UNIVERSITY OF SOUTHAMPTON

THE ACTIONS OF ANGIOTENSIN ON RAT INTESTINE AND KIDNEY

A thesis submitted for the degree of
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To those whom I love

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UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE

PHYSIOLOGY AND PHARMACOLOGY

Master of Philosophy

THE ACTIONS OF ANGIOTENSIN ON RAT INTESTINE AND KIDNEY

by Jane Winifred Evans

A study has been made of the actions of angiotensin on fluid transport in the rat intestine and the rat kidney cortex. Sensitisation of the animals to angiotensin, by removal of endogenous hormone has been shown to be not strictly necessary. Descending but not ascending colon *in vitro*, showed enhanced fluid transport in the presence of angiotensin. However consistent stimulation by angiotensin required adrenalectomy and nephrectomy 48 hours previously.

Fluid transport and sensitivity to angiotensin showed an annual rhythm in both the jejunum *in vivo* and the descending colon *in vitro*. When the rate of fluid transport was low, sensitivity to angiotensin was lost or attenuated.

Chloride ions have been shown to be necessary in order to demonstrate the angiotensin stimulation of fluid transport in the descending colon. This observation is consistent with the view that the effect of the hormone on intestinal transport is the result of an action by angiotensin on electro-neutral sodium-chloride linked processes.

An investigation of the effects of angiotensin on ion transport in rat kidney cortex slices has confirmed that the hormone stimulates the rate of sodium extrusion via a chloride-linked process. Further studies using this preparation have implicated cyclic GMP as an intracellular messenger of angiotensin action. A model has been proposed to explain this and previous observations. Angiotensin may primarily increase intracellular calcium concentrations, which activate guanylate cyclase leading to enhanced ribosomal production of proteins involved in sodium-chloride transport.

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Abbreviations Used.

ACTH	adrenocorticotrophic hormone
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ATPase	adenosine 5'triphosphate phosphohydrolase
Butyl PBD	2 (4' tert. - Butyl phenyl) - 5 - (4" Biphenylyl) - 1, 3, 4, - oxidiazole
C	degrees centigrade
cyclic AMP	cyclic 3'5' adenosine monophosphate
cyclic GMP	cyclic 3'5' guanosine monophosphate
Ci	Curies
cms.	centimetres
DNA	deoxyribose nucleic acid
d.p.m.	disintegrations per minute
equiv	equivalents
ether	diethylether
fig.	figure
g	gram
GMP	guanosine monophosphate
hr	hour
l	litre

M	molar
min	minutes
mg	milligrams
ml.	millilitres
mm	millimetres
mRNA	messenger ribose nucleic acid
osc.	oscillations
pd	potential difference
RNA	ribose nucleic acid
scc	short circuit current
sec	second
tris	tris hydroxymethylaminomethane
μ	micro
μ equiv	microequivalents
V/V	volume per volume
wt.	weight

General Introduction

Of primary importance to animals is the need to maintain a constant ionic and osmotic internal environment. The ability to regulate body salt and water balance provides the optimal conditions necessary for metabolic and biochemical functioning of all tissues. Specialised organs have been evolved to maintain this constant internal environment. Of these the kidney is the most important but the small and large intestines and the skin play auxiliary roles in this homeostatic control.

Certain hormones can influence the metabolism of the specialised organs mentioned above exerting a control over salt and water homeostasis. These hormones include aldosterone, released from the adrenal cortex, which is generally agreed to be the major sodium retaining hormone and antidiuretic hormone, which is released from the neurohypophysis and is primarily involved in the control of water balance.

Recently, the importance of other hormonal factors has been suggested. Among those implicated are prolactin, which causes renal retention of water, sodium and potassium in man (Horrobin, Lloyd, Lipton, Burstyn, Durkin & Muiruri, 1971), a salt excretion hormone (Cort, Lichardus, Pliska, Uhrin, Barth & Rudinger, 1969) and of particular interest to this study angiotensin.

Evidence for the existence of extra-adrenal hormones exerting an influence over sodium balance comes from the observations that the enhancement of intestinal sodium absorption following salt depletion is not prevented by adrenalectomy (Ross & Spencer, 1954), or by administration of the aldosterone inhibitor, spironolactone (Clarke, Miller & Shields, 1967). Furthermore, Crocker & Munday (1970) showed that after adrenalectomy and therefore the removal of endogenous aldosterone, fluid transport in the rat jejunum could not be reduced to the low values observed in animals maintained on a high sodium diet; again inferring that an extra-adrenal factor is involved. In further experiments with adrenalectomized and nephrectomized rats, treated with or without a prepared kidney extract, Crocker & Munday (1970) observed that fluid transport was significantly increased in those to which the extract had been administered, demonstrating that a renal factor is indeed involved in maintaining intestinal sodium and fluid transport. The hormone present in the extract has since been shown to be angiotensin, establishing its role as a salt and water retaining hormone.

Angiotensin does not only exert its role over salt and water homeostasis by direct actions on the epithelial cells of the jejunum (Crocker & Munday, 1970) (Bolton, Munday, Parsons & Poat, 1974) and other regions of the small and large intestines (Davies, Munday & Parsons, 1970). Low infusion rates of angiotensin have also been shown to have antinatriuretic and antidiuretic actions on the kidney. It is, however, uncertain whether or not these actions are due to direct stimulation of tubular transport mechanisms or are secondary to changes in renal blood flow (Bonjour & Malvin, 1969; Barraclough, Jones & Marsden, 1967). Apart from its actions on intestine and kidney, angiotensin and peptide precursors of the hormone are known to control homeostasis centrally by acting on the thirst centre of the brain (Fitzsimons & Simons, 1969; Fitzsimons, 1971) and also on the hypothalamus to stimulate ADH release (Mouw, Bonjour, Malvin & Vander, 1971). In addition to the above effects the renin-angiotensin system has also been shown to be one of the primary mechanisms concerned in the control of aldosterone secretion (Davis, Hartcroft, Titus, Carpenter, Ayers & Spiegal, 1962). Thus angiotensin can be said to have multiple control over salt and water metabolism.

Studies on the effects of angiotensin on salt and water transport in the intestine and kidney have shown that a variety of conditions are necessary to elicit a response. Davies et al (1970) showed that it was necessary to remove endogenous angiotensin and aldosterone before an angiotensin transport effect could be obtained in everted sacs of stripped rat colon. By nephrectomizing and adrenalectomizing their rats 24 hours prior to use, they obtained dose-dependent actions of angiotensin. Low doses of the hormone (10^{-11} and 10^{-12} g/ml.) were found to stimulate while high doses (10^{-9} and 10^{-8} g/ml.) inhibited transfer across the tissue. A dose-dependent response to angiotensin on fluid transport across the rat jejunum in vivo was also obtained by Bolton et al (1974). However, in this study no pre-treatment of the animals proved to be necessary. Again no preliminary treatment of the animal is required to obtain an angiotensin response by the in vivo kidney, although it is of general practice to salt load the animals in order to sensitize them to the action of the hormone. No pre-treatment of the animals is required to demonstrate angiotensin in stimulation of sodium transport by rat kidney cortex slices.

In addition to the various conditions required to obtain a response to angiotensin in different preparations, the magnitude of the angiotensin stimulation of fluid transport in the *in vivo* rat jejunum preparation and the *in vitro* rat colon preparation appears to be somewhat irregular. An explanation of the variability of the responses of rat tissues to angiotensin may be found in the observations of McAfee & Locke (1967) and Coviello (1970) that amphiba show seasonal sensitivity to the transport effects of the hormone. In view of these observations it was decided to carry out a comprehensive study investigating the rate of fluid and ion transfer in the rat kidney cortex slices, rat colon *in vitro* and rat jejunum *in vivo* and to monitor the sensitivity of these tissues to angiotensin. Using these techniques the optimum conditions and requirements for eliciting an angiotensin stimulated transport response were also studied.

Generally hormones exert their influences by altering the activities of rate limiting enzymes in metabolic pathways. They are believed to do this in two ways. Some hormones induce the synthesis of enzymes at the transcription stage (e.g. the action of aldosterone on toad bladder, (Sharp & Leaf, 1966) or at the translation stage (e.g. protein synthesis by insulin, Wool, Stirewalt, Kurihara, Low, Bailey & Oyer, 1968). In contrast most polypeptide hormones appear to alter the activity of existing enzymes directly or indirectly via a secondary messenger. Cyclic AMP has been established as a secondary messenger for a number of hormones (Sutherland & Robison, 1966) and has been implicated in the stimulation of active sodium transport across toad bladder by ADH (Orloff & Handler, 1964). Unlike most polypeptide hormones angiotensin does not, except for its action on the adrenal medulla (Peach, Bumpus, & Khairallah, 1971), appear to act through the secondary messenger, cyclic AMP. The stimulatory effect of angiotensin on fluid and ion transfer by the intestinal sac and kidney slice preparations does not appear to be mimicked by the addition of cyclic AMP or by phosphodiesterase inhibitors (Davies, Munday & Parsons, 1972; Munday, Parsons & Poat, 1972). Further studies using these two preparations show that protein synthesis inhibitors such as cycloheximide and puromycin inhibit the stimulatory response of the hormone, whereas actinomycin D is without effect. These observations suggest that a protein synthesis event, at a later stage than transcription is, in some way, necessary for the response (Davies *et al*, 1972; Munday *et al*, 1972). These

findings were confirmed by Bolton, Munday & Parsons (1975) using an in vivo jejunum preparation.

