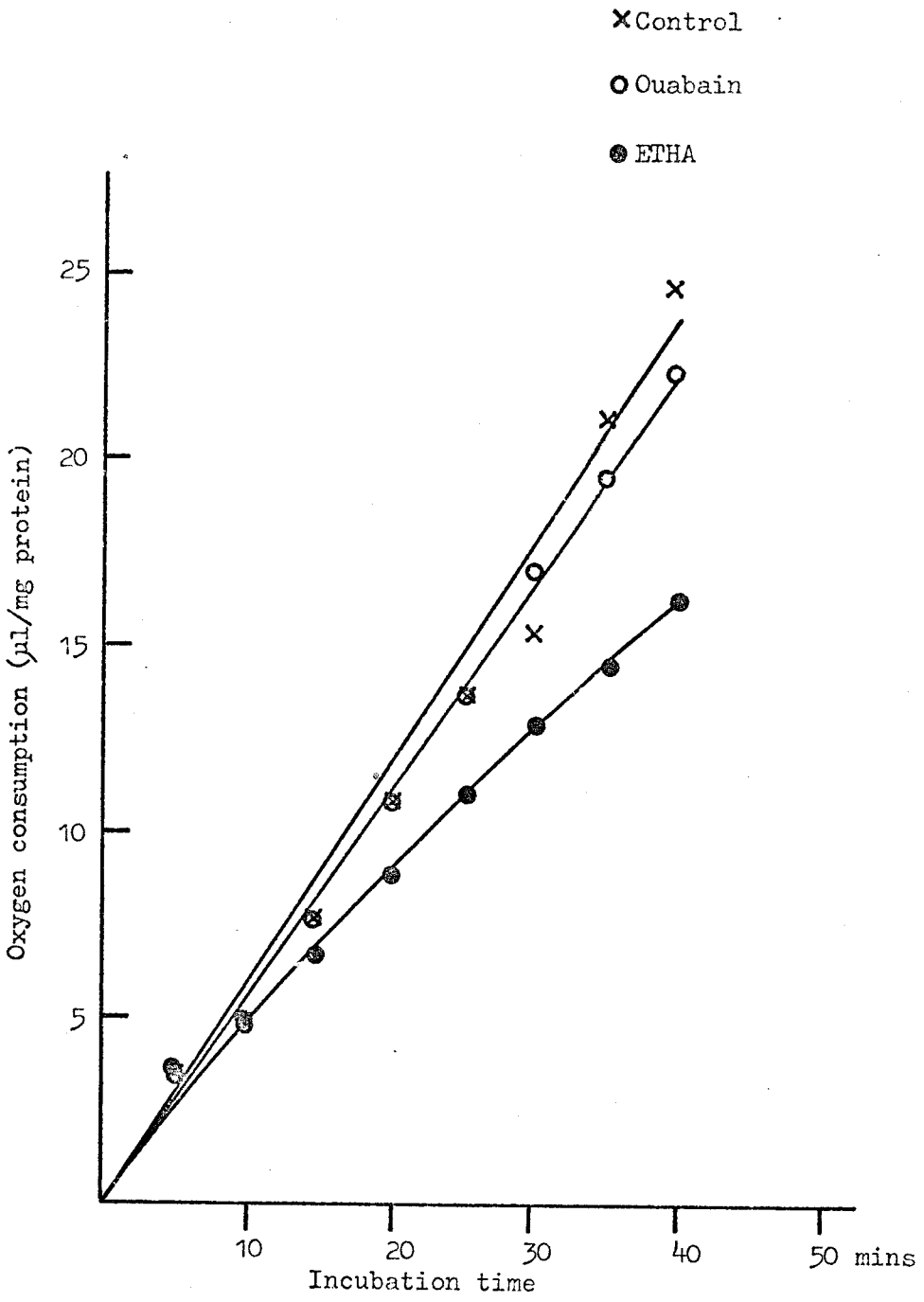
The effects of ethacrynic acid and ouabain on oxygen consumption by slices and homogenates of renal cortex taken from rat, guinea-pig or rabbit kidney

An attempt was made to determine the metabolic actions of ouabain and ethacrynic acid by studying the effects of these two compounds on the consumption of oxygen by slices and homogenates of kidney cortex prepared from the three species used before. The concentrations of the inhibitors used were also the same as in the previous study, that is, concentrations which lead to comparable reductions in sodium transport.

The rates of oxygen consumption by slices and homogenates of kidney cortex prepared from the three species, and the effects of the two inhibitors on these rates are shown in Figs 17-23.

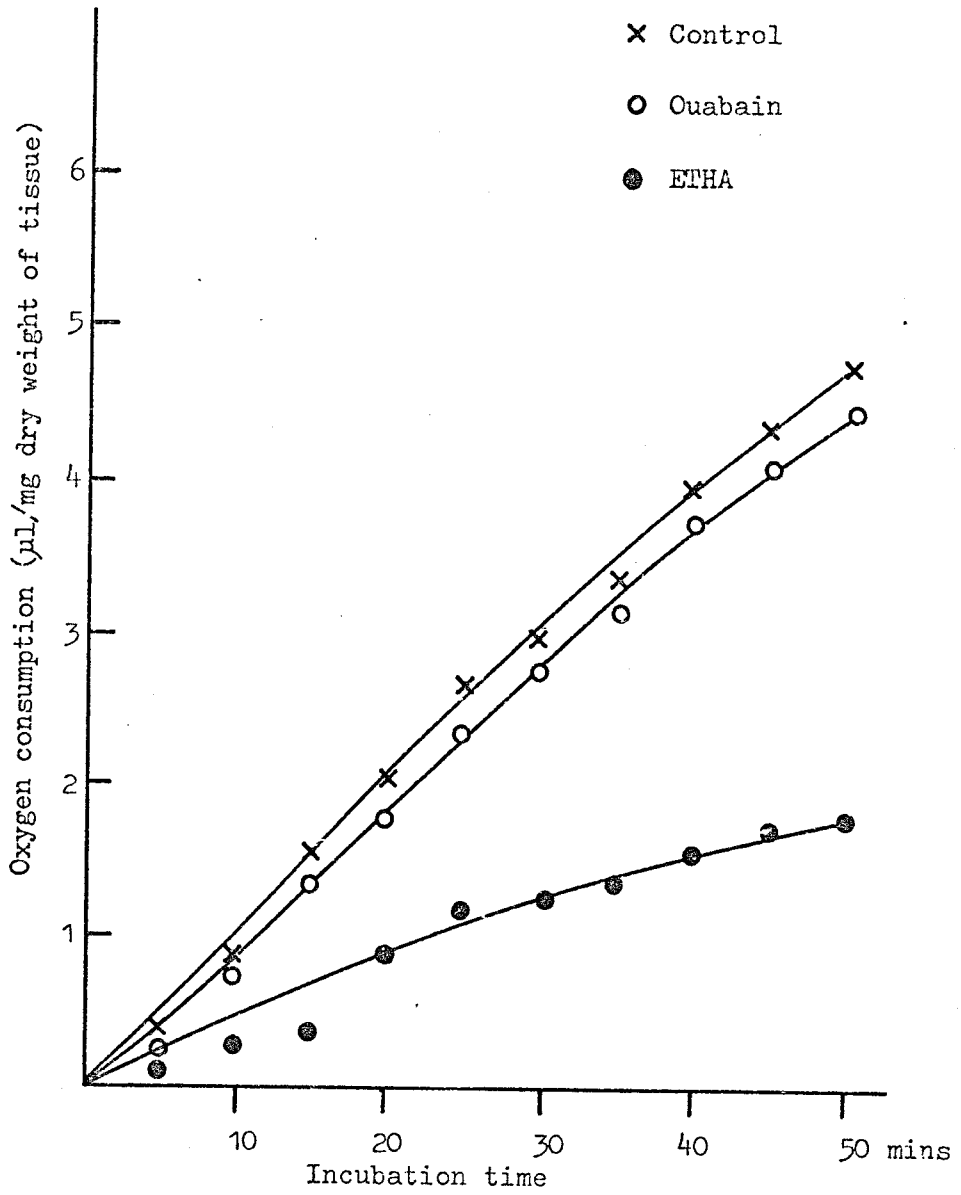
Figs 17-19 show the effects of ouabain and ethacrynic acid on the rate of oxygen consumption by homogenates and slices of rat kidney cortex. These results demonstrate that ouabain has no significant effect on the rate of oxygen consumption by kidney homogenates, whereas, the rate of oxygen consumption by kidney slices is inhibited in proportion to the concentration of ouabain present in the medium, this inhibition being significant at the 0.1% level at both concentrations of inhibitor. In contrast, ethacrynic acid causes a marked inhibition of oxygen consumption both by homogenates and slices, and, at a lower concentration (Fig 19) is more effective as an inhibitor of oxygen consumption by cortex slices than is the highest concentration of ouabain used in this experiment (P in all case being < 0.001), a concentration which would lead to a much greater inhibition of sodium transport than would the lower concentration of ethacrynic acid. However, the effect of ethacrynic acid is greater when added to cortex slices than when added to cortex homogenates.

Fig. 17 The effect of ethacrynic acid (3mM) and ouabain (4mM) on oxygen consumption by rat kidney cortex homogenates



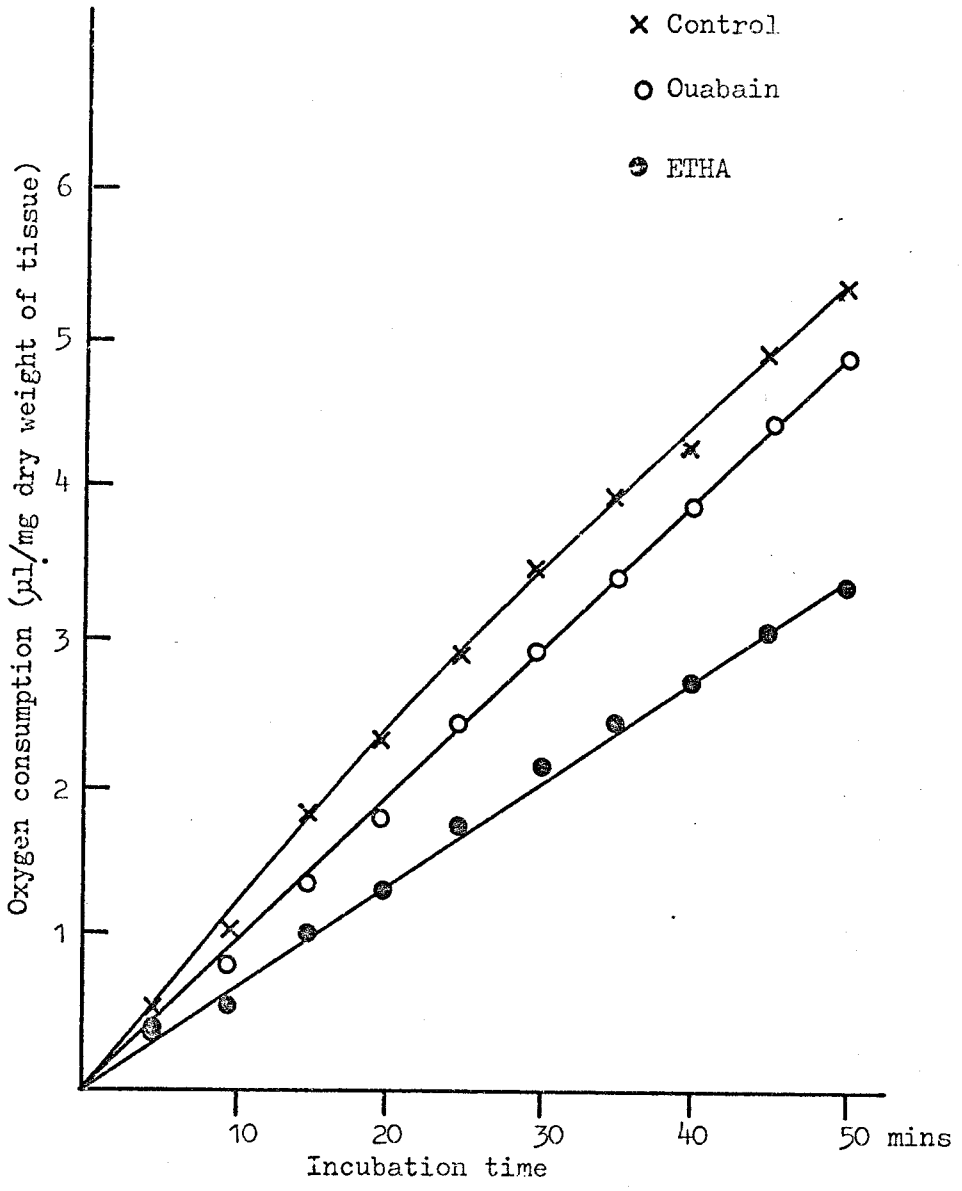
Each value is the mean of two observations

Fig 18. The effect of ethacrynic acid (3mM) and ouabain (4mM) on oxygen consumption by rat kidney cortex slices