The mechanism of action of angiotensin still remains far from clear. At present cyclic AMP is the only compound that has been fully established as a secondary messenger in the action of hormones. Recently cyclic GMP has also been investigated with respect to hormone action (Bourgoignie, Guggenheim, Klahr, 1969), although it is too early at this time to ascribe the role of secondary messenger to this compound, cyclic GMP has been implicated in the control of ribosomal protein synthesis (Varrone, Di Lauro & Macchia, 1973) and consequently an investigation was carried out in an attempt to implicate cyclic GMP in the response of rat kidney cortex slices to angiotensin.

Chapter 1

Review of the literature

Section 1

The Renin-Angiotensin System

a. Historical Account

Pressor responses to intravenous injections of crude saline extracts of rabbit kidney were first described by Tigerstedt & Bergman (1898), who named the active substance in their extracts, renin. Later Kohlstaedt, Helmer & Page (1938) found that plasma or blood is necessary with renin to obtain vasoconstrictor activity and concluded that plasma contains a substance that reacts with renin. The plasma factor was called renin substrate. It has since been shown that renin is a proteolytic enzyme that acts on a substrate in the plasma to produce a pressor substance, which was named hypertensin by Braun-Menendez, Fasciolo, Leloir & Munoz (1940) and angiotonin by Page & Helmer (1940). The name of the pressor substance was later changed to angiotensin by agreement.

b. Renin

Renin, a proteolytic enzyme with a molecular weight of approximately 40,000, transforms angiotensinogen (renin substrate) into angiotensin 1. Although renin has been found in many tissues of the body, it is predominately located in the juxtaglomerular apparatus of the kidney, (Page & McCubbin, 1968). The juxtaglomerular apparatus includes the macula densa, which is the specialised area of the kidney tubule located at the transition between the ascending loop of Henle, the distal tubule and the granular cells.

After much controversy the granular cells were shown by Cook (1968) to be the site of renin production, storage and release. He found that the degree of juxtaglomerular cell granulation was increased by adrenalectomy and sodium depletion and that these granules proved capable of generating angiotensin on incubation with a partially purified renin substrate. Moreover, Edelman & Hartcroft (1961) using fluorescein-labelled antirenin were able to show specific staining in the juxtaglomerular cells and Granger, Dahlheim & Thurau (1972), using a microdissection technique provided additional evidence for this location of renin.

Renin activity, similar to that produced by the kidney, has also been observed in the large arteries and veins of the dog, in the uterus, placenta and liver (Gould, Skeggs, & Kahn, 1964). Activity has also been reported in the adrenals and brain (Ganten, Minnich, Granger, Hayduk, Brecht, Barbeau, Boucher & Gernest, 1971) and, since renin is incapable of passing the blood-brain barrier Ganten et al (1971) suggest that it

is synthesized in the brain itself. Renin has a half life of 15 to 20 minutes and is inactivated by both the liver and the kidney (Lee, 1969).

Factors effecting Renin Release

The factors effecting renin release have been reviewed by Vander (1967), Lee (1969) and more recently by Stein & Ferris (1973).

1. Haemodynamic factors

A reduction in blood volume due to haemorrhage has been shown by many investigators to be a potent stimulant of renin release (McKenzie, Cook & Lee, 1966), whereas rapid plasma expansion quickly lowers circulating renin levels. Moreover, drug induced hypotension, without haemorrhage has been shown to stimulate release of renin (Kaneko, Ikeda, Takeda & Ueda, 1967). Mild chronic haemorrhage, where there is no decrease in arterial blood pressure, appears to activate renin release by way of the renal sympathetic nerves. In support of this theory is the evidence of Hodge, Lowe & Vane, (1966), who were able to block the increased plasma angiotensin concentrations of chronic haemorrhage, in the dog, by anaesthetising the renal nerves with lignocaine. In severe haemorrhage, the direct hypotensive stimulus and renal sympathetic discharge almost certainly summate to increase renin release to levels never observed in mild haemorrhage in the absence of any fall in blood pressure.

Early experiments of Goldblatt, Lynch, Hanzal & Summerville (1934) suggested that renal ischemia could be an important stimulus for renin release. Today, however, there seems little question that ischemia is not necessary for enhanced renin release and the strongest evidence existing against this concept has been put forward by Skinner, McCubbin & Page (1963), who demonstrated that very small reductions in renal perfusion pressure, which do not reduce renal blood flow, stimulate renin secretion. Again Skinner, McCubbin & Page (1964) observed no increase in the rate of renin release following constriction of the renal vein, a procedure which reduced renal blood flow by 50%.

2. Sodium Balance

Acute administration of natriuretic drugs increases plasma renin levels in both man and dog within 2 hours of administration (Frazer,

James, Brown, Isaac, Lever & Robertson, 1965). At this time the sodium loss persists, the distal tubular load of this ion is still high and the increased level of renin in the plasma does not effect the kidney stores of this enzyme (Brown, Davis & Johnston, 1966). When sodium depletion is produced by diet or natriuretic agents on a chronic basis, the plasma renin level rises in man and dog with parallel changes in the juxtaglomerular cell granulation (Tobian 1964). It must be emphasised that in the acute situation this correlation is not observed. Conversely, increased dietary intake of sodium, in man, reduced renin secretion from the granular cells (Brown, Davies, Lever & Robertson,1964 (1)).

3. Other factors which cause changes in renin release

In addition to those previously described, other factors are known which cause changes in renin secretion. In humans increased plasma renin levels have been observed during pregnancy (Ferris, Gorden & Mulrow, 1967) and in the luteal phase of the menstrual cycle (Brown, Davies, Lever & Robertson, 1964 (2)). Exercise (Castenfors, 1967), the assumption of an upright posture or tilting result in a rapid increase in plasma renin activity (Conn, 1964). The latter are thought to be associated with increased sympathetic tone (Gordon, Kuchel, Liddle & Island, 1967). Deep sleep also causes increased renin secretion but this is probably a consequence of a fall in blood pressure. There is also evidence of a diurnal rhythm in plasma renin activity (Brown, Davies, Lever & Robertson, 1966). Again, increased sodium retention observed after operations in man and experimental animals has been attributed to increased renin secretion. For example; McKenzie, Ryan & Lee, (1967) produced a three fold increase in plasma renin activity within 4 hours of opening the peritoneum.

Evidence is now accumulating to suggest that angiotensin itself inhibits renin secretion by acting through a negative feedback loop. It has been demonstrated (Vander & Geelhoed, 1965) that angiotensin exerts negative feedback control over renin release and that this is independent of changes in renal arterial pressure or aldosterone secretion. Kamura, Niwa, Skelton & Bernadis (1966) have shown that the effect of angiotensin administration depends upon the method by which the peptide is given. A single injection of angiotensin in the

rat depresses granulation in the juxtaglomerular cells, but prolonged infusion increases granulation. Although the specific mechanism by which negative feedback is exerted is unknown, it is evident that the plasma angiotensin concentration may have an important role in renin release.

A finding by Tagawa, Vander, Bonjour & Malvin (1971) showed that infusions of vasopressin in dogs depleted sodium inhibits renin secretion and is unaccompanied by either changes in plasma sodium or in arterial pressure. In addition it has been demonstrated that there is a reciprocal relationship between plasma potassium and renin release (Sealey, Clark, Bull & Laragh, 1970), although it is not clear whether this occurs independently of changes in sodium delivery to the distal tubule.

Theories for Renin Release

There are three proposed mechanisms by which renin release from the kidney may be regulated. The theories are as follows:-

1) The intrarenal baroreceptor theory.
2) The macula densa theory.
3) The sympathetic nervous theory.

These three theories are not mutually exclusive and probably several, at any one time, may be involved in the control of renin secretion.

The Baroreceptor Theory

The concept of the renal baroreceptor theory has been developed by Tobian (1960). Tobian hypothesises that, since juxtaglomerular cells are located in the wall of the afferent arteriole, they would be sensitive to the same stresses that effect the vessel. Therefore the juxtaglomerular cells could act as stretch receptors, and increased arterial pressure would stretch the cells and inhibit renin release, whereas decreased pressure would reduce cell distention and enhance renin secretion. Lack of responsiveness to pulse pressure would be considerably dampened at the level of the juxtaglomerular apparatus. From the described investigations into haemorrhage and hypotension where the fall in arterial pressure is the stimulus for renin release, it is clear that there is ample evidence to suggest that the juxtaglomerular cells could indeed act as the baroreceptors of this theory.