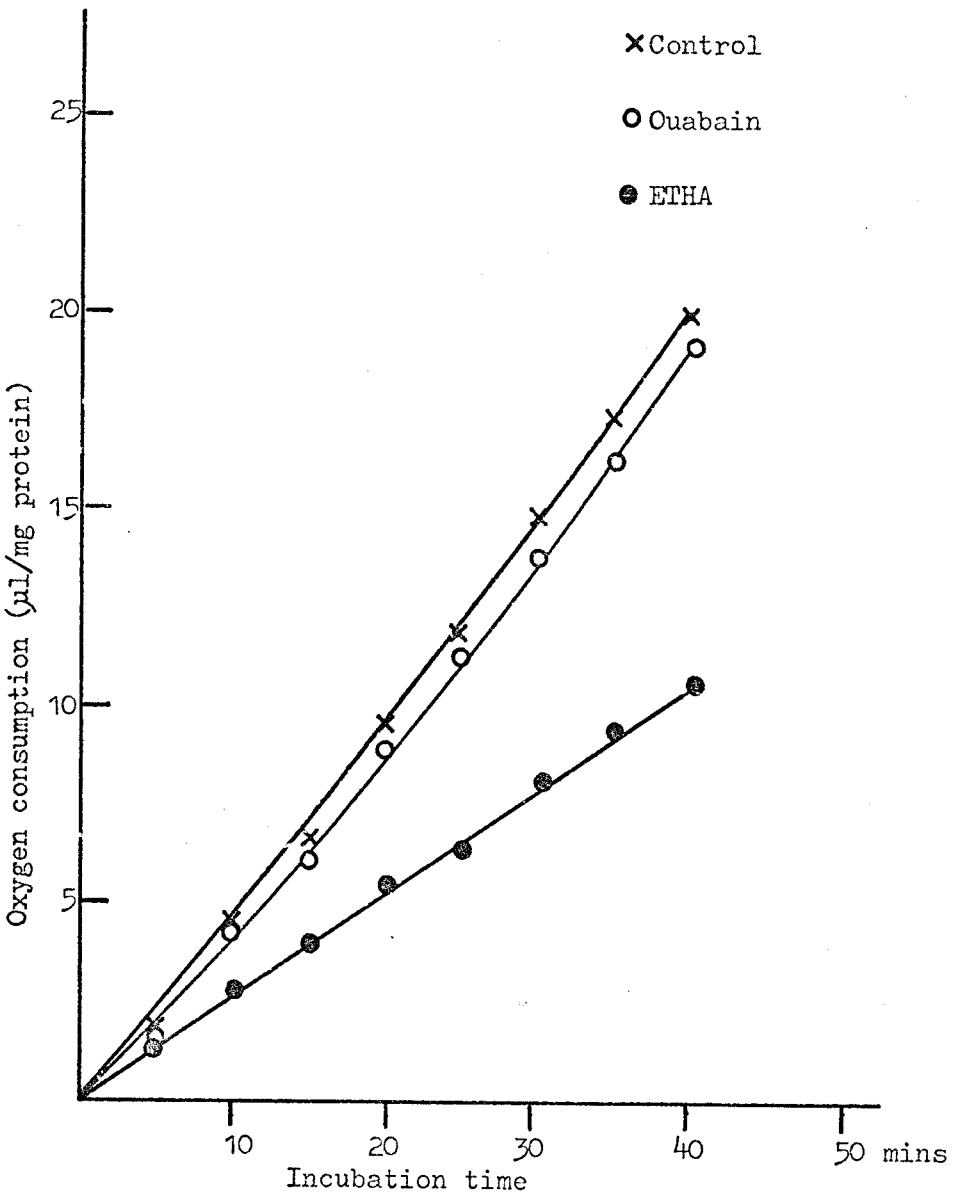
Each value is the mean of two observations

Fig. 19 The effect of ethacrynic acid (2mM) and ouabain (8mM) on oxygen consumption by rat kidney cortex slices



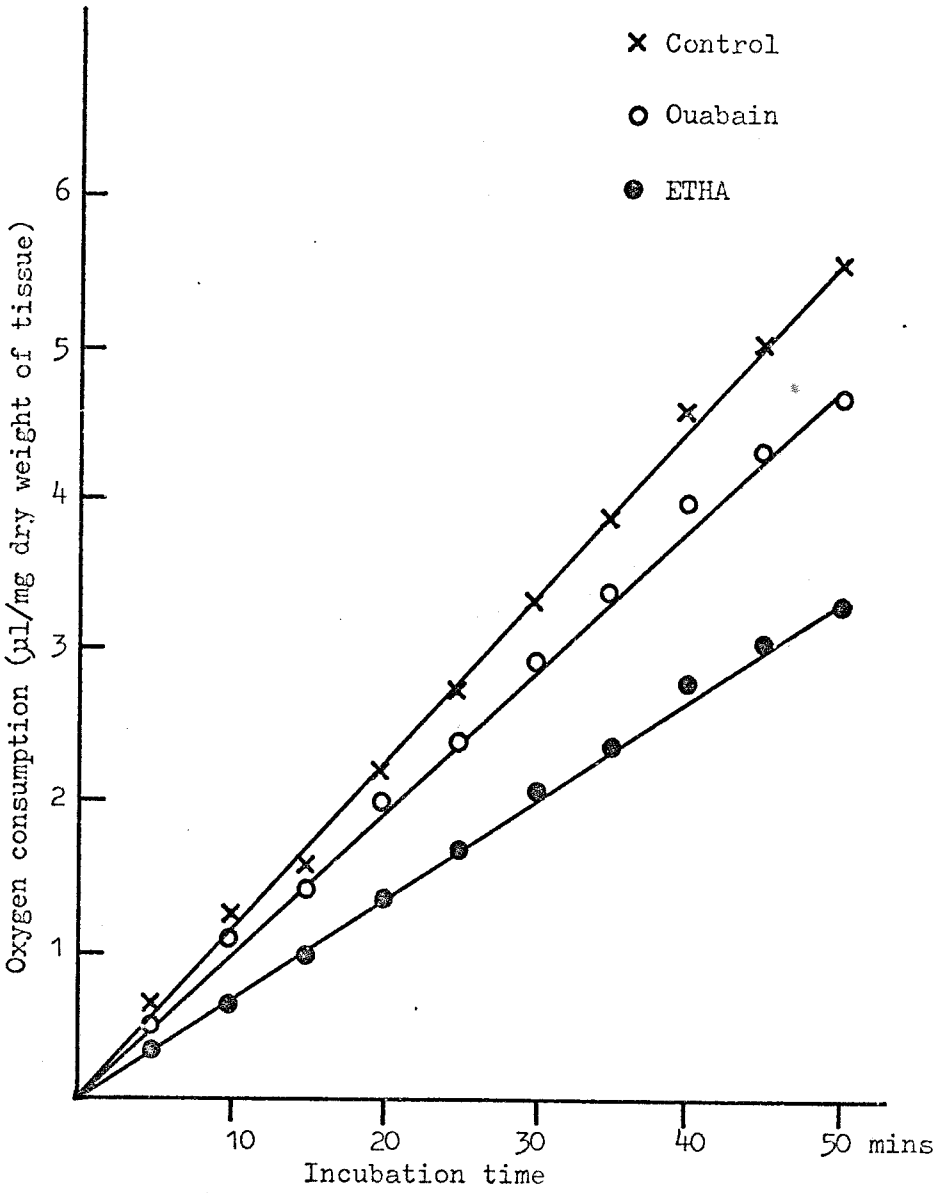
Each value is the mean of two observations

Fig. 20 The effect of ethacrynic acid (2mM) and ouabain (10μM) on oxygen consumption by guinea-pig kidney cortex homogenates



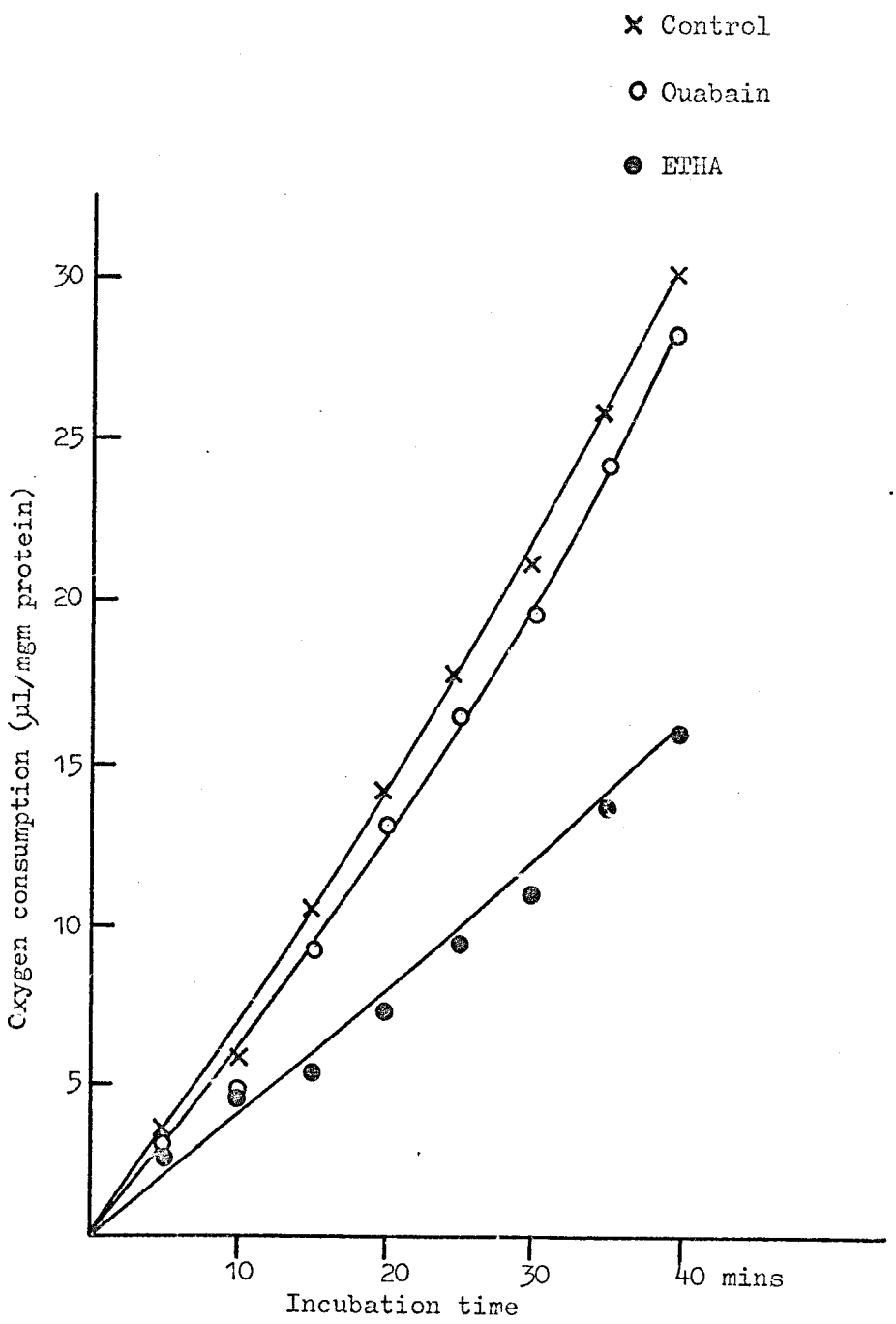
Each value is the mean of two observations

Fig. 21 The effect of ethacrynic acid (2mM) and ouabain (10μM) on oxygen consumption by guinea-pig kidney cortex slices



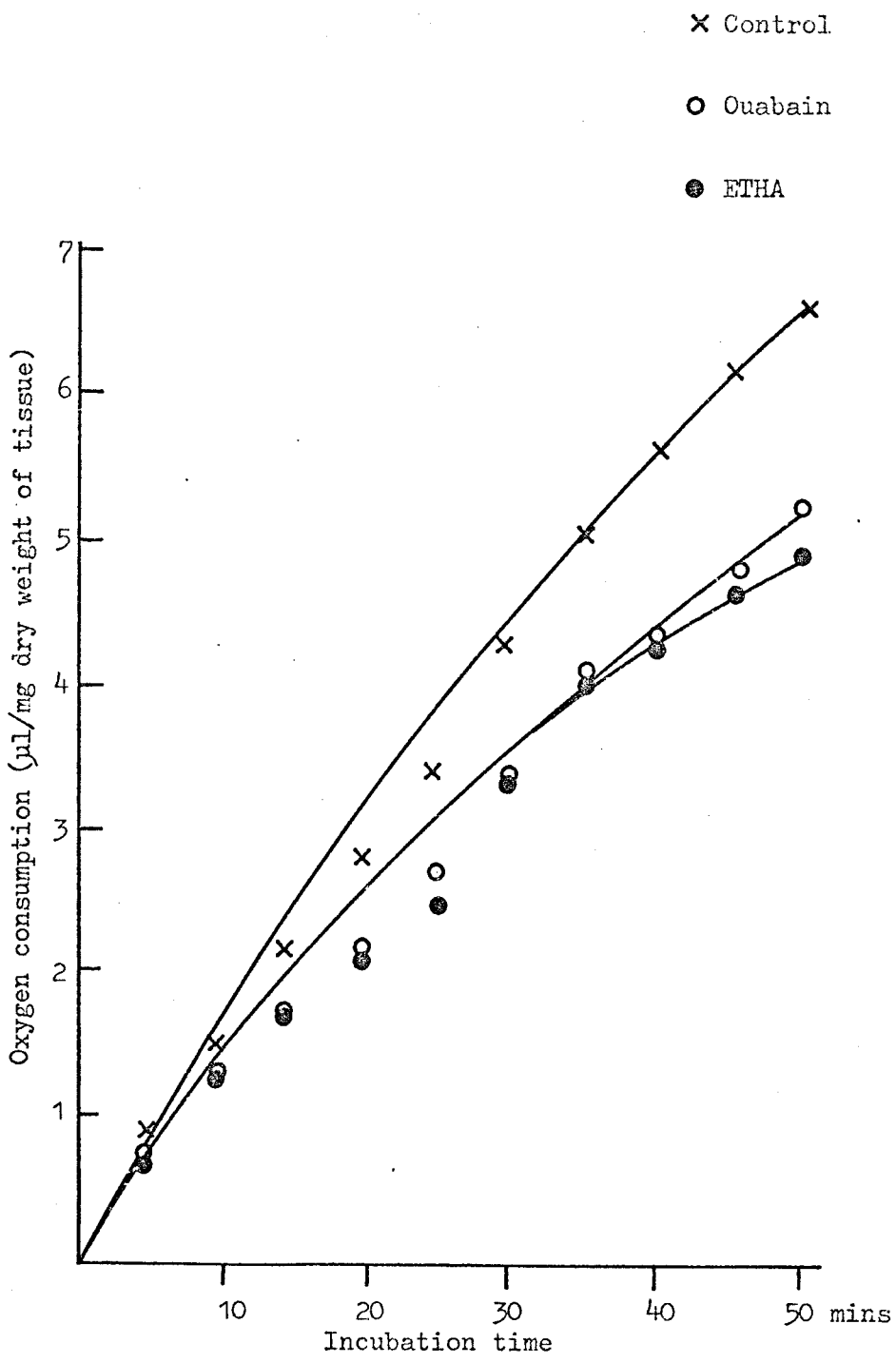
Each value is the mean of two observations

Fig. 22 The effect of ethacrynic acid (2mM) and ouabain (15μM) on oxygen consumption by rabbit kidney cortex homogenates



Each value is the mean of two observations

Fig. 23 The effect of ethacrynic acid (2mM) and ouabain (15μM) on oxygen consumption by rabbit kidney cortex slices



Each value is the mean of two observations

These results suggest that, in rat kidney cortex, both ethacrynic acid and ouabain reduce oxygen consumption via an inhibition of a membrane bound energy requiring transport process. In addition, ethacrynic acid appears to further reduce oxygen consumption by an inhibitory effect on cellular respiration.