Macula Densa Theory

There are several anatomical observations (Vander, 1967) as well as physiological studies which suggest that the macula densa plays an important role in the control of renin release. Vander & Miller (1964) in an extensive series of studies, showed that aortic constriction, elevation of ureteal pressure, renal nerve stimulation and the infusion of catecholamines all increase renin secretion, whereas natriuresis, induced by diuretics such as mannitol, will reverse this elevation in release. They, therefore, propose a reciprocal relationship between sodium load at the macula densa and renin release. That is a high sodium load will inhibit and a low sodium load will stimulate the rate of renin secretion. Cook, Brown, Zacherle & Walker (1970), working with the potent diuretic ethacrynic acid, found that with the increase in sodium delivery at the macula densa, there is an increase in renin release. Although this might be construed as evidence against the hypothesis that increased sodium delivery to the macula densa will inhibit renin release there is an alternative interpretation. As ethacrynic acid inhibits sodium transport across epithelial tissue it might also inhibit sodium transport across the macula densa. If this inhibition is evident then the findings of Cooke et al (1970) agree with the proposed hypothesis. In contrast with the studies of Vander, it has been suggested by Thurau (1964) and Thurau, Schnermann, Nagel, Horster & Wahl (1967), using micropuncture techniques, that an increased sodium concentration at the macula densa increases renin release. Although these two hypotheses are mutually contradictory it would appear that at this time the available data favours the Vander theory but the actual mechanism whereby alterations in sodium delivery lead to changes in renin release is as yet unknown.

Sympathetic Nervous Theory

Apart from the existence of a completely intrarenal mechanism which can alter rates of renin release is the recent accumulated evidence for an extrarenal system which may exert reflex neural control over the secretion of renin. Direct electrical renal nerve stimulation has been shown to increase renin release (Vander, 1965), infusion of catecholamines into the renal artery causes renin secretion (Bunag, Page & McCubbin, 1966) and denervation of the kidney decreases its renin content

(Tobian, Brandon & Maney, 1965). Moreover, it is known that sympathetic nerve fibres supply the afferent and efferent arterioles and that the juxtaglomerular apparatus is supplied by non-myelinated sympathetic nerve fibres (Barajas, 1964). All this data indicates the participation of the sympathetic nervous system in the control of renin release.

It is clear that the control of renin release is complicated and at present neither the intrarenal baroreceptor nor the macula densa theories can explain all experimental observations. It is likely that both types of receptor exist and can influence each other. Furthermore, superimposed on this is sympathetic neural control of renin secretion.

Inhibitors of Renin

Over the years many claims have been made for the existence of renin inhibitors in many tissues including the kidney. Smeby, Sen & Bumpus (1967) have succeeded in preparing a phospholipid from the kidney which prevents an in vivo response to injected renin. The phospholipid is not the active inhibitor but is a preinhibitor which is converted to the active form by hydrolysis. Recently Rakhit (1971) determined that the structure of the renin inhibitor is phosphatidylethanolamine with a large percentage of polyunsaturated fatty acids. It must be noted however that some investigators can find no evidence for an inhibitor of the reaction between renin and its substrate (Blaquier, 1965; Poulsen 1971). Apart from the possible existence of specific inhibitors, renin activity has also been shown to be supressed by heparin (Sealey, Gerten Ledingham & Laragh, 1967) and small peptides (Kokubu, Ueda, Fujimoto, Hiwada, Kato, Akutsu & Yamamura, 1968).

Pseudorenin

Angiotensin 1 may also be produced by the enzyme pseudorenin (Skeggs, Lentz, Kahn, Dorer & Levine, 1969). This enzyme has been found in the plasma and is in high concentration in the salivery glands, thymus and spleen of the rat. Pseudorenin is believed to be an intracellar enzyme, which can exist in several forms with molecular weights ranging between 43,000 and 158,000 (Skeggs, Lentz, Kahn, Levine & Dorer, 1972). The source of its substrate and the function of the angiotensin which may be produced are not known.

Angiotensinogen (renin substrate)

Renin substrate, a glycoptrotein was originally found in the a_2 globulin fraction of plasma. (Plentl, Page & Davis, 1943). It has more recently been found in the lymph (Horky, Rojo-Ortega, Rodriguez, Boucher & Gernest, 1971) and in the dog brain (Ganten et al, 1971). A number of early experiments involving hepatectomy in animals provided evidence that the liver is the principal source of plasma renin substrate (Page, McSwain, Knapp & Andrus, 1941). Levels of angiotensinogen in the plasma may be decreased by adrenalectomy (Braun-Menendez, Fasciolo, Leloir, Munoz & Taquini, 1946) and increased in pregnancy, malignant hypertension (Gould, Skeggs & Kahn, 1966), after the administration of adrenal cortical hormones (Haynes, Forsham & Hyme, 1953) and oestrogens (Helmer & Griffith, 1952). After 24 hour bilateral nephrectomy of the rat the plasma concentration of renin substrate increases dramatically by a factor of 4 to 15 times (Blaquier, 1965).

Angiotensin 1

Skeggs, Marsh, Kahn & Shumway (1954) discovered that there are two forms of angiotensin that can be separated by countercurrent distribution. One of these the decapeptide, angiotensin 1, is the initial produce of the action of renin on renin substrate. Skeggs, Kahn & Shumway (1956) found that, in saline perfused rat kidneys angiotensin 1 was ineffective, whereas angiotensin 11 had vasoconstrictor activity. It is generally agreed that angiotensin 1 has no angiotensin 11 like activity (Helmer, 1957) although it does appear to have potent stimulatory effects on catecholamine secretion from cat adrenal medulla (Peach, Bumpus & Khairallah, 1971). As well as a variable degree of conversion of angiotensin 1 to angiotensin 11 there is also direct inactivation of angiotensin 1, possibly by the same enzymes that inactivate angiotensin 11.

Converting Enzyme

The peptidase in plasma that converts angiotensin 1 to the octapeptide, by removal of a dipeptide has been termed converting enzyme (Lentz, Skeggs, Woods, Kahn & Shumway, 1956). Although the two forms of angiotensin were first distinguished by their behaviour

in counter-current distribution and their differing biological effects, a much more significant physiological difference was later found. Helmer (1955), working with an isolated rabbit aortic strip, had observed a factor in plasma that enhances the activity of angiotensin and suggested that it might be the angiotensin-converting enzyme. The possibility was tested in the isolated rat kidney, perfused with physiological salt solution (Skeggs et al, 1956). As described in the previous section, it was found that angiotensin 11 is vasoconstrictor whereas angiotensin 1 in the absence of plasma is not, thus inferring the presence of converting enzyme in plasma.

The isolation of the two forms of angiotensin and of a plasma converting enzyme led to the belief that conversion of angiotensin 1 to angiotensin 11 in circulating blood was sufficiently rapid to account for the vaso-active effects of the latter in vivo. The importance of the plasma converting enzyme has, however, been questioned. Ng & Vane (1967, 1970) found that conversion of angiotensin 1 to angiotensin 11, in dog blood is too slow to account for the rapid pressor effects of angiotensin 11 and demonstrated rapid conversion in the lungs. Now although converting enzyme activity has been found in the plasma of all species investigated including man (Poulsen & Poulsen, 1971) it is thought to be of little physiological importance owing to its slow converting ability. Consequently the tissue conversion of angiotensin 1 to angiotensin 11 is thought to be of greater significance. The existence of converting enzyme in many tissues is now evident and the rate of conversion in most organs has been established, although considerable variation is shown from organ to organ within a species and also between species. However, whether measured in vivo, by perfusion in vitro or after tissue maceration, the lung displays high converting enzyme activity in all species so far studied. With the possible exception of the testes, the lung shows higher converting enzyme activity than any other tissue in the body (Ng & Vane, 1967). After considering the fact that the pulmonary circulation handles the total cardiac output a second or so before the blood is pumped to the target organs, it becomes evident that the lungs must play a predominant role in the formation of angiotensin 11 and thus the arterial circulating levels.

Conversion of angiotensin 1 to angiotensin 11 by converting enzyme has also been reported in the hindquarters of the dog (Aiken & Vane, 1972) and sheep (Osborn, Tildesley, O'Gorman & Mahler, 1971), in the intestine (Aiken & Vane, 1970) and to a small degree in the kidney (Aiken & Vane, 1972). The very small proportion of angiotensin 1 converted to angiotensin 11 in kidney, with high conversion in the lungs and considerable conversion in other vascular beds, suggest that the kidney is exceptional for its low converting enzyme activity. This may serve a protective function; for release of renin from kidney stores could then take place without local generation and consequent local action of angiotensin 11. The finding of Itskovitz and Odya (1971), that concentrations of angiotensin 1 in renal blood are forty five times higher than those of angiotensin 11, supports this concept. Therefore without effective converting enzyme, the renal vascular bed, which is highly sensitive to angiotensin 11 will receive only the hormone formed primarily by lung converting enzyme.

Angiotensin 11

As previously described angiotensin 11 is an octapeptide produced by the action of converting enzyme on angiotensin 1. It has been tested as a pressor or vasoactive material but it now appears that in the physiological dose range the actions of angiotensin on the adrenal gland, the central nervous system and the kidney are important both in normal homeostasis and in pathological states. Its action on the above and other tissue will be discussed in the following section of this review.

The pressor actions of angiotensin last only a few minutes which implies that it is either rapidly destroyed or bound to its sites of action in the body. Its half life has been assessed as less than 120 seconds in the rat (Regoli, Riniker & Brunner, 1963) and 113 to 280 seconds in the dog (Hodge, Ng & Vane, 1967). However the observations of Hodge et al (1967) show that up to 75% of the activity of angiotensin is removed in one circulation through the liver, head, kidneys and hindquarters, which indicates that the half life of angiotensin in the dog is within the range of 15 to 30 seconds. In the rat the half life of angiotensin has been estimated to be approximately 15 seconds (Chapman, 1975).

There are many enzymes in the body which are capable of destroying angiotensin. These enzymes are called angiotensinases and are mainly peptidase enzymes such as aminopeptidase A, leucine aminopeptidase and carboxypeptidase. A specific angiotensinase has, however, been isolated from kidney and red blood cells by Khairallah, Bumpus, Page & Smeby, (1963) which is called angiotensinase A. The angiotensin is removed from the circulatory system by specific binding was suggested in a study made by Bumpus, Smeby, Page & Khairallah (1964). They infused tritiated angiotensin at a high dose rate for twenty minutes into experimental dogs, and killed the animals immediately or after a further thirty minutes. Organs were removed and assayed for radioactivity. High levels of radioactivity were found in the uterus, kidneys and adrenals of the animals killed immediately. In those animals killed thirty minutes after stopping the infusion of angiotensin highest radioactive content was found in the urine, brain and adrenals, which suggests that the brain and adrenals have little angiotensinase so that angiotensin remains bound to these tissues. Recent studies using labelled angiotensin have provided little evidence of an uptake system for angiotensin by cells, or of a transfer from the vascular lumen to other extracellular spaces. (Bailie, Rector & Seldin, 1971).

Section 11

The Actions of Angiotensin 11

1. The Circulatory System

a. The effects of angiotensin 11 on the heart

Angiotensin 11, has been shown to increase the rate and the myocardial contractile force of the heart. The positive chronotropic action induced by the hormone is thought to be produced by a sympathetic mechanism, on the basis that this effect is reduced by beta adrenergic blockade, pretreatment of the animal with reserpine and by surgical decentralisation of the heart (Farr & Grupp, 1967). The increase in myocardial contraction is probably also mediated via the sympathetic nerves, although it has been suggested that augmentation of the stroke volume in intact animals is due to a Frank-Starling effect secondary to a rise in systemic pressure (Krasney, 1968). Farr & Grupp (1967) have postulated that angiotensin 11 stimulates an extra-cardial site, the caudal cervical ganglia, to induce both its positive inotropic and chronotropic effects.

b. The effects of angiotensin 11 on the regional blood flow

The effects of angiotensin on regional blood flow to the organs of the body has been reviewed by Bunag (1974). When angiotensin is injected intravenously blood flow is markedly reduced in the splanchnic area, liver and skin because of predominant vasoconstriction and is increased passively in skeletal muscles, the brain, heart and uterus because of elevated perfusion pressure. The overall effect is an increase in total peripheral resistance which is primarily responsible for the elevation in systemic arterial pressure.

The intravenous injection or infusion of angiotensin produces a marked decrease in renal blood flow. In response to low doses of angiotensin (1 - 50 ng/kg/min) renal vasoconstriction is apparent leading to the decrease in renal blood flow, concommitant with a lesser decrease in glomerular filtration rate in many species including man (Torres, Lozano, Whittembury & Monge, 1970). High doses of angiotensin (250 ng/kg/min) cause much greater initial vasoconstriction and there is a sharp fall in urine output, but the responses are less well sustained. The initial response is followed by a reversal in urine output, which increases, and a diuretic and natriuretic response occurs (Cambridge, Chapman & Munday, 1976).

c. Effects of angiotensin II on the systemic circulation

Angiotensin-induced elevation of the systemic arterial pressure is the best known of the many effects of the hormone and angiotensin is generally thought to be the most potent pressor substance now available. In all animal species, including man, the characteristic response to a single intravenous injection is a sharp rise in arterial pressure, which begins within 20 to 30 seconds and reaches a peak in 1 to 2 minutes. Pressure then returns to its initial level in 3 to 5 minutes. Responses to small doses are fairly reproducible but large doses can cause tachyphylaxis. The mechanism of the systemic pressure response is complicated but increased central and peripheral sympathetic activity, an increased release of catecholamines plus angiotensin's direct effect on the vascular smooth muscle all contribute to this effect of the hormone. (Reviewed by Bunag, 1974).

2. Smooth muscle contraction

Angiotensin is a potent agonist that causes contraction to vascular smooth muscle. Consequently this action of the hormone has been used as a basis for assaying angiotensin and its analogues. When investigating the vasoconstrictor action of angiotensin it is important to consider whether the hormone is acting indirectly on the muscle via influence on the nervous system, or directly on the smooth muscle cell itself. Angiotensin has been shown to contract guinea pig ileum by both an indirect liberation of acetycholine from nerve terminals and by stimulating the muscle cells directly (Khairallah & Page, 1961). Attempts to demonstrate a similar acetycholine-mediated effect of angiotensin on rat uterus, stomach and colon have however been unsuccessful, although experiments performed on a variety of pharmacological preparations have demonstrated the importance of the neurotransmitter, noradrenaline, in the indirect vasoconstrictor action of angiotensin.

Angiotensin could indirectly induce contraction of smooth muscle in three different ways:-

a. by causing the release of noradrenaline or other neurotransmitters from nerve terminals (reviewed by Zimmerman, Gomer & Liao, 1972).

b. by inhibiting the uptake of noradrenaline by the nerve

endings, and thereby potentiating its own action by allowing a higher concentration of catecholamines to have access to the receptors on the muscle cell (reviewed by Khairallah, 1972).

or c. by accelerating the biosynthesis of noradrenaline from its precursors (reviewed by Roth, 1972).

Bohr (1974) claims that the contraction of vascular smooth muscle in response to angiotensin is primarily due to direct action of the hormone on the muscle cell, and that the indirect neurogenic effects of angiotensin, at least in acute studies, are relatively minor. The mechanism of the direct action of angiotensin on smooth muscle has been reviewed by Regoli, Park & Rioux (1974). It is believed that Ca^{2+}, acts as a cofactor for the direct myotropic action of angiotensin, The hormone caused mobilisation of Ca^{2+} ions firmly bound to the muscle cell membrane to increase the intracellular concentration of the ion, which then activates the ATPase of the contractile protein. In addition to the above, studies of the action of angiotensin on fragments of rabbit aorta (Baudouin-Legros & Meyer, 1973) also suggest that the hormone action could be partially mediated by the translocation of calcium from binding stores in the muscle cell membrane.

It is evident that further studies are needed to understand the mechanism of action of angiotensin on smooth muscle and to explain the relative importance of the direct and indirect actions of this potent vasoconstrictor.

3. a. The effects of angiotensin 11 on the peripheral nervous system

In addition to the actions of angiotensin on nerve terminals as described in the previous section, the hormone also exerts effect on the adrenal medulla and sympathetic ganglia. Khairallah & Page (1961) have postulated that angiotensin is able to modulate nervous transmission through sympathetic ganglia by stimulation of postganglionic nerve cells leading to a release of acetycholine from nerve endings, Feldberg & Lewis (1964) have demonstrated angiotensin's action on the adrenal medulla. They suggest that the angiotensin induced release of catecholamines from the medulla could be due either to a stimulatory action of the hormone on the medullary cells or could be caused indirectly through excitation of the cholinergic sympathetic nerve endings in the medulla. Severs, Daniels, Smookler, Kinnard & Buckly (1966) also found that angiotensin may initiate stimulation of the

adrenal medulla but via an indirect centrally mediated mechanism. There is also increasing evidence to support the theory that the direct action of angiotensin on the medulla is initiated by depolarisation of the chromaffin cell membrane by the hormone (Douglas, Kanno & Sampson, 1967), which results in a net transfer of extracellular Ca^{2+} into the cell.

b. Central actions of angiotensin on the nervous system

The central actions of angiotensin on the nervous system have been reviewed by Severs & Daniel-Severs (1973). It was originally assumed that angiotensin is unable to penetrate the blood-brain barrier and would therefore be unlikely to have central effects. However, Fischer-Ferraro, Nahmod, Goldstein & Finkielman (1971) and Ganten et al (1971) have independently shown that renin, renin substrate and angiotensin are all endogenous to the brain. Their data suggests that angiotensin may be formed locally and could therefore exert effects on the central nervous system. Fischer-Ferraro et al (1971) observed that the angiotensin in the dog brain is concentrated in the brainstem and especially in the hypothalamus whereas Ganten, Marquez-Julio, Granger, Hayduk, Karsunky, Boucher & Gernest (1971) found no localisation of renin in dog brain, although both groups observed that most of the angiotensin in the brain is in the form of angiotensin 1. These studies implicate a physiological role for the renin-angiotensin system in brain tissue and have prompted the investigation of the central actions of angiotensin 11.