When these experiments were repeated with slices and homogenates prepared from guinea-pig kidney cortex (Figs 20 and 21), it was found that ethacrynic acid gave comparable reductions in the consumption of oxygen by both preparations ($P < 0.001$). Ouabain caused a considerable reduction in oxygen consumption by cortex slices, and a slight, but significant, reduction in oxygen consumption by cortex homogenates (P in both case being < 0.001). These results support the proposition that ouabain acts via an inhibition of a membrane bound energy consuming process, however, it is not possible to say whether the effect of ethacrynic acid is due to an action at the cell membrane in addition to a metabolic action, or solely a metabolic action.

The effects of ethacrynic acid and ouabain on the consumption of oxygen by slices and homogenates of rabbit cortex are shown in Figs 22 and 23. These results show that the effects of ouabain on the two rabbit kidney preparations are similar to those observed in the two guinea-pig preparations, again indicating that the effects of ouabain are due to an action at the cell membrane. The effects of ethacrynic acid on homogenates of rabbit kidney cortex are again similar to that observed in the guinea-pig kidney cortex homogenate, the drug causing a marked reduction in oxygen consumption, this reduction being significant at the 0.1% level. However, in rabbit kidney cortex slices, the effects of ethacrynic acid are similar in magnitude to the effects of ouabain. Whether these effects of ethacrynic acid on oxygen consumption by cortex slices are due to an action at the cell membrane

or due to a low cellular concentration of the drug cannot be determined from the results obtained. However, as the two drugs caused a similar degree of inhibition of both sodium transport and oxygen consumption by rabbit kidney cortex slices,at the concentrations used, a specific action at the cell membrane cannot be discounted.

These results suggest that in all three species, ethacrynic acid will inhibit cellular respiration once it penetrates the cell membrane, however, it also appears to have actions at the cell membrane, and it appears likely that this latter effect is the predominant mechanism of action in rabbit kidney cortex, and to a lesser extent in guinea-pig kidney cortex. The effect of the inhibitor in rat kidney cortex, on the other hand, would suggest that the main action of ethacrynic acid is on cellular respiration.

The effect of incubating fresh kidney cortex slices for 1hr in the presence or absence of ethacrynic acid or ouabain

Following the measurement of oxygen consumption by kidney cortex slices, the sodium and potassium content of these slices was determined and the results presented in Tables 26 to 28.

In all three species, the effects of the two inhibitors are quantitively similar. In the presence of ethacrynic acid, the tissue gains sodium and loses potassium, indicating a general inhibition of sodium pump activity in all three species. After incubation with ouabain, potassium is lost from the tissue, yet, sodium is not taken up, suggesting that the sodium content of the tissue is being maintained by a potassium independent sodium pump.

Table 26. The cation content of rat kidney cortex slices after incubation at 25°C for 1hr in the presence or absence of ouabain (4mM) or ethacrynic acid (3mM)

	Tissue sodium	Tissue potassium	
Control	588±18	180±5	(4)
Ouabain	608±27	116±7	(4)
ETHA	741±11	121±8	(4)

All values expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Table 27. The cation content of guinea-pig kidney cortex slices after incubation at 25°C for 1hr in the presence or absence of ouabain (10μM) or ethacrynic acid (2mM)

	Tissue sodium	Tissue potassium	
Control	459±37	229±4	(4)
Ouabain	477±32	175±15	(4)
ETHA	595±31	165±13	(4)

All values expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Table 28. The cation content of rabbit kidney cortex slices after incubation at 25°C for 1hr in the presence or absence of ouabain (15μM) or ethacrynic acid (2mM)

	Tissue sodium	Tissue potassium	
Control	623±13	306±18	(4)
Ouabain	612±7	148±3	(4)
ETHA	906±14	123±4	(4)

All values expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Section 3

The effect of angiotensin on sodium transport in kidney cortex slices

Section 3. The effect of angiotensin on sodium transport in kidney cortex slices

It is well documented that angiotensin has a biphasic action on the handling of sodium by the kidney (Barraclough et al., 1967 and others). It has been suggested that the antinatriuretic effect occurring with low doses of the hormone (Malvin & Vander, 1967; Bonjour & Malvin, 1969) is secondary to renal vasoconstriction, although Baraclough et al. (1967) suggest that this effect is due to a direct tubular action. The results of experiments carried out by Munday, Parsons & Poat (1971) support this view. They observed that when sodium loaded, potassium depleted rat kidney cortex slices were reincubated in the presence of angiotensin at physiological concentrations (10^{-12}M), sodium extrusion from these slices was stimulated, and potassium uptake inhibited. They also observed that in the absence of potassium or in the presence of ouabain in the second incubation buffer angiotensin again stimulates sodium extrusion, suggesting that angiotensin has a direct action on a potassium independent sodium transport mechanism.

Experiments were carried out to further elucidate the mechanism of action of angiotensin, and to determine whether a similar response to the hormone could be obtained in other species.

The effect of angiotensin on sodium extrusion from rat kidney cortex slices incubated in the presence of ouabain or in the absence of potassium

Rat kidney cortex slices were loaded with sodium and depleted of potassium in the usual way. These slices were reincubated in the absence of potassium, or in the presence of a saturating concentration of ouabain (7mM). The results in Table 29 show that under both these

Table 29. The effect of angiotensin (10^{-12}M) on sodium extrusion and potassium uptake by 12min sodium loaded, potassium depleted rat kidney cortex slices during a 10min incubation in the absence of potassium, or in the presence of ouabain (7mM)

	Na loss	P	K gain	P	
Potassium-free					
Control	115±8	< 0.01	2±3	N.S.	(6)
Angiotensin	153±7		6±3		(6)
Ouabain					
Control	96±9	< 0.05	2±6	N.S.	(7)
Angiotensin	138±18		-12±3		(7)

All values expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

conditions, the addition of angiotensin (10^{-12}M) to the second incubation buffer causes a significant stimulation of sodium extrusion.

The effect of angiotensin on potassium linked, and chloride linked sodium extrusion

To determine whether the action of angiotensin is via a stimulation of the chloride linked, potassium independent sodium pump, slices were loaded with sodium and depleted of potassium by a 20min preincubation in buffer containing chloride, or buffer containing succinate in place of this ion. Those slices preincubated in the presence of chloride were incubated, in a similar buffer, for 10mins, in the absence of potassium to inhibit potassium linked sodium pump activity. Those slices preincubated in the absence of chloride were incubated, for 10mins, again in the absence of chloride, but in the presence of potassium.

It was found that when angiotensin (10^{-12}M) was added to the second incubation buffer, an increase in sodium extrusion was only observed in the presence of chloride, no stimulation occurring in the presence of potassium but the absence of chloride. The results of these experiments, shown in Tables 30 and 31, suggest that angiotensin stimulates sodium retention by the kidney through, at least in part, a stimulation of chloride linked sodium pump activity.

The effect of angiotensin on sodium extrusion and potassium uptake by guinea-pig kidney cortex slices

It has been demonstrated (Munday et al., 1971) that angiotensin, at physiological concentrations, stimulates sodium extrusion and inhibits potassium uptake by sodium loaded, potassium depleted rat kidney cortex slices. To investigate whether this effect could be

Table 30. The effect of angiotensin (10^{-12}M) on sodium extrusion and potassium uptake during a 10min incubation in buffer containing potassium, but with succinate added in place of chloride. The slices had been loaded with sodium and depleted of potassium by a 20min preincubation in a similar buffer in the absence of both potassium and chloride.

	Na loss	P	K gain	P	
Control	74±15	N.S.	70±5	N.S.	(4)
Angiotensin	73±10		65±4		(4)

All values expressed as μEq of ion/g dry weight of tissue

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Table 31. The effect of angiotensin (10^{-12}M) on sodium extrusion and potassium uptake during a 10min incubation in buffer containing chloride, but in the absence of potassium. The slices had been loaded with sodium and depleted of potassium by a 20min preincubation in a similar buffer, in the presence of chloride, but in the absence of potassium.

	Na loss	P	K gain	P	
Control	90±15		-8±3		(4)
		< 0.01		N.S.	
Angiotensin	168±14		-10±3		(4)

All values expressed as μEq of ion/g dry weight of tissue

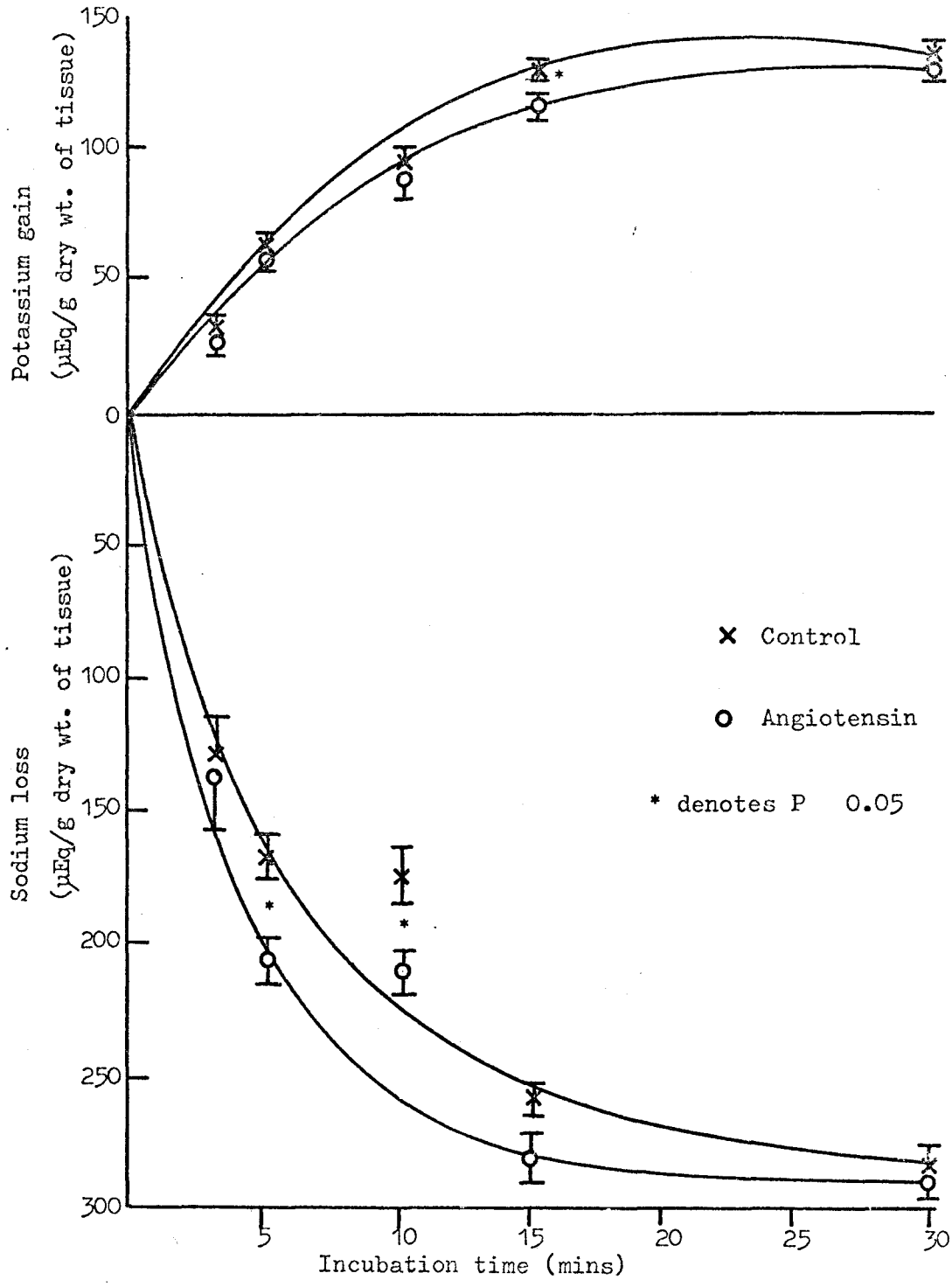
Results expressed as Means ± S.E.M.

No. of observations in parenthesis

observed in another species, guinea-pig kidney cortex slices were loaded with sodium and depleted of potassium by a 12min preincubation. These slices were then incubated in the presence or absence of 10^{-12}M angiotensin. Fig 24 shows the time course of sodium extrusion and potassium uptake under these conditions, and demonstrates that angiotensin causes an increase in the rate of sodium extrusion, this being significant at 5 and 10mins from the beginning of the second incubation. This is associated with a slight, but significant, decrease in potassium uptake after 15mins incubation.

These results are similar to those obtained by Munday et al. (1971), indicating that in the guinea-pig, as in the rat, angiotensin, at physiological concentrations, causes a stimulation of potassium independent sodium pump activity.

Fig. 24 The effect of angiotensin (10^{-12}M) on sodium extrusion and potassium uptake by guinea-pig kidney cortex slices. The slices were loaded with sodium and depleted of potassium by a 12min preincubation.



Results expressed as Mean of 3 observations ± S.E.M.

Section 4

Sodium pump activity in intestinal rings

SECTION 4. Sodium pump activity in intestinal rings

In an attempt to compare the effects of angiotensin on sodium pump activity in the kidney with that in the intestine, everted intestinal rings were prepared and used for the following study.

The effects of ouabain and dinitrophenol (DNP) on the cation content of everted intestinal rings prepared from a range of species

Table 32 shows the effect of incubating everted rings of jejunum prepared from rats, hamsters, guinea-pigs and mice, and everted colon rings prepared from rats, in the presence or absence of 5mM ouabain or 2mM DNP. These experiments were carried out to determine which of the above intestinal preparations was most suitable for further study. DNP would be expected to inhibit sodium transport as a consequence of its inhibitory action on aerobic respiration so that a comparison between the tissue ion contect in the presence or absence of this compound should give an indication of the ability of intestinal rings to maintain their sodium and potassium balence. The ability of ouabain, through its effect on the potassium dependent sodium pump, to increase tissue sodium and reduce tissue potassium was also measured.

The response of intestine to these two inhibitors depends very much on the tissue under study. Rat and mouse jejunum ring sodium levels are almost unaffected by ouabain, whereas this compound causes an increase in the sodium content of rings prepared from the jejunum of hamster and guinea-pig. In contrast, guinea-pig intestine appears to be insensitive to DNP whereas, hamster intestine shows a considerable reduction in the tissue potassium/sodium ratio in the presence of this inhibitor, consequently, hamster jejunum was chosen for the following study, as it appears to be highly sensitive to both metabolic and membrane inhibitors.

Table 32. The effect of DNP (2mM) and ouabain (5mM) on the ion content of various intestinal ring preparations incubated aerobically for 30mins at 25°C

Species	Tissue	Tissue Na			Tissue K		
		Control	DNP	Ouabain	Control	DNP	Ouabain
Rat	jejunum	628	732	636	366	315	316
	colon	732	770	772	326	309	239
Hamster	jejunum	463	614	528	337	310	234
Guinea-pig	jejunum	465	475	531	346	313	144
Mouse	jejunum	667	869	674	380	313	231

All values expressed as μEq of ion/g dry weight of tissue

All results are the means of 2 observations

The effect of potassium, ouabain and ethacrynic acid on sodium extrusion and potassium uptake by sodium loaded, potassium depleted hamster jejunum rings.

Everted hamster jejunum rings were prepared and incubated at 0°C for 2hrs in Krebs' bicarbonate buffer in the absence of potassium and glucose. Under these conditions, the tissue takes up sodium and loses potassium. These sodium loaded, potassium depleted rings were then reincubated at 25°C for 15mins in the presence of glucose, and in the presence or absence of potassium, ouabain and ethacrynic acid.

It can be seen (Table 33) that on reincubation, the rings lose sodium, and in the presence of potassium, accumulate potassium. The addition of ouabain or the omission of potassium during the second incubation causes a further loss of potassium, and a reduction in sodium extrusion, although in the absence of potassium this reduction is not significant. Ethacrynic acid causes a significant reduction in potassium uptake ($P < 0.02$) with no change in sodium extrusion.

Since tissue potassium does not drop to a low level after the first incubation, tissue wet weights and dry weights were compared in order to determine the degree of loading of the tissue. It was found that the tissue sodium concentration after incubating for 2hrs at 0°C was 57.4 ± 2.8 µEq/ml ($n = 9$) compared with a buffer concentration of 143µEq/ml. Thus the tissue is not well loaded with sodium, and consequently tissue potassium remains at a high level.

The effect of incubating sodium loaded, potassium depleted jejunum rings from hamster, on intracellular sodium and potassium

Hamster jejunum rings were loaded with sodium and depleted of potassium for 2hrs at 0°C and reincubated at 25°C or 37°C for up to 14mins in the presence of H^3 Inulin to measure extracellular space.

Table 33. The effects of omitting potassium or adding ouabain (5mM) or ethacrynic acid (1mM) to the incubation buffer, on sodium extrusion and potassium uptake by everted hamster jejunum rings incubated for 15mins. The rings had been loaded with sodium and depleted of potassium by a 2hr preincubation at 0°C.

	Tissue Na	Na loss	Tissue K	K gain	
Control	564±22	172±22	368±11	72±11	(5)
Potassium-free	615±20	121±20	261±15	-35±15	(5)
Ouabain	668±26	68±26	165±9	-131±29	(5)
ETHA	550±50	186±50	326±9	30±9	(5)

After sodium loading, the rings contained 736±68µEq of sodium and 104±2µEq of potassium/g dry weight of tissue, these values being the means of 6 observations.

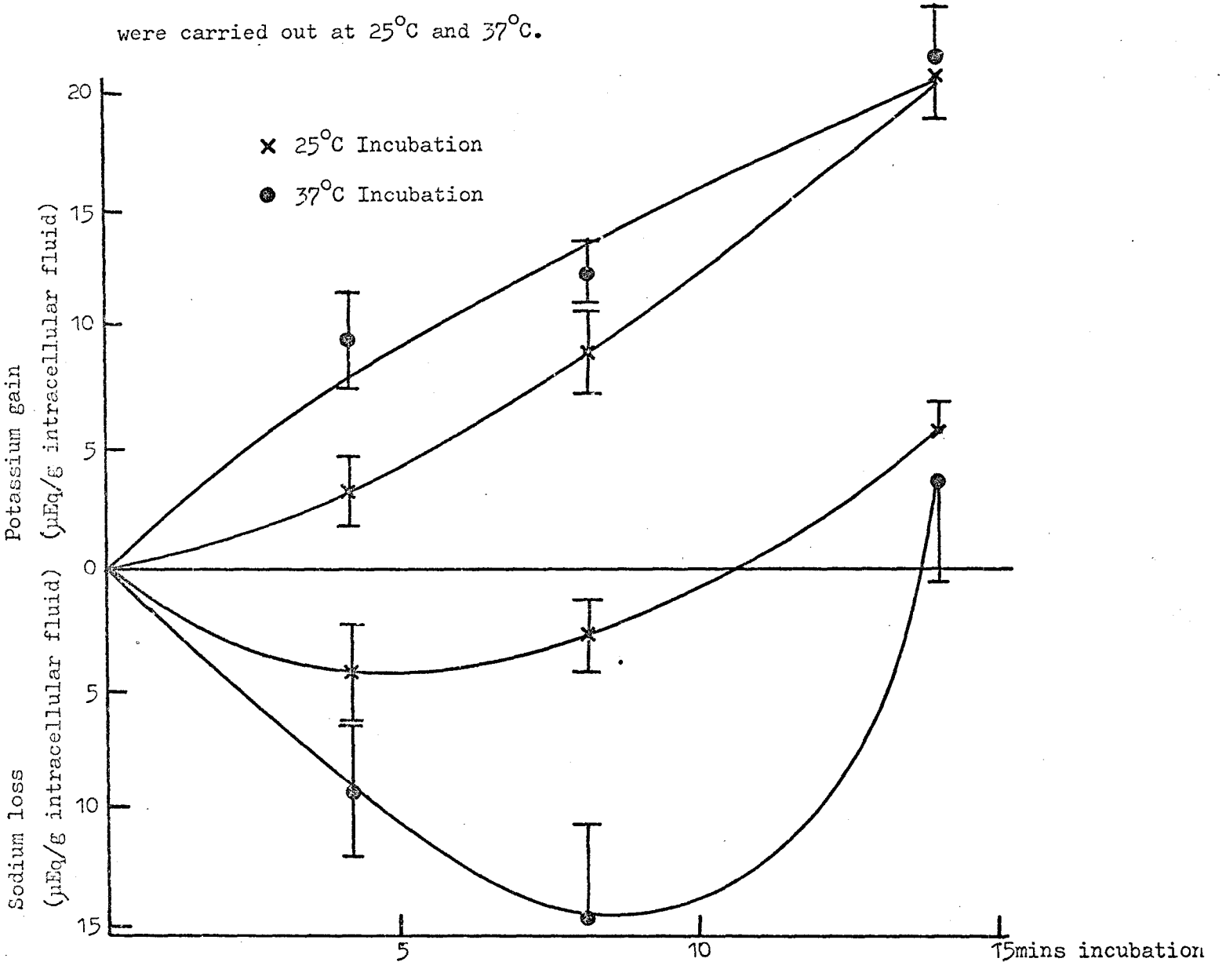
All values expressed as µEq of ion/g dry weight of tissue
Results expressed as Means ± S.E.M.
No. of observations in parenthesis

It was found that, on reincubation, the extracellular space of the tissue decreases. When the intracellular ion changes are calculated, it is found that the decrease in cell sodium is not maintained and, after 8mins, the cells begin to take up sodium from the buffer, indicating that the cells become leaky to this ion. In contrast, potassium is taken up throughout the 14min period. The time course of these movements is shown in Fig 25, and demonstrates that the initial rates of sodium extrusion and potassium uptake are higher at 37°C than at 25°C. However, whilst the rates of potassium uptake are maintained, the rate of sodium uptake, occuring after 8mins, at the higher temperature is faster than at the lower temperature, indicating that the stability of the tissue is reduced by increasing the temperature from 25°C to 37°C.

The results presented suggest that, unlike the cellular sodium and potassium pools of rat kidney cortex slices, those of hamster jejunum rings do not readily equilibrate with buffer sodium and potassium. In addition, again in contrast to kidney cortex slices, the two incubation procedures lead to a considerable change in the extracellular space of the tissue, complicated by the secretion of mucus from the mucosa.

These findings indicate that intestinal rings are not amenable to the method of study designed for kidney cortex slices.

Fig. 25 The time course of sodium and potassium movements between hamster jejunum rings and the incubation medium after a 2hr loading procedure at 0°C. Incubations were carried out at 25°C and 37°C.



Section 5

The involvement of divalent cations in the actions of angiotensin

SECTION 5. The involvement of divalent cations in the actions of angiotensin

Divalent cations, particularly calcium, are required for the action of several polypeptide hormones. The enhancement of steroid production in the adrenal cortex by ACTH (Birmingham, Elliott & Valère, 1953) or cyclic AMP (Birmingham, Kurlents, Lane, Muhlstock & Traikov, 1960) and the vasopressin stimulation of short circuit current across toad bladder epithelium (Bentley, 1959) all require the presence of calcium ions in the incubation medium.

Calcium has also been implicated in the contractile effects of angiotensin on smooth muscle (Tikka & Bohr, 1972) and in this tissue, Boudouin & Meyer (1972) have evidence to suggest that calcium release follows the interaction of angiotensin with its receptor

The effect of divalent cations on the stimulation of colon fluid transport by angiotensin

It has been demonstrated by Davis, Munday & Parsons (1970), that angiotensin, at physiological concentrations, stimulates the transport of fluid by colon sacs prepared from rats nephrectomized and adrenalectomized 24hrs previously. Consequently, experiments were carried out to determine the involvement of divalent cations in this angiotensin stimulation of fluid transport.

(I) Calcium

Preliminary experiments were carried out to determine the effect of altering the incubation buffer calcium ion concentration on the transport of fluid by colon sacs prepared from untreated rats. The results in Table 34 show that omitting calcium from both the mucosal and serosal fluids causes a slight, but significant, reduction in fluid

Table 34. The effect of calcium on fluid transport by colon sacs prepared from untreated rats

Buffer Calcium	Mucosal fluid transport (mls/g wet wt. of tissue/hr)	P	
2.5mM	0.820±0.057	< 0.05	(12)
Calcium-free	0.670±0.041		(15)
2.5mM	0.612±0.080	< 0.05	(3)
5.0mM	0.975±0.086		(3)

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

transport ($P < 0.05$). Conversely, doubling the buffer calcium concentration results in a marked increase in fluid transport ($P < 0.05$).

To test whether the presence of calcium ions is required for angiotensin to cause an increase in fluid transport, colon sacs were prepared from 24hr nephrectomized and adrenalectomized rats and then incubated for 1hr in Krebs' bicarbonate buffer in the presence of 2.5mM calcium in both serosal and mucosal solutions. From the results presented in Table 35, it may be observed that only in the presence of calcium does angiotensin significantly stimulate fluid transport across the colon, suggesting that this effect of angiotensin on the colon requires the presence of calcium ions.

In order to determine whether calcium is necessary on both sides of the colon to support increased fluid transport following the administration of angiotensin, calcium ions were omitted from either the mucosal or serosal solution, while retaining the normal calcium concentration (2.5mM) in the other solution.

It may be seen (Table 36) that in the absence of calcium in the serosal fluid, angiotensin does not significantly stimulate fluid transport by colon sacs, whereas, in the absence of mucosal calcium, the normal response is observed in the presence of the hormone. These results show that calcium ions must be present in the serosal solution but are not required in the mucosal solution, in order that a stimulation of colon fluid tranport by angiotensin may be obtained.

(II) EGTA

Calcium is known to be transported by the colon, from the mucosal to the serosal solution (Harrison & Harrison, 1969), so that the removal of serosal calcium does not necessary imply the absence of serosal calcium. Consequently, ethylene glycol tetracetic acid (EGTA), a

Table 35. The effect of omitting calcium from both the mucosal and serosal fluids on the stimulation by angiotensin of fluid transport by colon sacs prepared from nephrectomized, adrenalectomized rats.