In addition to the well established peripheral pressor response to angiotensin 11, Bickerton & Buckley (1961) provided the first evidence that angiotensin could produce centrally mediated hypertensive responses. The findings of Joy & Lowe (1970, 1) confirm those of Gildenberg (1969) in demonstrating that the area responsible for the central hypertensive effects of angiotensin lies in the lower medulla and both groups suggest that the area postrema, where the blood-brain barrier is deficient, is the most probable site of angiotensin action (Joy & Lowe, 1970, 2) (Gildenberg, 1971). Joy (1971) suggests that the central haemodynamic response to angiotensin usually involves widespread constriction of the arterioles, mediated through the sympathetic nervous system, although a rise in cardiac output,

mediated by decreased vagal sympathetic activity might also be responsible for a small part of the response.

There is also growing literature which suggests that when angiotensin is injected or infused into the central nervous system, marked changes in the animal's state of hydration occur. Fitzsimons (1972) reports that many species respond to C.N.S. injections of angiotensin by increasing water intake without any apparent initiating stimulus. Epstein, Fitzsimons & Rolls (1970) observed that large doses of angiotensin cause changes in drinking behaviour when injected locally into the nucleus accumbens, septum, preoptic area, anterior and lateral hypothalamic areas, the ventromedial nucleus and the amygdala. Several investigators have attempted to identify potential neurotransmitters associated with the angiotensin-induced drinking response and data at present suggests an aminergic mechanism is probably involved. Intrahypothalamic administration of 6-hydroxydopamine (haloperidol) inhibits the drinking response when injected at the same site as angiotensin. Consequently a dopaminergic mechanism has been suggested by Fitzsimons & Setler (1971).

The mechanism by which angiotensin induces the drinking response is at present hypothetical. Andersson, Eriksson & Fernandez (1971) report that when angiotensin dissolved in hypotonic saline is infused into the third ventricle of the goat's brain, drinking behaviour is evident. Conversely, if the peptide is administered in a solution of glucose or surcose the drinking response is absent. They postulate the existence of sodium sensitive receptors in the brain, which are activated by changes in sodium concentration of the cerebro-spinal fluid and conclude that angiotensin stimulates these receptors to induce drinking. These remain only hypotheses and clearly further investigations are necessary to elicit the mechanism of the angiotensin-induced drinking response.

In addition to causing marked changes in drinking behaviour, angiotensin, when administered to the central nervous system, also appears to release ADH from the neurohypophysis, as reported by Mouw et al (1971). The latter two central actions of angiotensin, that is the angiotensin-induced drinking response and ADH secretion, emphasise the importance of the hormone in the physiological control of salt and water homeostasis.

Recently, it has been suggested that angiotensin acts centrally to release adrenocorticotropic hormone (ACTH) (Daniels-Severs, Ogden & Vernikos-Danellis, 1971). It is not known at present whether this is due to a direct effect of angiotensin, or a secondary release of ACTH associated with the pressor response and/or ADH release, both of which release ACTH. However, brain ablation studies (Gann, 1969) predict that angiotensin may well act at the median eminence to release ACTH.

The central actions of angiotensin may contribute to the maintenance of hypertensive states, fluid and electrolyte balance. How these central effects are integrated with peripheral effects of angiotensin is not known and much research is still necessary to expand and evaluate the role of the hormone in the brain.

4. The effects of angiotensin on salt and water balance

Angiotensin is now becoming established as a major salt and water retaining hormone. As already described, it acts centrally to induce drinking and the release of vasopressin. Furthermore at physiological concentrations it has a controlling role in the release of aldosterone from the adrenal cortex, acts directly on the epithelial cells of the intestine to alter fluid transfer and has a major action on the tubular resorption of sodium in the kidney. Since these actions of the hormone must be of physiological significance to salt and water homeostasis it is important to this study to examine them in some detail.

a. Effects of angiotensin on aldosterone secretion

In early investigations it was recognised that the immediate stimulus to aldosterone production might be either nervous or hormonal or perhaps both. Subsequently, several groups of workers demonstrated that the nerves to the adrenals are unnecessary for enhanced secretion of aldosterone to occur and this provided indirect evidence for a hormonal mechanism in the control of aldosterone biosynthesis. In the 1960's two new findings suggested that the renin-angiotensin system is important in the control of aldosterone secretion. It was discovered that a potent aldosterone stimulating hormone is produced by the kidney (Davis, 1961) and that synthetic angiotensin II increases the rate of aldosterone secretion (Laragh, Angers, Kelley & Liebermann,

1960). Bumpus et al (1964) provided important evidence to link the renin angiotensin system with the adrenal cortex. In studies investigating the metabolic fate of (H^3) angiotensin 11, they found that, after injection of this compound, it is taken up more readily by the adrenals than by any other organ. Davis (1962) also provided evidence for a direct action of angiotensin 11 on the adrenal cortex to increase the biosynthesis of aldosterone, when he infused the hormone into the arterial supply of isolated adrenal gland.

In addition there is now evidence to suggest that the renin-angiotensin system functions as a negative feedback mechanism in controlling aldosterone secretion. A decrease in the concentration of sodium in the plasma causes an increase in renin release (Vander, 1967) and hence a rise in angiotensin production. The hormone, in its turn, acts directly on the zona glomerulosa to stimulate the rate of aldosterone biosynthesis from cholesterol leading to an increase in the aldosterone levels of the blood. The aldosterone thus produced acts upon various target tissues including the kidney distal tubule to promote sodium reabsorption and hence fluid retention. In normal animals retained fluid increases the circulating blood volume, the arterial pressure, renal blood flow and glomerular filtration rate. The increase in blood volume and these changes secondary to it, lead to decreased renin release by a negative feedback mechanism (Lee, 1969).

Although it has become clear after many clinical investigations, that the renin-angiotensin system is very important in the pathogenesis of hyperaldosterism in disease, it has been difficult to obtain evidence to the extent of its importance in normal homeostasis. It has been suggested by Davis (1971), that if the renin-angiotensin system controls aldosterone secretion in primitive mammals and lower vertebrates then it may be of importance in homeostasis in man. But although observations to support this hypothesis have been reported in the North American opossum (Johnston, Davis & Hartcroft, 1967) where a renin-angiotensin system is evident, it is unclear under normal physiological conditions how important the part that angiotensin plays in the secretion of aldosterone.

b. Effects of angiotensin 11 on transporting epithelial tissues

From the investigations of Crocker & Munday (1970), who showed

that adrenalectomy reduces fluid transport across rat jejunum to a lesser extent than maintaining the rats on a high sodium diet, and the observations of Clarke et al (1967) that the aldosterone inhibitor, spironolactone fails to inhibit increased sodium absorption in sodium-depleted dogs, the existence of a major extra-adrenal salt and water retaining hormone is inferred. Crocker & Munday (1970) were also able to demonstrate, using nephrectomised and adrenalised rats that a renal factor is important in maintaining rat jejunal fluid transport and hence showed that angiotensin, produced as a result of renin secretion from the kidney, stimulates intestinal sodium and fluid transport when applied directly to the serosal surface of the intestine. Thus angiotensin seems to be responsible in part, for the control of intestinal sodium and water transport. This statement is supported by the observations that angiotensin stimulates the active transport of sodium by frog skin preparations (McAfee & Locke, 1967), sodium and fluid transport by the rat colon in vitro (Davies et al, 1970) and the rat jejunum in vivo (Bolton et al, 1974) and sodium extrusion in rat kidney cortex slices (Munday, Parsons & Poat, 1971). In addition the actions of angiotensin on intestinal transport have been shown to be dose dependent (Davies et al, 1970) (Bolton et al, 1974), low doses stimulating and high doses inhibiting intestinal fluid transfer. Although low doses of the hormone used in these studies are within the normal physiological range, the significance of the response to high doses of angiotensin is doubtful but may be important under pathological conditions. These actions are similar to those described by Barraclough et al (1967) and Bonjour & Malvin (1969) on kidney in vivo. They found that low doses of angiotensin produce an antinatriuretic/antidiuretic effect, whereas high doses result in diuresis and natriuresis. There is still, however, much controversy as to whether angiotensin induces the diuretic and antidiuretic responses of the kidney by direct stimulation of tubular transport mechanisms or indirectly through changes in renal blood flow. Barraclough et al (1967) attribute both the natriuretic and antinatriuretic effects to the direct action of angiotensin on tubular transport, while Bonjour & Malvin (1969) postulate that only the natriuretic effect is a result of direct tubular action and that the antinatriuretic response is secondary to changes in blood flow. In kidney cortex slices, however, angiotensin clearly increases activity of the second sodium exchange pump (Munday et al, 1971), and Davies et al (1970) suggest that both the stimulatory

and inhibitory actions of angiotensin on colonic fluid transfer are due to direct actions on the epithelial cells.