	Mucosal fluid transport (mls/g wet wt. of tissue/hr)	P	
Buffer + 2.5mM Calcium			
Control	0.404±0.049	< 0.01	(9)
Angiotensin 10^{-11} g/ml	0.636±0.037		(7)
Calcium-free buffer			
Control	0.413±0.049	N.S.	(9)
Angiotensin 10^{-11} g/ml	0.394±0.046		(9)

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Table 36. Effect of angiotensin, in the absence of mucosal or serosal calcium, on fluid transport by colon sacs prepared from nephrectomized, adrenalectomized rats

	Mucosal fluid transport (mls/g wet wt. of tissue/hr)		
Calcium (Serosal fluid only)			
Control	0.366±0.014	<0.01	(6)
+ Angiotensin 10^{-11} g/ml	0.524±0.039		(7)
Calcium (Mucosal fluid only)			
Control	0.360±0.035	N.S.	(6)
+ Angiotensin 10^{-11} g/ml	0.437±0.047		(6)

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

chelator of calcium ions, was added in excess (5mM) to the serosal incubation solution in an attempt to remove calcium ions transported across the colon wall from the mucosal solution.

The effect of EGTA on the transport of fluid and alkali cations was investigated by preparing colon sacs from untreated rats and incubating these in the presence or absence of serosal EGTA, both the mucosal and the serosal buffers containing 2.5mM calcium. When the transport of fluid, sodium and potassium across the mucosal and serosal membranes of the colon was compared, it was found (Table 37) that the addition of EGTA to the serosal solution causes a significant reduction in the serosal tranport of fluid and sodium ($P < 0.05$). No reduction in either serosal potassium transport or mucosal fluid, sodium or potassium transport is observed. In the absence of any reduction in mucosal transport following the addition of EGTA, the effects of angiotensin on mucosal fluid transport in the presence or absence of EGTA in the serosal solution were compared. Table 38 presents the results of these experiments. It can be seen that the stimulation, by angiotensin, of fluid transport across colon sacs prepared from 24hr nephrectomized and adrenalectomized rats is completely abolished by the addition of 5mM EGTA to the serosal incubation medium. These results support the previous findings that angiotensin requires the presence of calcium ions in the serosal solutionin order to exert its stimulatory effect on fluid transport.

An attempt was made to confirm that angiotensin does not require calcium ions in the mucosal solution in order to exert its effect. However, the decrease in basal fluid transport resulting from the addition of EGTA to the solution bathing the mucosal surface of colon sacs prepared from untreated rats (Table 39) was such as to preclude any further studies with this compound present in this compartment.

Table 37. The effect of EGTA (5mM), in the serosal solution, on water, sodium and potassium transport by colon sacs prepared from untreated rats.

	Water transport (mls/g wet wt. of tissue/hr)		Sodium transport (µEq/g wet wt. of tissue/hr)		Potassium transport (µEq/g wet wt. of tissue/hr)		
	Mucosal transport	Serosal transport	Mucosal transport	Serosal transport	Mucosal transport	Serosal transport	
Control	0.675±0.039	0.356±0.047	119±8	86.2±5.2	3.37±0.55	2.25±0.39	(6)
+ EGTA	0.636±0.056	0.265±0.059	111±10	68.4±5.9	3.95±0.40	2.44±0.29	(6)
Difference from control (P)	N.S.	<0.05	N.S.	<0.05	N.S.	N.S.	

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Table 38. The effect of EGTA (5mM), in the serosal fluid only, on the angiotensin stimulation of fluid transport by colon sacs prepared from nephrectomized, adrenalectomized rats

	Mucosal fluid transport (mls/g wet wt. of tissue/hr)	P	
Normal buffer			
Control	0.357±0.022	< 0.01	(7)
+ Angiotensin 10^{-11} g/ml	0.507±0.029		(11)
Buffer + EGTA			
Control	0.399±0.010	N.S.	(5)
+ Angiotensin 10^{-11} g/ml	0.375±0.032		(10)

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Table 39. The effect of EGTA (5mM), in the mucosal fluid only, on fluid transport by colon sacs prepared from untreated rats

	Mucosal Fluid transport (mls/g wet wt. of tissue/hr^{-1})	P	
Normal buffer	0.442±0.019	<0.01	(3)
Buffer + EGTA	0.252±0.024		(3)

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

(III) Magnesium

The possibility that magnesium ions are required for the action of angiotensin on colon fluid transport was investigated by omitting this ion from both the mucosal and serosal solutions and then testing the effect of the hormone in the usual way. Table 40 shows that in the presence of angiotensin, a significant stimulation of fluid transport is observed in the absence of magnesium ($P<0.05$). Furthermore, in the presence of 6mM magnesium, a concentration at which magnesium ions would be expected to displace calcium ions from certain binding sites, angiotensin causes a highly significant increase in fluid transport ($P<0.001$) as shown by the results presented in Table 41. These results indicate that magnesium ions are not involved in the action of angiotensin and are unlikely to displace calcium ions from those sites involved in the action of this hormone.

(IV) Strontium and Barium

In many biological systems, calcium can be replaced by strontium and barium. To test whether this is so in the case of the angiotensin stimulation of fluid transport by the colon, the effect of angiotensin was measured using colon sacs, prepared from nephrectomized and adrenalectomized rats, incubated in the absence of added calcium. Strontium or barium was present in the serosal solution only at a concentration of 2.5mM. From the results presented in Table 42, it may be seen that in the absence of calcium, angiotensin will stimulate fluid transport by the colon, providing that strontium or barium ions are present in the serosal solution. Thus strontium, and to a lesser extent barium, will act as replacements for calcium ions.

Table 40. The effect of omitting magnesium from the incubation fluids on the angiotensin stimulation of fluid transport by colon sacs prepared from nephrectomized, adrenalectomized rats

	Mucosal fluid transport (mls/g wet wt. of tissue/hr)	P	
Control	0.370±0.020	< 0.05	(4)
+ Angiotensin 10^{-11} g/ml	0.541±0.054		(5)

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Table 41. The effect of increasing the concentration of buffer magnesium on the angiotensin stimulation of fluid transport by colon sacs prepared from nephrectomized, adrenalectomized rats

	Mucosal fluid transport (mls/g wet wt. of tissue/hr)	P	
Buffer + 6mM Magnesium			
Control	0.437±0.011	<0.001	(5)
+ Angiotensin 10^{-11} g/ml	0.632±0.008		(6)

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Table 42. The effect of angiotensin on fluid transport by colon sacs prepared from nephrectomized, adrenalectomized rats, in the absence of calcium, but in the presence of strontium (2.5mM) or barium (2.5mM) in the serosal solution only

	Mucosal fluid transport (mls/g wet wt. of tissue/hr)	P	
Serosal Strontium			
Control	0.326±0.040	< 0.05	(5)
+ Angiotensin 10^{-11} g/ml	0.513±0.052		(6)
Serosal Barium			
Control	0.309±0.016	< 0.02	(7)
+ Angiotensin 10^{-11} g/ml	0.411±0.030		(9)

Results expressed as Means ± S.E.M,

No. of observations in parenthesis

The action of angiotensin on sodium transport by rat kidney cortex, and its dependence on calcium ions

The observation that angiotensin will stimulate sodium extrusion from sodium loaded, potassium depleted rat kidney cortex slices in the absence of potassium (Munday et al., 1971) has been confirmed. Sodium loaded, potassium depleted rat kidney cortex slices were prepared and reincubated in Krebs' phosphate buffer containing glucose, calcium (2.5mM) but no potassium, and in the presence or absence of angiotensin at a concentration of 10^{-12}M. To test whether the action of angiotensin in this tissue is also dependent upon calcium ions, EGTA was added to some flasks at a concentration of 5mM. The possibility that EGTA may have effects other than those resulting from the chelation of calcium ions was tested by studying the effect of angiotensin in the presence of EGTA (5mM) and excess calcium (12.5mM). The results presented in Table 43 show that in the presence of calcium ions, angiotensin significantly stimulates sodium extrusion from sodium loaded, potassium depleted kidney cortex slices ($P<0.02$). This stimulation is abolished in the presence of EGTA, suggesting that, as in the colon, calcium is required for angiotensin to exert its effect on ion transport by kidney slices. Following the addition of angiotensin in the presence of EGTA and excess calcium ions, the normal stimulation of sodium extrusion is restored, indicating the dependence of the angiotensin effect on calcium in both colon and kidney.

The site of action of calcium in the angiotensin response

Two mechanisms may be proposed for the involvement of this ion in angiotensin action. Firstly, that calcium ions are required for the interaction of angiotensin with its serosal membrane receptor, or secondly that the interaction of angiotensin with its receptor initiates

Table 43. The effect of EGTA and calcium on the angiotensin stimulation of sodium loss from rat kidney cortex slices loaded for 12mins, during a 10min. incubation

	Sodium loss	P	Potassium gain	P	
	μEq/g dry weight of tissue				
2.5mM Calcium					
Control	104±10	< 0.02	-6±2	N.S.	(13)
+ Angiotensin 10^{-12}g/ml	143±11		-11±2		(13)
2.5mM Calcium + 5mM EGTA					
Control	120±10	N.S.	-15±3	N.S.	(8)
+ Angiotensin 10^{-12}g/ml	120±7		-16±3		(8)
12.5mM Calcium + 5mM EGTA					
Control	106±4	< 0.05	-9±3	N.S.	(4)
+ Angiotensin 10^{-12}g/ml	141±13		-10±1		(5)

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

calcium movements, either its uptake into the tissue, or its release from the cells of the tissue into the extracellular space, an effect which has been observed in smooth muscle (Baudouin & Meyer, 1972).

The effect of angiotensin on calcium fluxes in kidney cortex slices

Two series of experiments were carried out to test the possibility that calcium movement is induced during the action of angiotensin. In one series, the rate of calcium uptake by slices loaded in buffer containing unlabelled calcium was measured during a second incubation in a buffer containing ^{45}Ca. Table 44 shows that sodium loaded, potassium depleted rat kidney cortex slices, reincubated in the presence of ^{45}Ca, take up radioactivity into the tissue at the same rate in the presence or absence of angiotensin.

The second series of experiments was carried out to test whether calcium loss from sodium loaded, potassium depleted slices is affected by angiotensin during a reincubation period. These slices were loaded with sodium in the presence of ^{45}Ca and reincubated in buffer containing unlabelled calcium for 5 and 10mins in the presence or absence of angiotensin. The results presented in Table 45 show that at neither time period are calcium movements affected by the presence of angiotensin.

It was found, that although no change in calcium ion movement was observed with angiotensin, the normal effects of this hormone were observed on sodium and potassium transport.

These results indicate that calcium movement is not induced following the addition of angiotensin to kidney cortex slices, and suggest that calcium ions may be involved in the in-teraction of angiotensin with its receptor.

Table 44. The effect of angiotensin (10^{-12}g/ml) on ^{45}Ca uptake by sodium loaded, potassium depleted rat kidney cortex slices during a 10min. reincubation in the presence of ^{45}Ca

	Counts/mg dry weight of tissue		
Control	849±67	N.S.	(3)
+ Angiotensin	844±49		(3)

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Table 45. The effect of angiotensin (10^{-12}g/ml) on ^{45}Ca loss from slices loaded with sodium and depleted of potassium, in the presence of ^{45}Ca, during a 5, or 10min reincubation in the presence of unlabelled calcium

	% Loss of radioactivity	
Incubate 5"		
Control	65.1±2	(4)
+ Angiotensin	65.1±3	(4)
Incubate 10"		
Control	71.6±1	(4)
+ Angiotensin	72.0±1	(4)

Results expressed as Means ± S.E.M.

No. of observations in parenthesis

Chapter 4

Discussion

Section 1

The dependence of sodium transport by rat kidney cortex slices on the presence of other ions

SECTION 1. The dependence of sodium transport by rat kidney cortex slices on the presence of other ions.

The requirement for cations

POTASSIUM

Results obtained from studies of ion movements, cell membrane permeabilities and electrical potentials lead to the proposal of a general mechanism of sodium transport by the kidney proximal tubules. This mechanism requires the presence of a sodium pump at the peritubular cell membrane (Geibisch, 1960; 1961; Whittembury, 1968; Whittembury, Sugino & Solomon, 1961) which transports sodium, against an electrochemical difference, from the interior of the cell towards the intercellular space. If sodium extrusion is coupled to potassium uptake, this pump will maintain a high level of intracellular potassium and a low intracellular sodium concentration (Whittam & Wheeler, 1970). It is also possible that the exchange of sodium for potassium, together with ionic leaks, may regulate cell volume (Tosteson & Hoffman, 1960).

The model of a tightly coupled sodium for potassium exchange pump is difficult to reconcile with more recent observations. It has been shown that the ratio of potassium uptake to sodium extrusion is variable under a number of conditions (Whittembury, 1968; Burg, Grollman & Orloff, 1964). A key observation in this respect is that sodium loaded, potassium depleted kidney cortex slices are capable of extruding sodium on reincubation in a medium without potassium (Macknight 1968; Whittembury 1968; Kleinzeller & Knotkova 1964). These results, which are in complete agreement with those reported in this study, may be interpreted in two ways.

An explanation postulating the existence of one pump was put forward by Klainzeller & Knotkova (1964) and formulated into the "cryptic pump" hypothesis by Willis (1968). This hypothesis suggests

that the potassium extruded from a tubule cell remains in the intercellular spaces, in a region where fluid flow is slow, permitting sodium potassium exchange to continue.

Whittembury (1968) and Macknight (1969) prefer the concept of a second, chloride linked sodium pump which regulates cell volume. Some observations by Whittembury & Fishman (1969) and Whittembury & Proverbio (1969) are important. They showed that ouabain reaches the pumps facing the intercellular spaces, and that ethacrynic acid reduces sodium extrusion without inhibiting potassium uptake. In order to explain these results in terms of a single pump, it must be postulated that ouabain cannot reach the proposed cryptic region, whereas ethacrynic acid can act in this concealed compartment leaving the superficial sites uninhibited, possibilities which seem less than likely. Furthermore, if the two mechanisms are different modes of the same pump, then they would have the same energy source. Ouabain is known to inhibit the Na-K ATPase, and consequently the utilization of energy for sodium potassium exchange and would, therefore, also be expected to inhibit potassium independent sodium transport. Such inhibition is not observed. Thus the majority of evidence available favours the existence of two modes of sodium transport, one pump exchanging sodium for potassium, the second being independent of potassium, but involving the movement of sodium, chloride and water.