From the evidence presented it is clear that there is a role for angiotensin in salt and water homeostasis. Its central actions to induce drinking (Fitzsimons & Simons, 1969) and to secrete vasopressin (Mouw et al, 1971) serve to control the extracellular fluid volume of the body, as do its actions on the kidney (Barraclough et al, 1967) and on intestinal absorption (Davies et al, 1970). Although angiotensin has been cited as one of the primary mechanisms in the control of aldosterone secretion, this seems at the present time to be more evident in disease than in normal homeostasis. However this important action cannot be overlooked when examining the long term control of salt and water balance by the hormone.

Section 111

Transport Mechanisms across Epithelial Tissue

It is evident that transporting epithelial tissues such as kidney, intestine and amphibian skin are of extreme importance in the control of salt and fluid homeostasis. The mechanisms of salt and water transfer by all these tissues have many characteristics in common so that this present review will primarily consider fluid transport across the intestine with references to kidney epithelial transport.

Since the studies of Ussing & Windhanger (1964) it has become increasingly clear that fluid transport across all epithelia may occur by two routes; the first, a transcellular route that involves movements across at least two membranes arranged in series, usually the mucosal or brush border membrane and the serosal/lateral membrane; the second an extracellular route that circumnavigates the membranes surrounding the epithelial cells. The latter route is referred to as the "shunt pathway" and evidence has been put forward to suggest that this pathway comprises the tight junctions and the underlying lateral intercellular spaces (Frömter & Diamond, 1972). Although the relative contributions of the transcellular and intercellular routes to ion and water transport differ widely, the shunt pathway has an important role in the movement of ions, small non electrolytes and water across the intestine. Both mechanisms have been reviewed by Schultz, Frizzell & Nellans (1974).

Transcellular Transport

Both active and passive processes are responsible for the movement of ions across cell membranes. Passive transport is a result of movement of ions down an electrochemical gradient, while active transport refers to net movement of ions against an electrochemical gradient. Ions traversing the intestinal membranes have two membranes over which they must be transported, the mucosal and the serosal/lateral membrane.

Sodium Transport and the Sodium Dependent ATPase

Most cells are able to maintain a higher internal potassium and a lower internal sodium concentration than the concentration of these ions in the extracellular fluid. They achieve this by transporting potassium into the cell and sodium out of the cell against electrochemical gradients, utilising a process which requires the expenditure of metabolic energy. It has been shown by experiments on nerve (Caldwell, 1960) and red blood cell membranes (Hoffman, 1960) that the energy for this active transport

of sodium is supplied by the hydrolysis of adenosine triphosphate (ATP). This active transport of sodium is also dependent upon the concentration of potassium in the extracellular fluid, strongly suggesting that there is a coupling between the active extrusion of sodium and the inward transport of potassium ions (Glynn, 1956). This process has been called the "sodium potassium exchange pump" or more simply the "sodium pump". It has now been shown that the coupling between the sodium efflux and the potassium influx can vary in different tissues and with ionic concentration from a 1:1 ratio, when the pump is operating in an electrically neutral manner, to a 3:2 ratio when it becomes more electrogenic (reviewed by Thomas, 1972).

The molecular mechanism coupling the utilisation of ATP to active transport was first described by Skou (1957), who identified an enzyme system present in the peripheral nerve of the crab capable of hydrolysing ATP. This system, which is located in the cell membrane, is similar to other ATP hydrolysing enzymes in that it has an absolute requirement for magnesium ions. It is also, however, totally dependent upon the addition of both sodium and potassium ions for maximum activity. As a result of these additional requirements the system has been called the Na-K dependent ATPase. The details of the precise biochemical mechanisms, by which the hydrolysis of ATP is linked to the active transport of sodium and potassium remain obscure. Post (1968) postulates that a cyclic sodium dependent phosphorylation and potassium dependent dephosphorylation of a membrane carrier occurs and suggests that sodium has a high affinity for the non-phosphorylated carrier, whilst potassium has a higher affinity for the carrier in the phosphorylated form. It has been shown that a membrane fraction exists which can be phosphorylated by ATP in the presence of Na- ions and rapidly hydrolysed in the presence of K- ions, and that the ATPase activity is sensitive to inhibition by cardiac glycosides, particularly ouabain.

A model for Transcellular Sodium Transport

A mechanism proposed by Koefoed-Johnsen & Ussing (1958) for explaining active sodium transfer by frog skin has now been accepted as a general model for sodium transport in a variety of epithelial tissues including intestinal mucosa and kidney. The model, in its simplest form, proposes that sodium ions enter the cell passively at the mucosal border down an

electrochemical gradient, thus raising the intracellular sodium levels. Sodium ions are then extruded at the serosal/lateral borders by the sodium pump, whose activity is related to the intracellular concentration of sodium. Energy for the active extrusion of sodium ions comes from the hydrolysis of ATP by the Na-K dependent ATPase system. Commonly observed characteristics of the sodium pump in epithelial tissues are that it:-

a) requires the presence of potassium in the serosal fluid for optimum activity
b) pumping activity is inhibited by the presence of cardiac glycosides in the serosal medium
c) it is inhibited by many metabolic inhibitors.

It seems reasonable to infer that the extrusion of sodium by a Na-K dependent ATPase system is found in virtually all epithelial tissues and this inference is supported by studies on fractionated small intestine cells (Quigley & Golterer, 1968) as well as autoradiographic studies (Stirling, 1972), which indicates that most of the Na-K stimulated, ouabain sensitive ATPase is located on the basolateral membranes of epithelial cells.

Electrical Measurement of Sodium Transport and the Difficulty in its Interpretation

One experimental approach that has contributed greatly to the investigation of ion transport and, which has also been applied to studies of intestinal transfer, is the measurement of transmural potential difference (pd) and short circuit current (scc). The advantage of scc studies is derived from the relationship between the net ionic fluxes and the magnitude of the external current necessary to reduce the electrical pd across the tissue to zero. The current applied is essentially equal to the current transversing the tissue and as this is produced by the net ionic flow, it must represent the sum of the net fluxes of all ions which flow across the tissue. In the absence of an electrochemical gradient, a single charged species will undergo net transport only by active processes and the scc therefore provides a simple and reliable method for the monitoring of the net active ion flux. It must be emphasised that the agreement between scc and the net flux of each ion must be established for each condition of investigation. For example, in most epithelial cells sodium is the most commonly transported ion species, but if an experimental condition induces the transfer of a second ion, which is not normally transported, the change

in pd and scc cannot be attributed to sodium transport alone.

Leaf, Anderson & Page (1958) found that in the urinary bladder of Bufo Bufo, the net sodium flux from the lumen to the blood equalled the scc. A similar equality between scc and net sodium flux was also found by Ussing & Zerahn (1951) using frog skin, and from most of the cases reviewed by Keynes (1969) it is evident that intestinal net sodium transport and scc are equal. However, for a number of cases cited in the literature this correlation between scc and net sodium flux does not exist. Barry, Smyth & Wright (1965), who studied the relationship between net sodium movement, pd and scc in rat jejunum in the presence of hexose sugars, found that scc and net sodium flux correlate when glucose or mannitol, but not galactose or fructose, are present in the mucosal solution. These findings have been confirmed in studies on rat and rabbit ileum by Taylor, Wright, Schultz & Curran (1968), where a lack of correlation between sodium fluxes and scc was also observed. These examples illustrate the danger in assuming that scc is always equal to net sodium flux and it is evident that under certain experimental conditions there can be significant differences in these two factors.

Modes of Active Sodium Transport

Electrical activity has been observed in many transporting epithelial tissue, including the intestine and this can be related to the movement of ions. Ion pumps in transporting epithelia may either be electrogenic, which means that there is a net transfer of charge across the tissue generating a change in transmural pd, or non-electrogenic where there is no generated change in pd as there is no net transfer of charge. Non-electrogenic transfer is achieved by either the transport of two ions of opposite charge in the same direction or by the movement of two ions of similar charge in opposite directions at the same time. Evidence has now accumulated to suggest that in the kidney, toad bladder and intestinal epithelial cells, there are at least two modes of active sodium transport. These have been identified by use of electrical measurements and inhibitory agents such as ethacrynic acid and ouabain.