The observations, presented here, that sodium loaded and potassium depleted rat kidney cortex slices, when incubated in the absence of potassium or in the presence of ouabain, are capable of sodium extrusion strongly support the view that two sodium pumps exist in the membrane of kidney cortex cells.

A third, but unlikely, explanation of the results summarised above is that the apparent changes in ion transport in kidney slices are due to alterations in the extracellular fluid volume of the tissue.

However, this suggestion is untenable since it has been demonstrated that, under the conditions employed, the extracellular fluid volume of kidney cortex slices remains at a constant level (Whittembury & Proverbio, 1970; Munday et al., 1971).

CALCIUM

Although calcium ions are not thought to be directly involved in the transport of sodium, some evidence is available to implicate calcium in the maintenance of the transporting ability of certain epithelia. For example, work carried out with frog skin suggests that changes in tissue calcium alter the rate of sodium transport (Curran & Gill, 1962), and calcium must be present in the incubation fluids of isolated toad bladder to support the stimulation of sodium transport by ADH (Bentley, 1959). On the other hand, the increase in water permeability caused by ADH may be partially reversed by adding calcium (Petersen & Edelman, 1962), a similar observation being recorded in Necturus kidney slices (Whittembury, Sugino & Solomon, 1960). In mammalian kidney, Kleinzeller (1961) noted that the near steady state tissue water level does not differ markedly in the presence or absence of calcium in the incubation buffer, whereas, there is a considerable change in the tissue cations.

Results presented here demonstrate that potassium independent sodium transport by rat kidney cortex slices is not inhibited when calcium ions are omitted from the incubation medium. However, under the conditions employed, the amount of potassium lost by the tissue is increased in the absence of calcium. If it is proposed that one pump controls the level of cell cations, while a second pump maintains tissue water content, then these results give considerable support to those of Kleinzeller (1961) and the results obtained with amphibian epithelia. A possible interpretation of these observations is that

sodium transport by the potassium independent pump is calcium independent, whereas sodium transport by the sodium potassium exchange pump may be altered by changes in the concentration of buffer calcium.

Studies carried out on rat colon, and reported later in this thesis, demonstrate that fluid transport is slightly reduced in the absence of calcium. These results may be interpreted on the basis of a calcium dependent, fluid linked sodium pump. However, the transporting ability of rat colon is dependent on the integrity of the brush border membrane, and experiments in which EGTA was added to the mucosal solution demonstrate that this agent causes a marked sloughing of the mucosal layer. Consequently, the reduction in colon fluid transport may reflect a decrease in the stability of the mucosal epithelial cells, and not a reduction in sodium pump activity.

The requirement for anions

Whittembury (1968) and Whittembury & Proverbio (1970) concluded, from the results of inhibitor studies, that the two mechanisms for the transport of sodium, which have been proposed to exist in the kidney proximal tubule, have the following characteristics. The first mechanism, the sodium potassium exchange pump is expected to be electrically neutral, as it involves a cation for cation exchange, whilst the second sodium pump should be electrogenic, since no cation is exchanged for sodium. These proposals must be viewed with caution, since only if a 1:1 exchange occurs between sodium and potassium will the first pump be electrically neutral. In addition, if sodium transport by the second pump is tightly coupled to the transport of an anion, then this mode of transport will be electrically neutral. Such a neutral sodium chloride transport mechanism will generate an osmotic gradient, resulting in the net movement of fluid from the tubular lumen to the peritubular space. There is evidence, from the work performed

with Necturus kidney proximal tubules (Whittembury et al., 1960), that the cell membranes of this tissue are more permeable to potassium than to chloride, so that the transport of sodium is more likely to be balanced by the opposite movement of potassium than a parallel movement of chloride. The transport of sodium in exchange for potassium could then generate a potential which would depend on the ratio of potassium ions exchanged for sodium, this potential being zero under conditions of a 1:1 sodium, potassium exchange.

A neutral sodium chloride pump has been demonstrated conclusively in the gall bladder (Diamond,1962; 1969; Martin & Diamond,1966), an epithelium which does not generate a potential. The arguement for or against coupled sodium chloride transport by the mammalian kidney proximal tubule may be clarified by determining whether or not this portion of the nephron generates a potential difference. If a potential exists due to the transport of sodium, as proposed by Burg & Orloff (1970) and Malnic & Aires (1970), then this may be sufficient to induce passive chloride movements. However, if this potential is insufficient or absent , as Frömter & Hegel (1966) and Maude (1970) believe, then this is indicative of a neutral sodium chloride pump, since 80% or more of the sodium and chloride in the glomerular filtrate is reabsorbed in this region.

There is some evidence of chloride transport not coupled to sodium movements. Malnic et al. (1970) have shown that in the convoluted proximal tubules of rats undergoing a metabolic acidosis, acetazolamide reduces chloride transport, whereas in the straight proximal tubule and loop of Henle, this transport is increased. In addition, Malnic & Aires (1970), measuring chloride movements into segments of proximal tubule perfused with sodium bicarbonate or sodium sulphate, demonstrated a net secretion of chloride into the tubule which was dependent on the acidification of the proximal urine. From these results, a coupled HCl

secretory mechanism, or possibly a chloride bicarbonate exchange, is proposed. What relevance these latter observations have to the reabsorption of large quantities of sodium chloride is not clear. In the study reported in this thesis, it has been demonstrated that sodium transport is not reduced in the absence of bicarbonate although potassium uptake is slightly decreased. On the addition of acetazolamide, no reduction in sodium or potassium transport is observed, and, although not measured directly, that portion of chloride transport which may be linked to sodium movement is presumably unchanged. There is also an indication, from these results, that high concentrations of acetazolamide may have a stimulatory effect on sodium potassium exchange activity. Thus it would seem likely that, in normal rats, chloride linked sodium pump activity is not dependent upon bicarbonate, although secondary mechanisms involving chloride movement may be in evidence during periods when the body's acid base balence is upset. Consequently, sodium transport from the proximal tubular lumen to the peritubular fluid, independent of the opposite movement of potassium, may be linked to the movement of chloride in the same direction.

The involvement of chloride in potassium independent sodium pump activity

Diamond (1962) demonstrated that replacing buffer chloride by sulphate causes a marked reduction in sodium transport by rabbit gall bladder. These results are in agreement with those reported here, and with similar observations by Maude (1970). When rat kidney cortex slices are sodium loaded and subsequently reincubated in buffer containing an alternative anion to chloride, then the ratio of sodium extrusion to potassium uptake is greatly reduced. In most cases, this is brought about by a significant inhibition of sodium extrusion. There are several ways of interpreting the results obtained with this

preparation.

Firstly, all changes in sodium transport observed when substituting chloride are a consequence of changes in extracellular and not intracellular sodium. This may be the case if replacement of chloride leads to a redistribution of extracellular volume. However, this explanation would require that this parameter increases during the loading procedure by 50% or more. It is possible that the extracellular space may change in the presence of certain anions such as acetate and bicarbonate. A general explanation of the results on this basis seems unlikely, but cannot be ruled out, since extracellular space measurements were not carried out in this study.

Secondly, the uptake rates of the sodium salts of many of the replacement anions employed are lower than the rate of uptake of sodium chloride during a loading procedure, due to either the low permeability of the tissue to these anions, or to a low degree of ionization of the sodium salts of the replacement anions leading to a low activity of sodium. The consequently low sodium extrusion rates during the second incubation period would be an inevitable reflection of the reduced uptake rates. However, if an anion is chosen which has a similar tissue permeability to chloride and, in addition, the sodium salt of this anion has a degree of ionization similar to that of sodium chloride, then the tissue sodium concentration would be expected to be similar to that in the presence of chloride. The anion most closely fitting these requirements is succinate, and when the results of experiments carried out in the presence of this anion are compared with those carried out with chloride, it may be observed that the loaded sodium values are indeed similar. However, on reincubation, the tissue extrudes only half the quantity of sodium extruded in the presence of chloride. Thus it appears that explanations based solely on reduced permeability of the replacement anion are inadequate to

explain all the results presented.

Thirdly, there is the possibility that active sodium transport independent of potassium movement is absolutely dependent on chloride ions, so that this mode of transport is not maintained in the presence of alternative anions. This is by far the most likely explanation of these results, and requires the existence of a sodium pump having a highly specific requirement for chloride, a proposition further supported by the following observation. When sodium loaded, potassium depleted rat kidney cortex slices are reincubated in the presence of chloride but in the absence of potassium, potassium uptake is abolished and sodium extrusion reduced by half. If, in addition, chloride is replaced by succinate in both incubation buffers, the remaining fraction of sodium extrusion is lost.

In summary, these findings are incompatible with the "cryptic pump" hypothesis of Willis (1968) and support the view that, in rat kidney proximal tubules, there exists, in addition to a sodium potassium exchange pump, a potassium independent sodium pump which is tightly coupled to chloride. Although potassium linked and potassium independent sodium pumps have comparable activities under the conditions employed in this study, in free-flow conditions, with the proximal cells in normal intracellular cation balance, the coupled transport of sodium and chloride may well be of greater significance.

The chloride dependence of potassium independent sodium pump activity in species other than the rat

The existence of two modes of sodium transport in kidney cortex was proposed mainly on the basis of inhibitor studies, carried out on guinea-pig kidney cortex slices (Whittembury, 1968; Whittembury & Proverbio, 1970). However, results discussed later in this thesis demonstrate that the effects of the inhibitors in question, namely

ethacrynic acid and ouabain, show a significant degree of species variation. It is possible,therefore, that potassium independent sodium extrusion in different species could have differing anion requirements. When this possibility was investigated, it was found that, in three species, namely rat, guinea-pig and rabbit, the replacement of chloride by succinate causes a reduction in the sodium loss to potassium gain ratio of about 50%. In both the guinea-pig and rabbit, the responses to chloride depletion are more complicated. Sodium extrusion is maintained at about the normal rate, while potassium uptake is significantly increased, indicating that potassium linked sodium extrusion is stimulated. This observation could be explained either by assuming that inhibition of Pump 2 activity increases the activity of Pump 1, or that succinate, a metabolisable anion, is utilized by the tissue to direct more energy to Pump 1. A further study carried out with rabbit kidney cortex slices, demonstrated that the replacement of chloride by sulphate, a non-metabolisable anion, increased sodium potassium exchange activity in a manner which is qualitatively similar to that observed with succinate. Consequently, in all three species studied potassium independent sodium transport in kidney proximal tubules appears to be chloride dependent. Furthermore, the movement of this anion appears to be tightly coupled to sodium movement, and not secondary to the development of an electrical gradient.

The study, reported here, demonstrates that chloride linked sodium transport can occur in the absence of sodium potassium exchange activity, and vica versa. If it is assumed that the sodium potassium exchange pump is concerned with the maintenance of cell cation content, then it would appear that chloride linked sodium transport can occur in a tissue in which the intracellular cation balance is severely upset. This proposition is supported by the observations of the calcium requirements of cation transport reported, both here, and by Kleinzeller (1961).

The location of these two modes of sodium transport may now be considered. If mode 1 maintains cell sodium and potassium content, then this pump must be situated on the epithelial membranes, probably on the basal and lateral membranes as described in the Ussing model (Koefoed-Johnsen & Ussing, 1958). Mode 2, however, may have two functions, the mainte nance of cell water content and the transepithelial transport of large quantities of sodium, chloride and water. It would seem likely that the extrusion of sodium and chloride from the cortical cells is by a mechanism identical with that which may be proposed to transport large quantities of these materials from the tubular lumen to the peritubular space. However, in this preparation, the kidney tubules are collapsed such that no transepithelial transport will occur. Consequently, it is not possible, with this preparation, to determine whether the movement of sodium with chloride represents a mechanism of cell water regulation, transcellular sodium, chloride and water transport or a secondary function of an extracellular salt pump.

Although there is considerable evidence to suggest that transepithelial sodium transport occurs via an extracellular route (Geibisch, Boulpaep & Whittembury, 1971; Cereijido & Rotunno, 1968), the classical model of sodium transport in exchange for potassium requires the existence of an intracellular sodium pool (Koefoed-Johnsen & Ussing, 1958). To propose an extracellular route for chloride linked sodium transport would require that different pools supply sodium ions for the two routes described. The observation reported in this study that inhibition of one mode of sodium transport leads to increased activity of the other, is in agreement with the findings of Munday et al. (1971). They showed that, on treating sodium loaded, potassium depleted rat kidney cortex slices with angiotensin, potassium independent sodium transport is stimulated, whereas potassium linked sodium transport is inhibited. These results are best explained on the assumption that both

sodium pumps are associated with the same intracellular pool. A possible alternative explanation not requiring the proposition of a single sodium pool is that under certain conditions, both transport mechanisms can utilize energy from the same ultimate source.

Section 2

The mechanism of action of ouabain and ethacrynic acid in the kidney

SECTION 2. The mechanism of action of ouabain and ethacrynic acid in the kidney

The study of potassium linked and potassium independent sodium pump activities is greatly simplified if a reliable method exists for the separation of these two modes of transport. It has been demonstrated in the previous section that the replacement or omission of certain key ions will result in the inhibition of one or other of the mechanisms described. This has allowed estimates to be made of the relative contributions of the two pumps to total sodium transport. An alternative method of separating the activities of these two transport modes is the use of specific inhibitors, if such compounds exist.

The specificity of ouabain

It has been well documented that a group of compounds, namely the cardiac glycosides, have a highly specific inhibitory action on the potassium linked sodium pump (Proverbio et al., 1970; Whittam & Willis, 1963). Ouabain, or strophanthin G, is the cardiac glycoside most widely used for this purpose, and appears to exert its effect by a specific interaction with the potassium site of the transport ATPase, thereby, inhibiting the dephosphorylation of the E_2 phosphate form of the enzyme (Glynn, 1957; Post et al., 1969).