Barry et al (1965) utilised scc techniques in experiments on sodium transport across the intestine. They postulate the existence of two pumps in rat jejunum, one being electrogenic and closely concerned with transporting hexose sugars and the other, an electroneutral pump concerned with bulk

transfer of fluid and sodium. Evidence for the existence of this second sodium pump has been extended by Binder & Rawlins (1973). They propose that this neutral pump is sodium-chloride coupled and that sodium and chloride absorption may represent two related ion exchanges; Na - H and Cl - HCO_3. Their model of ion transport is consistent with electrolyte movement in vivo. Additional evidence to support the neutral pump concept has been put forward by Bolton, Munday, Parsons & York (1974) and Levens, Munday & York (1975), who from work on the in vivo jejunum and colon respectively, suggest that angiotensin-stimulated fluid transport across the intestine can be explained in terms of tightly coupled Na-anion pump or by independent sodium and anion transport mechanisms. A similar non-electrogenic sodium pump in gall bladder (Diamond, 1962 (1)) appears to transport sodium and chloride ions across the cells in a tightly coupled one to one ratio which results in no net transfer of charge.

Two modes of sodium extrusion have also been described in slices of outer cortex of guinea pig kidney (Whittembury, 1968) (Whittembury & Proverbio, 1970). The first mechanism is linked to potassium uptake and is inhibited by ouabain. The second mechanism is refractory to ouabain, sensitive to ethacrynic acid and low temperature and is independent of potassium. The latter has also been called the "second sodium pump" and is thought to extrude sodium with chloride as an accompanying ion with the net movement of ions being linked to cell water loss. Whittembury & Proverbio (1970) predict that the first sodium pump they describe is electroneutral since it extrudes one sodium ion for each potassium ion taken up, a 1;1 exchange, and that the second sodium pump is electrogenic. This prediction is, however, rather speculative since the electrical properties of the pumps depend upon the stoichiometry associated with ion transport and the degree of sodium and chloride coupling. In contrast Smith (1975) demonstrated that the second sodium pump has an absolute requirement for chloride ions. This observation together with the findings of Munday et al (1971) that angiotensin stimulates the second sodium pump and Bolton et al (1974) that angiotensin enhances non-electrogenic sodium transfer, suggests that the second sodium pump is tightly coupled to chloride ions in an electroneutral manner.

Sodium Dependent Transport

Many observations suggesting that sodium influences the transport of

solutes across cell membranes occur throughout the early literature. Ricklis & Quastel (1958) reported that the absorption of glucose by isolated guinea pig small intestine depends markedly on the presence of sodium in the solution bathing the mucosal surface. Crane (1962) reported that sodium is required for the uptake of actively but not passively transported sugars by the mucosal cells of hamster small intestine and postulated that sodium and actively transported hexoses share a common carrier across the mucosal membrane. This sodium dependence also appears to be closely related to the ability of these cells to transport other solutes against an electrochemical potential difference (reviewed by Schultz & Curran, 1970).

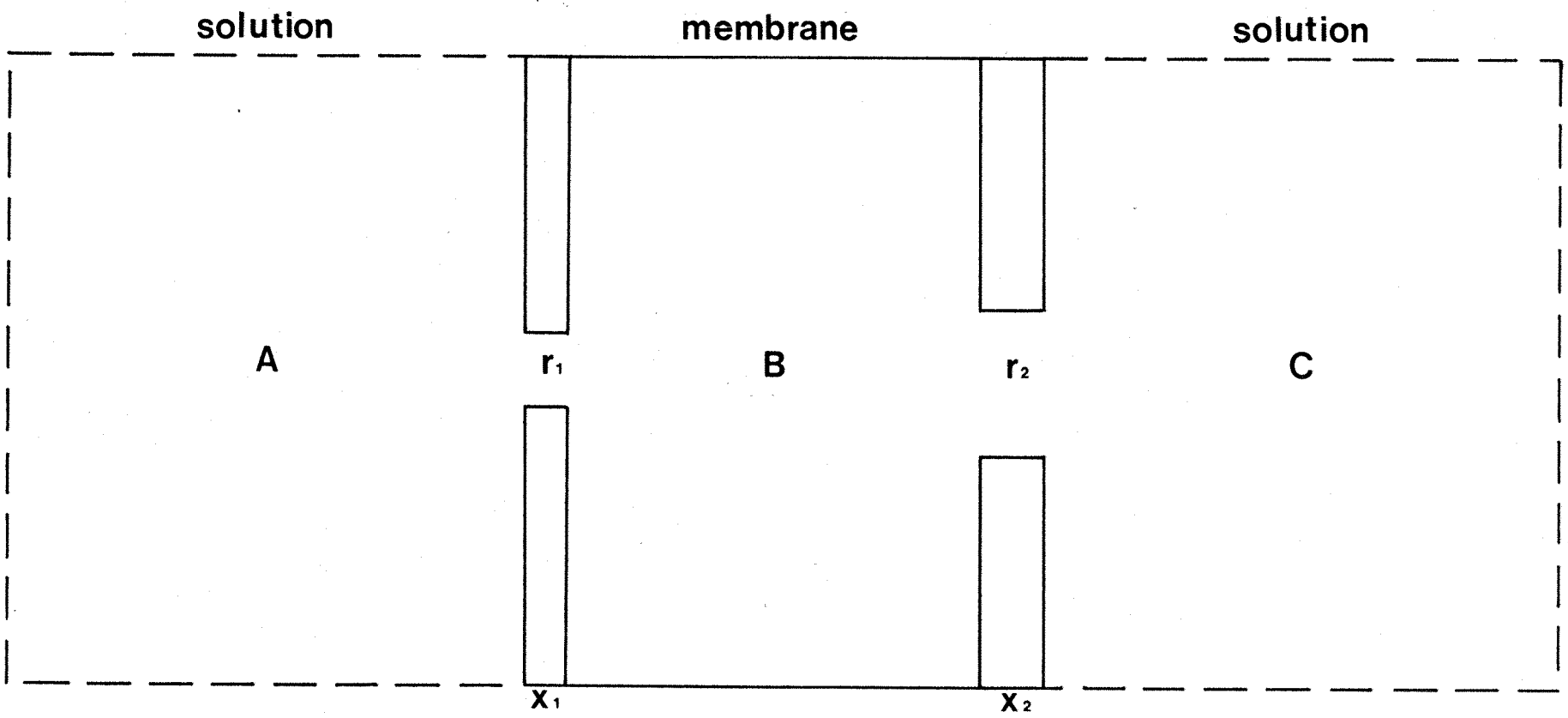
It is clear that active transport of a wide range of solutes by many tissues is sodium dependent although the role of sodium in these processes is far from clear. Crane (1965) concluded that the sodium gradient across cell membranes produced by the activity of the sodium pump supplies the energy for active transport by increasing the affinity of carriers on the outside of the membrane for substrates, where the sodium concentration is high, and reducing the affinity on the inside of the membrane where sodium concentration is low. This hypothesis has been generally accepted for many years but has been questioned by Kimmich (1973) although a reasonable alternative mechanism has not been developed.

Transcellular Water Transport

In early experiments on rat ileum in vivo, Curran & Solomon (1958) obtained evidence indicating that water absorption is a passive process which takes place as a result of active solute transport. Conversely, Smyth & Taylor (1957) found that water absorption by isolated rat small intestine is inhibited by the absence of glucose in the mucosal solution and by metabolic inhibitors suggesting that water transfer might be an active process. Although water absorption can take place against the water activity gradient it is now generally agreed that water transport itself is not an active process but is linked to the movement of solutes, usually sodium and chloride. In the ileum and colon water absorption is dependent upon the rate of net sodium movement and a reduction in sodium concentration in the lumen, resulting in decreased net sodium absorption, causes decreased net water transport. (Curran & Schwartz, 1960).

Curran (1960) has proposed a three compartment model to explain water absorption in transporting epithelia and this is illustrated in figure 1.

Fig. 1 A SCHEMATIC MODEL SYSTEM FOR WATER TRANSPORT



KEY

A and C	external solution
r	pore radius
m	membrane thickness

In the model two solutions of equal strength (A & C) are separated by a complex membrane which is considered as a separate compartment (B). The membrane is made up of two porous structures in series, a thin membrane with small pores and a thicker membrane with large pores. It is assumed that solute is actively transported across the thin membrane from region A to B. In the intestine in vitro, the thin membrane might be represented by one of the epithelial cell membranes (serosal/lateral) while the thick membrane might be the basement membrane of the epithelia. Active solute transport will cause a relatively high solute concentration in region B, and the resulting osmotic pressure gradient will cause water to move across the membrane from A to B. This gradient will cause little water movement from C to B because of the large pores in this region. Thus the osmotic pressure gradient between B and C will be small if the pores are sufficiently large. Entrance of water into region B from A will, however, cause an increase in hydrostatic pressure unless the compartment can freely expand. This pressure would then drive water through the large pores from B to C, but would have little effect on water flow from B to A, because of the much smaller radius of the pores between these regions, the net result would be movement from A to C.

An alternative model proposed by Diamond (1962) is a special case of the Curran model. In this model it is suggested that active solute transport (NaCl) occurs at the lateral membrane into the intercellular channels; that is the long channels which are closed at the luminal end. This transport establishes a standing osmotic gradient with a progressive approach to equilibrium along the channels length. Thus the water transfer allowed by the favourable osmotic gradient, emerges as a flow from the open end of the channel, the fluid of which is iso-osmotic with the initial luminal fluid.