If the sodium potassium exchange pump is stochiometric, as proposed in guinea-pig kidney cortex (Whittembury & Proverbio, 1970), then ouabain should cause comparable reductions in both sodium extrusion and potassium uptake. However, observations reported here demonstrate that ouabain has a far greater effect on sodium extrusion than potassium uptake by kidney cortex slices prepared from rat, guinea-pig and rabbit. The effect is most noticeable in the rabbit and guinea-pig, the inhibition of sodium extrusion being more than twice as great as the

inhibition of potassium uptake. In the rat, the ratio of inhibition is closer to unity, but again, sodium extrusion is reduced to a greater extent than potassium uptake. These findings alone suggest that ouabain has actions other than inhibiting the sodium potassium exchange pump, or that the exchange of sodium for potassium is not stochiometric. In contrast, it has been shown that in the species studied, the omission of potassium causes a reduction in sodium extrusion and potassium uptake in a ratio close to unity. Consequently, a non specific membrane action of ouabain would seem to be a possible explanation of the results presented above. However, this suggestion does not take into account the possibilities that potassium may be recycled at the lateral and basal membranes, thus maintaining an elevated sodium extrusion rate or, alternatively, a change in the passive leak of potassium out of the cells which may be greater in the absence of potassium than in the presence of ouabain. Support for the view that ouabain is a specific inhibitor of sodium potassium exchange comes from the work of Jones & Landon (1967), who showed that ouabain inhibits oxygen consumption of slices but not homogenates of kidney cortex, in agreement with results reported here, and who found that the inhibition of cation transport, induced by ouabain, can be reversed by the addition of elevated levels of potassium. The weight of evidence, therefore, supports the view that ouabain is a highly specific inhibitor of potassium linked sodium transport.

The specificity of ethacrynic acid

Ethacrynic acid is the parent molecule of a group of synthetic compounds which have, in certain species, including man, a profound diuretic action. It has been shown by Beyer et al. (1965) that, in the dog, rabbit and to a lesser extent, the guinea-pig, a marked diuresis and natriuresis results from the intravenous administration of ethacrynic acid, whereas, no such response has been obtained in the rat.

A specific inhibitory action of this drug on potassium independent sodium pump activity has been suggested from work carried out with guinea-pig kidney cortex slices (Whittembury, 1968; Whittembury & Proverbio, 1970), a species which exhibits a diuretic response to ethacrynic acid. However, other workers have demonstrated a range of other actions, both membrane linked and metabolic. Duggan & Noll (1965), Duggan et al. (1965) and Bannerjee et al. (1970) have shown an inhibition of sodium potassium exchange which, they suggest, may be due to interaction with the potassium site of the Na-K ATPase in competition with ouabain (Binder, 1966). Macknight (1969) made an alternative suggestion that this effect may be due to an increase in the passive leak of potassium ions. In addition to membrane actions, studies of cellular metabolism have demonstrated that ethacrynic acid will inhibit both oxidative phosphorylation and glycolysis (Daniel et al., 1971; Poat et al., 1970; Macknight, 1969). A possible explanation of the above conflicting results is that Whittembury (1968), and Whittembury & Proverbio (1970) obtained their data, indicating that ethacrynic acid is a specific inhibitor of potassium independent sodium transport, from work carried out on guinea-pig kidney, a species which is sensitive to the diuretic actions of the drug, whereas, the non-specific actions were observed in rat tissue, a species which does not respond to the diuretic. Consequently, it would seem likely that the non-specific actions of ethacrynic acid are unrelated to the in vivo diuretic effect.

It is reported here that the effects of ethacrynic acid on ion transport by kidney cortex slices differ in the three species studied. The reduction in kidney potassium transport induced by ethacrynic acid in the rat does not suggest a specific action on potassium independent sodium movement. Furthermore, the limited effect of this drug in the presence of ouabain or absence of potassium again suggests an action on sodium potassium exchange pump activity in this species. In contrast,

in the rabbit and dog, the reduction in potassium transport is slight, as is the interaction with ouabain. In these two species, ethacrynic acid inhibits sodium transport both in the presence and absence of potassium, indicating some relatively specific affinity of the drug for the potassium independent, chloride linked sodium pump. The guinea-pig occupies a position intermediate between the rat and the rabbit. Clearly the specificity of ethacrynic acid as an inhibitor of the potassium independent pump is highly species dependent.

It should be emphasized that, in none of the species described, is ethacrynic acid a completely specific inhibitor of sodium transport, as in all cases potassium uptake is reduced to a varying extent. This reduction in potassium uptake may be due to one or more of the following possibilities. An inhibition of the Na-K ATPase, as has been demonstrated in the rat (Duggan & Noll, 1965), an increase in tissue permeability to potassium, giving an increased passive leak of potassium out of the tissue as has been suggested by Macknight (1968) or an inhibition of energy production leading to reduced activity of the sodium-potassium exchange pump, in addition to the potassium independent pump. There has been no comparative study of the actions of ethacrynic acid on passive ion permeabilities and Na-K ATPase activities, so that the contribution of these effects to the inhibition of potassium movements cannot be determined. However, the effects of ethacrynic acid on cellular metabolism have been studied. It is observed that, in the three species tested, ethacrynic acid causes a marked reduction in oxygen consumption when added to homogenates of kidney cortex. Although these effects were not studied in great detail, they appear to be not inconsistent with the observations of Poat et al. (1970) on rat kidney cortex homogenates that this is due to a direct inhibition of NAD and flavin linked mitochondrial oxidations, and possibly glycolysis (Jones & Landon, 1967).

Using kidney cortex slices, different responses are observed.

In the rat and guinea-pig, ethacrynic acid is more effective than ouabain in reducing oxygen consumption, whereas in the rabbit the reduction in oxygen consumption, brought about by ouabain and ethacrynic acid, are similar. As the concentrations of the inhibitors employed result in comparable reductions in sodium transport, and ouabain is known to inhibit oxygen consumption solely by this reduction in ion transport (Jones & Landon, 1967), it is tempting to propose that the reduction in oxygen consumption by rabbit kidney cortex slices, caused by ethacrynic acid, is secondary to the inhibition of an energy requiring process. Confirmation that this reduction in oxygen consumption by cortex slices is secondary to a membrane effect, in the rabbit, would require that ethacrynic acid is excluded from the cells. No studies of tissue permeability have been carried out in the present study, but Epstein (1972) observed an accumulation of labelled ethacrynic acid by rabbit kidney cortex, reaching a maximum after one hour. Whether this is by cellular uptake, or by membrane binding has not been elucidated. Daniel et al. (1971) suggest that in rat uterus, ethacrynic acid actually enters the cells, binding to mitochondria. However, this entry was demonstrably slow, tissue ethacrynic acid concentration still increasing even after three hours incubation.

In the studies reported in this thesis, the tissue was only in contact with the drug for ten minutes, so that entry into the cells would be expected to be low. Not surprisingly, incubating or preincubating rat kidney cortex slices with ethacrynic acid for longer time periods results in increased inhibition of active sodium and potassium transport. Again, incubating fresh slices of rat, guinea-pig and rabbit kidney cortex for one hour leads to a rise in tissue sodium and a fall in tissue potassium content, which is compatible with an inhibition of cellular metabolism. Comparable incubations with ouabain, lead to a fall in tissue potassium while, tissue sodium is maintained at a low level,

presumably by the action of the second pump.

The reported effects of ethacrynic acid on membrane and metabolic processes are not as inconsistent with one another as first appeared. It seems probable that intracellular ethacrynic acid will inhibit respiration as reported by Macknight (1969), Jones & Landon (1967), Poat et al. (1970) and Daniel et al. (1971). However, the entry of ethacrynic acid into the cells may be restricted, slow and, in addition, may be species dependent.

There is evidence that in the rat, ethacrynic acid inhibits Na-K ATPase activity (Duggan & Noll, 1965 and others). Support for this suggestion is provided by the observation that ethacrynic acid causes a marked inhibition of potassium uptake in rat kidney cortex. A further study reported here investigates the activity of ethacrynic acid on sodium potassium exchange, under conditions which block the chloride linked pump. The results of this study indicate that ethacrynic acid causes a considerable reduction in sodium potassium exchange in the rat, less in the guinea-pig and only a slight effect in the rabbit. Consequently, there is no positive correlation between the inhibition of Na-K ATPase activity and the induction of diuresis and natriuresis.

The statement that ethacrynic acid is a specific inhibitor of chloride linked, potassium independent sodium transport appears to be erroneous, particularly when applied to the rat. In the guinea-pig kidney cortex, the inhibitor has a range of actions, which are not fully in accord with the conclusions of Whittembury (1968) and Whittembury & Proverbio (1970). However, in rabbit and dog kidney, ethacrynic acid appears to have a higher degree of specificity for the potassium independent, chloride linked sodium transport mechanism, and in addition, causes a marked diuresis and natriuresis in response to small oral doses of the drug. Even so, it must be emphasized that in none of the three species can ethacrynic acid be considered to be a specific inhibitor of

the potassium independent sodium pump. The work reported in the foregoing pages emphasises the dangers of applying observations obtained in one animal species to a different species.

The involvement of thiol groups in the action of ethacrynic acid

Ethacrynic acid, a molecule containing conjugated double bonds, has the structure of a compound which would be expected to react with thiol, that is sulphahydryl,groups. Several groups of workers have demonstrated such an interaction. The inhibitory effects of ethacrynic acid on renal (Duggan, Baer & Noll, 1965), and erythrocyte membrane ATPase (Smith, Czerwinski & Welt, 1967) and on kidney cortex sugar transport (Epstein,1972) have all been reversed by the addition of cysteine. Furthermore, Kramer & Kaiser (1970) have demonstrated a direct reaction between ethacrynic acid and enzyme thiol groups. These observations suggest that the action of ethacrynic acid involves the binding with tissue thiol groups at its site of action. However, results presented in this study, whilst supporting the observations that the addition of cysteine blocks the action of ethacrynic acid, demonstrate that the drug will react directly with cysteine at room temperatures to form a conjugate which is probably inactive. Poat et al. (1970),on the other hand, observed that the ethacrynic acid inhibition of NAD and flavin linked oxidations in mitochondria is not reversed by the addition of cysteine, glutathione or dithiothrietol, or by preincubation of the tissue with these thiol compounds. This latter observation suggests that, although metabolically active, either the binding of ethacrynic acid at membrane sites is not possible whilst the compound is in the conjugated form, or that in this form entry into the cell is prevented.

Section 3

The effects of angiotensin on sodium transport by kidney cortex slices

SECTION 3. The effects of angiotensin on sodium transport by kidney cortex slices

Results demonstrating that angiotensin, at physiological concentrations, will stimulate sodium extrusion from sodium loaded, potassium depleted rat kidney cortex slices confirm the observations of Munday et al. (1971). These observations, made in a preparation devoid of an intact blood supply, support the conclusions of Barraclough (1965) and Barraclough et al. (1967) that angiotensin exerts its effects on sodium transport by a direct tubular action, and are in conflict with the proposals of Malvin & Vander (1967) and Bonjour & Malvin (1969) that the antidiuretic, antinatriuretic actions of the hormone are secondary to renal vasoconstriction.

The experiments reported here were carried out in the absence of potassium, or in the presence of high concentrations of ouabain, conditions which inhibit sodium potassium exchange activity. Sodium extrusion, under these conditions, is significantly stimulated by angiotensin, in agreement with the work of Munday et al. (1971) who concluded that angiotensin exerts its effect by increasing the rate of a potassium independent sodium transport mechanism of the type proposed by Whittembury (1968) and Whittembury & Proverbio (1970). This conclusion is further supported by the observation that angiotensin is inactive when added to kidney cortex slices incubated in the presence of potassium but absence of chloride, conditions which inhibit chloride linked sodium transport. The conclusion that angiotensin specifically increases the activity of a potassium independent sodium pump while having no effect on the potassium dependent sodium pump is again reinforced by the observation of Shaikh (1972) that the hormone is capable of stimulating sodium transport by rat colon in the presence of high concentrations of ouabain.

The inability of several groups of workers to demonstrate a stimulation of Na-K ATPase activity by angiotensin, either in rat kidney (Bonting, Canady & Hawkins,1964), or in rat colon (Davies, Munday & Parsons, unpublished observations), adds credence to the view that angiotensin does not exert its effect through the potassium dependent sodium pump. However, Levin (1970), using very high concentrations (1.7×10^{-6} g/ml) of angiotensin, obtained a small but significant increase in the Na-K ATPase activity of rat kidney cortex homogenates and Gutman, Shamir, Glushevitsky & Hochman (1972) demonstrated a stimulation of Na-K ATPase activity in bovine adrenal cortex, rat hypothalamus and colon, with more physiological concentrations of the hormone. Again, Marc-Aurele & Bergeron (1966), using dogs, showed that, following the infusion of angiotensin into one renal artery in vivo, there is an increase in ATPase activity of microsomes prepared from that but not from the contralateral kidney. These latter workers concluded that there was no difference in Na-K ATPase activity of the two kidneys, but, analysis of their data on the basis of paired samples shows the effect of angiotensin to be highly significant ($P < 0.01$).

The concentrations of angiotensin used in the studies of Levin (1970) and Marc-Aurèle & Bergeron (1966) were such as would induce a diuresis and natriuresis (Baraclough et al., 1967). This would lead to an increased sodium load at the distal tubule, and might be expected to stimulate sodium potassium exchange activity. Consequently, there is little support for the view that angiotensin directly stimulates kidney cortex Na-K ATPase, while there is considerable evidence to suggest that, in this tissue, angiotensin acts by stimulating a potassium independent sodium pump. This potassium independent sodium pump appears to have an absolute requirement for chloride.

The effects of angiotensin on sodium extrusion and potassium uptake by guinea-pig kidney cortex slices

Most of the studies of the action of angiotensin on mammalian kidney, reported in the literature, have been carried out using either the rat (Barraclough et al., 1967; Munday et al., 1971; Bonjour & Malvin, 1969; Malvin & Vander, 1967; Vander, 1963) or the dog (Barraclough,1965; Louis & Doyle,1965; Urquhart, 1963). Therefore, it was of interst to study the effects of angiotensin on other species. When guinea-pig kidney cortex slices are incubated anaerobically for twelve minutes in the absence of potassium, tissue sodium rises and tissue potassium falls. On reincubation in an aerobic medium in the presence of potassium, these slices take up potassium and extrude sodium. The addition of 10^{-11} g/ml angiotensin to the second incubation buffer stimulates the rate of sodium extrusion after five and ten minutes exposure to the hormone. A significant decrease in potassium uptake is observed after fifteen minutes and, after thirty minutes, tissue sodium and potassium reach an equilibrium level which is unaffected by angiotensin. These results are qualitatively similar to those of Munday et al. (1971), using rat kidney cortex slices, and favour the view that the stimulation of the potassium independent sodium pump by angiotensin is a general phenomenum, since it has now been established in two species, namely rat and guinea-pig, and two tissues, namely kidney cortex and intestine.

Section 4

Sodium pump activity in intestinal rings

SECTION 4. Sodium pump activity in intestinal rings

Two tissue preparations were used in this study, namely the everted intestinal sac for measuring net transepithelial transport of fluid, and the kidney cortex slice preparation for the measurement of sodium and potassium fluxes into and out of the cells. An attempt was made to apply the kidney slice technique to intestinal rings in order to study cellular cation transport and thus correlate transepithelial water transport with the cellular movements of sodium and potassium. To these ends, everted intestinal rings, prepared from a number of species, were tested for their sensitivity to metabolic and membrane inhibitors. From the results of these studies, hamster jejunum was chosen as the tissue most amenable to further study. Utilising one of two loading procedures, rings were tested for their ability to recommence pump activity following a period of metabolic inhibition. The high ($37^{\circ}C$) temperature loading procedure resulted in rapid tissue death and was not pursued. However, the low ($0^{\circ}C$) temperature loading procedure appeared to be less destructive to the epithelium and appeared to be worth further investigation. The concentration of sodium in the tissue was well below that in the incubation medium following this treatment, indicating incomplete equilibration between these two compartments. It was found that, although the tissue is able to pump sodium and potassium across its cell membranes in an attempt to return towards normal intracellular cation balance, this ability is, at best, limited.

The extracellular space of kidney cortex slices does not change under a variety of incubation conditions so that the measurement of tissue ions truly reflects changes in cellular ion content. However, the situation is much more complex in the intestine. The extracellular space appears to increase during loading and decrease on reincubation.

Furthermore, the reduction in extracellular space during the second incubation is further complicated by the observation that during this period, considerable quantities of mucus are produced by the epithelial cells. The measurement of the size of the mucosal extracellular compartment with inulin will be rendered inaccurate if this compound either binds to, or is excluded from, this space. In addition, the flow of transported fluid along the intercellular channels may not allow the equilibration of the marker in this region. These points make it desirable for extracellular space changes to be small if an accurate measure of intracellular sodium ion concentration is to be made.

The results presented demonstrate that sodium loss from the calculated intracellular compartment is short lived, and rapidly followed by sodium uptake, whereas, the uptake of potassium is maintained. These results may be explained if the cells become leaky to sodium but not to potassium, or if a large number of cells die and subsequently load with sodium, whilst other cells maintain a high level of sodium potassium exchange. An alternative explanation which may be put forward is that, in addition to changes in the inulin space, there are increases in the size of extracellular compartments not measureable with this compound, thus giving a false value for the calculated intracellular space, and consequently for intracellular sodium.

The actions of the inhibitors tested in this situation are therefore of doubtful significance, although it does appear that ouabain and ethacrynic acid have a marked effect on potassium linked sodium transport by hamster jejunum. The observation that rat and mouse intestine are relatively insensitive to the effects of ouabain are in agreement with other observations (Robinson, 1970; Munday et al., 1971).

It must be reluctantly concluded that the tissue slice method, which works succesfully with kidney cortex, is not applicable to the

study of cellular cation transport by intestinal epithelial cells. Why the intestine will not respond in the same way as the kidney to the condition required for these experiments cannot be ascertained from the available data.

Section 5

The involvement of divalent cations in in the actions of angiotensin

SECTION 5. The involvement of divalent cations in the actions of angiotensin

Evidence is presented in the literature to support the proposition that the actions of certain polypeptide and catecholamine hormones are dependent upon the presence of divalent cations, particularly calcium. Bentley (1959), studying the action of ADH on toad bladder, demonstrated that omission of calcium ions from the incubation buffer abolishes the increase in scc normally observed with this hormone. Similarly, the actions of ACTH on the adrenal cortex (Birmingham et al., 1953), adrenaline (Rasmussen & Tenenhouse, 1968) and noradrenaline (Douglas & Poisner, 1963) on salivary secretions and LH on the corpus luteum (Hermier & Jutisz, 1969) all require the presence of calcium ions. In addition, the release of LH (Samli & Geschwind, 1968) and FSH (Jutisz & de la Llosa, 1970) from the pituitary gland are dependent upon the presence of calcium and possibly magnesium ions in the bathing medium.

I) The action of angiotensin on fluid transport by rat colon sacs and its dependence on divalent cations

The observations of Davies et al., (1970) that angiotensin stimulates fluid transport by colon sacs prepared from 24hr nephrectomized and adrenalectomized rats have been confirmed. The results of an investigation into the involvement of divalent cations in this response are discussed below.

The requirement for calcium

It is observed that the omission of calcium from the mucosal solution of rat colon sacs causes a slight reduction in mucosal fluid

transport, this reduction being far greater following the addition of EGTA to this compartment. The findings that rat intestine studied in vivo (Dumont, Curran & Solomon, 1960) and frog skin (Curran, Zadunaisky & Gill, 1961) and toad bladder (Hays, Singer & Malamed, 1965) studied in vitro, are also sensitive to reductions in buffer calcium are in agreement with these findings, suggesting that calcium ions are required in order to maintain the integrity of the mucosal epithelial cells.

The omission of calcium ions from the mucosal and serosal incubation fluids was found to block the angiotensin stimulation of fluid transport normally observed with angiotensin. Comparable results obtained following the omission of calcium from, or the addition of EGTA to, the serosal compartment only, indicate that the presence of serosal, but not mucosal calcium is necessary to support the action of angiotensin on this tissue. These observations are in agreement with similar findings of Khairallah, Vadaparampil & Page (1965), Tikka & Bohr (1962) and Hinke, Wilson & Burnham (1962), who all showed that calcium is essential for the action of angiotensin on smooth muscle.

The requirement for magnesium

It has been demonstrated that omission of magnesium ions does not inhibit the angiotensin stimulation of fluid transport by rat colon. In addition, angiotensin induced effects are abolished following the omission of calcium from the incubation buffer, a buffer which contains magnesium. Thus it may be concluded that magnesium ions are not required and will not replace calcium as an essential component of some aspect of the angiotensin response. As it has been shown that calcium and magnesium share a common transport mechanism in the intestine (Clarkson, Warren, McDonald & de Wardener, 1967; MacIntyre, 1963) and in addition, that these two cations

compete for binding sites on the adenyl cyclase of fat cell membranes (Birnbaumer, Pohl & Rodbell, 1969), it could be suggested that high levels of magnesium might displace calcium ions from the sites involved in the action of angiotensin. However, elevated levels of magnesium do not inhibit the response to angiotensin in rat colon. Thus the ability of magnesium to replace or compete with calcium depends on the system under investigation, and in this study, magnesium will neither support the action of angiotensin, nor will it inhibit the response to the hormone by competing with calcium.

From the results presented, it may be observed that elevated levels of either magnesium or calcium cause increases in the rates of basal fluid transport. There are no obvious explanations for these effects, although, this may be due to changes in membrane permeability, as has been reported to occur in Necturus kidney slices on increasing buffer calcium (Whittembury, Sugino & Solomon, 1961). In conclusion, both calcium and magnesium appear to affect basal fluid transport rates, but calcium ions only are required for angiotensin to exert its effect on fluid transport.

The effects of strontium and barium

The ability of strontium and barium to replace calcium in biological systems has been demonstrated. Studies carried out by Taylor, Bligh & Duggan (1962) have shown that calcium, strontium, barium and radium are all transported by the rat intestine. Furthermore, Palmer & Thompson (1961) propose that, in the intestine, strontium and calcium compete for a common transport mechanism.

The results presented in this thesis indicate that the stimulation of fluid transport by angiotensin can be maintained in the absence of calcium, providing strontium or barium ions are added in place of the more physiological cation. The order of activity observed

is similar to that obtained by Taylor (Taylor et al., 1962), namely, calcium > strontium > barium.

Studies of divalent cation transport by the kidney indicate the presence of a system comparable to that in the intestine. Thus the ability of strontium and barium to replace calcium in biological systems appears to be widespread.

II) The action of angiotensin on sodium transport by rat kidney cortex slices and its dependence upon calcium

The observations of Munday et al. (1971) that, at physiological concentrations, angiotensin will stimulate the rate of sodium extrusion from sodium loaded, potassium depleted rat kidney cortex slices has been confirmed, and found to occur only in the presence of calcium ions, and not under conditions where free calcium ions are removed by the addition of EGTA. The finding that high concentrations will reverse the inhibitory effects of this compound strongly suggest that the action of EGTA is, indeed, due to the removal of calcium ions.

In the presence of EGTA, the magnesium ion concentration is near normal showing that this ion does not maintain the angiotensin response. Thus it appears that in kidney cortex slices, as in rat colon, the angiotensin stimulation of sodium transport is dependent upon the presence of calcium, and independent of magnesium in the incubation buffer.

The site of action of calcium in the angiotensin response

Calcium ions have been implicated in the action of several polypeptide hormones, although the sites of action have not been fully resolved. Whether calcium is involved in the binding of the hormone

to its target tissue, or participates in some other part of the response needs much clarification. Several groups of workers suggest that calcium is required for ACTH binding to its receptor (Lopez, White & Engel, 1959; Bally & Tilbury, 1968; Birnbaumer & Rodbell, 1969). However, additional observations question this proposal. It has been shown that the stimulation of steroid production by ACTH (Birmingham et al., 1953) and cyclic AMP (Birmingham et al., 1960) both require the presence of calcium ions in the incubation medium. However, the sites of action of ACTH and cyclic AMP are at different loci, the former acting on the outer surface of the cell membrane, the latter acting within the cell. Further, Koritz & Peron (1959) have demonstrated a dramatic stimulation of steroid production by calcium in rat adrenal homogenates, results which would suggest that calcium is required for a cellular process and not for hormone binding. This is in agreement with results presented by Lefkowitz, Roth & Pastan (1970) who demonstrated that binding of this hormone to adrenal tissue occurs in the presence or absence of calcium, but that cyclic AMP is only produced in the presence of free calcium ions, suggesting a secondary intracellular action of calcium. The localization of this requirement is not clear, although Baudouin, Meyer, Fermandjian & Morgat (1972) and Auriac, Baudouin & Meyer (1972) suggest that the action of angiotensin on smooth muscle involves the release of calcium ions from the muscle cells. However, as calcium movement is an integral part of muscle contraction, this movement of calcium may not be involved in the action of angiotensin in other tissues.

Experiments using labelled calcium have been unable to demonstrate an increased rate of calcium loss or uptake by kidney tissue in response to angiotensin administration. Thus it appears, in the kidney, that calcium is required either for the binding of the hormone to the tissue, or for a secondary intracellular action, probably not

involving the movement of calcium ions in or out of the cell.

The binding of angiotensin to kidney cortex slices has been investigated in the presence and absence of calcium ions (Munday, Parsons & Poat, 1974) and an inhibition of binding was observed following the addition of EGTA to the incubation medium, suggesting that the calcium requirement for the action of angiotensin is at the cell membrane level. The elucidation of any further role of calcium ions is hindered by the limited knowledge of the intracellular processes following angiotensin administration to intestine or kidney tubular cells. It appears that the adenyl cyclase system is not involved (Munday et al., 1974) and thus an intracellular calcium requirement need not be proposed for angiotensin action in these epithelial tissues. However, not until a clearer picture of the mechanism of action of this hormone is forthcoming, is the localization of the calcium requirement likely to be fully resolved.

SUMMARY

1) Experiments reported in this thesis provide new evidence for the existence of two modes of sodium transport in rat kidney cortex. Mode I is potassium dependent but chloride independent, while Mode II is potassium independent, but has a specific requirement for chloride ions. Similar mechanisms are described in rabbit and guinea-pig kidney cortex.

2) Further evidence is presented for the involvement of angiotensin in the stimulation of sodium transport by rat kidney cortex and fluid transport by rat colon.

a) Angiotensin action on renal cortical sodium transport is shown to be via the stimulation of the chloride linked sodium pump.

b) The angiotensin stimulation of both colonic fluid transport and renal cortical sodium transport is found to require the presence of calcium ions in the incubation buffer.

c) Guinea-pig renal cortical slices exhibit a response to angiotensin similar to that obtained in the rat.

3) The effects of ouabain and ethacrynic acid on the two renal cortical sodium pump mechanisms are described. Results obtained with ethacrynic acid indicate that this drug exhibits marked species dependent effects, and under none of the conditions studied can this compound be considered a specific inhibitor of Mode II sodium transport.

4) Using the method utilized for kidney cortex slices, an attempt was made to study sodium efflux and potassium uptake by intestinal epithelia prepared from several rodent species. These preparations were found to be intolerant of sodium loading, potassium depleting procedures and are, thus, not amenable to the study of ionic fluxes.

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