Intercellular Fluid Transport

Epithelia are uninterrupted sheets of cells surrounding cavities and held together at the luminal surface by junctional complexes (Goodenough & Revel,1970). The transport of substances through the cells themselves has now been firmly established and the question now arises as to whether or not the junction complexes will allow intercellular transfer. While proteins and large collodial tracers are generally stopped at the zona occludens or "tight junction", the junctions could still be leaky to small non electrolytes and water.

It has been recognised that there are large differences between transmural resistance values among different epithelia. Moreover in epithelia of low resistance, the resistance of cell membranes is well above that for transepithelial resistance, indicating the presence of large paracellular shunt pathways in these tissues. The large shunt was proposed to be within the zonulae occludens.

Fromter (1972) and Fromter & Diamond (1972) have shown, using lanthanum, an ion whose size is not much larger than that of sodium, that the zonula occludens is a major route for intercellular permeation in the low resistance epithelia, Necturus gall bladder. Intercellular transport across the small intestine has been extensively reviewed by Schultz et al (1974), providing evidence for the presence of a relatively high conductance shunt pathway across in vitro preparations of small intestine. The investigations of Frizzell & Schultz (1972), on the effect of imposed transepithelial potential difference on unidirectional influxes of sodium, chloride and potassium across the luminal surface of rabbit ileum, have shown that the total influx of each ion is comprised of two components. The first behaves as if it crosses the brush border membrane and the second component behaves as if the ion enters a water filled pore. Machen, Erlij & Wooding (1972) have also demonstrated that the tight junctions of rabbit ileal mucosa are permeable to lanthanum in a study similar to that of Fromter (1972) and findings to support major tight junction permeability in the proximal tubule of the toad kidney has now been provided by Whittembury & Rawlins (1971). Thus it seems safe to conclude that the tight junctions are permeable to small ions and electrolytes and that the shunt pathway is indeed present in some epithelial tissues.

From the above and many other studies it has been established that in epithelia, where transmural resistance and potential difference are low, there are large shunt pathways and intercellular ion movement predominates. Examples of these "leaky epithelia" are kidney proximal tubule, gall bladder, jejunum and ileum. Conversely transporting tissues displaying high transmural pd are known as "tight epithelia", such as frog skin and toad bladder, and transport ions by primarily intracellular routes.

Finally it appears that the properties of these different epithelial tissues can be correlated with their physiological functions. The clearest implication concerns the function of junctional "tightness" in those epithelia which must maintain steep solute gradients by active transport. For example, frog skin absorbs ions from water with a sodium chloride concentration up to 10,000 times lower than that of frog plasma. The maintenance of

such gradients would be impossible if junctions were highly permeable to ions. Similar considerations apply to sodium chloride absorption from urinary bladder, salivary gland ducts and renal collecting ducts. The significance of junctional "leakiness" in epithelia such as intestine, gall bladder and renal proximal tubule can be related to isotonic water transport. Essential to the function of these tissues is water and salt absorption, water transport being a consequence of active solute transport into the lateral intercellular spaces. As junctions leaky to ions are also permeable to water (Tormey & Diamond, 1967), a significant fraction of osmotic flow is probably across the junctions, although achievement of complete osmotic equilibrium would still require part of the water flow to be across cells and lateral cell membranes into the spaces (Diamond, 1971).

Section 1V

The Mechanism of Action of Hormones

The mechanism of action of hormones, from their binding at the receptor site of the target cell to the change in cell function, is, in the majority of cases still far from clear. However the following theories have now been put forward to account for the actions of hormones on their target cells.

Mechanism of Action of Polypeptide Hormones

These react with membrane receptors and initiate a sequence of events leading to changes in target cell matabolism.

The Secondary Messenger Hypothesis:-

The adenyl cyclase system

Cyclic AMP has now been established as an intracellular messenger mediating many of the actions of a variety of hormones. This functional role of the compound has been extensively reviewed by Robi son, Butcher & Sutherland (1968) and by Hardman, Robison & Sutherland (1971). Cyclic AMP was discovered in the course of investigations into the mechanism of the hyperglycaemic action of adrenaline and glucagon (Sutherland & Rall, 1958). These hormones stimulate liver glycogenolysis by increasing the formation of cyclic AMP, which in turn increases the formation of active from inactive phosphorylase (Riley, 1963). The intracellular level of cyclic AMP, at any one time, depends on the activities of at least two enzymes, adenyl cyclase, which catalyses the formation of cyclic AMP from ATP in a reaction that requires magnesium and a specific phosphodiesterase, which inactivates cyclic AMP by catalysing its hydrolysis to 5′AMP. Hormones which act by increasing the intracellular levels of cyclic AMP namely, vasopressin, parathyroid hormone, prostaglandin and glucagon, have been found to stimulate the membrane bound adenyl cyclase, thus catalysing formation of cyclic AMP. Constantopoulis & Najjar (1973) have studied the activation of adenyl cyclase by hormone action at the cell membrane. They postulate that the hormone activates a membrane bound phosphatase, which dephosphorylates adenyl cyclase causing its activation. As all the mentioned polypeptide hormones have been shown to exert their action through cyclic AMP it seems probable that the adenyl cyclase system functions as a discriminator for environmental signals by demonstrating a certain specificity. It has been suggested (Robison, Butcher & Sutherland, 1967) that adenyl cyclase may be

composed of two types of subunit. According to this proposal the actual receptor for a hormone would be part of the regulatory subunit of the enzyme, a variable component of the system, differing to some extent from tissue to tissue. The binding process would cause activation of the second subunit, which is expected to be functionally the same in all tissues. This hypothesis is supported by the investigations of Schwartz & Hector (1966), who also favour the idea that hormones which effect adenyl cyclase do not directly effect the enzyme but act by way of a separate receptor.

Once the intracellular levels of the secondary messenger are determined by hormone action, cyclic AMP can activate a number of protein kinases (reviewed by McMahon, 1974). The mechanism by which cyclic AMP stimulates protein kinases activity is as yet unknown but studies indicate that it may act by binding to and relieving the repression produced by an inhibitory protein (Reiman & Walsh, 1970). The kinases in their turn transfer a phosphate group from ATP to various enzymes, an action which results in either an increase or decrease of enzyme activity. Enzymes which are activated by this phosphorylation include muscle phosphorylase and those which are inhibited include glycogen synthetase.

In addition to its proposed messenger action cyclic AMP can also alter the permeability of the cell membrane to certain inorganic ions, which are necessary for generation and destruction of cyclic AMP and for the action of its dependent protein kinases and phosphotases. Changes in permeability to potassium, calcium and chloride result from hormonally produced changes in cyclic AMP concentration or from application of the nucleotide or its derivatives (Prince & Berridge, 1973). Besides changing membrane permeability, cyclic AMP has been shown to liberate some cations including calcium from intracellular pools, thus changing ionic concentrations within the cell (Rasmussen, 1970) and evidence has now accumulated to show that in many of the systems, in which cyclic AMP has been implicated as a secondary messenger, calcium ions also have an important role in the cellular response to the stimulating hormone. Observations of the complex interrelations in the metabolism of cyclic AMP and calcium have now led to the formulation that calcium itself acts as a secondary messenger in hormone action and from work on a number of specific systems models have been suggested to interpret the inter-involvement of calcium and cyclic AMP in hormone action (reviewed by Rasmussen & Goodman, 1977).

Cyclic Guanosine 3'5' monophosphate (cyclic GMP) and Hormone Action

Although cyclic AMP has been established as a secondary messenger of hormone action, it seems probable that there may be a role for other messengers in cell activation. The cyclic nucleotide, cyclic GMP, has been investigated with respect to hormone action and there is now increasing evidence to suggest that this compound may be involved in the biochemical effect of some hormones. Cyclic GMP, which occurs widely in nature and is a natural constituent of mammalian tissues (Steiner, Parker & Kipnis, 1970) has been shown to possess similar pharmacological properties to cyclic AMP. They are produced and destroyed in the cell in an analogous manner, cyclic GMP being formed from GTP by the enzyme guanyl cyclase and degraded to 5'GMP by possibly the same phosphodiesterase that hydrolyses cyclic AMP. Although the biochemical mechanism of the effects of cyclic GMP has not been investigated as intensively as that of cyclic AMP, cyclic GMP dependent protein kinases similar to their cyclic AMP counterparts have been reported in the pancreas and the cerebellum of the rat (Van-Leeput-Coutrez, Camus & Cristophe, 1973). Moreover, physiological studies also indicate that the interaction of cyclic AMP and cyclic GMP is important. In a number of systems these cyclic nucleotides impose contrasting or opposing regulatory influences and in others act co-operatively. The interrelationship of cyclic AMP and cyclic GMP has been formulated in the Yin-Yang hypothesis (Goldberg, Haddox, Nicol, Glass, Sanford, Kuehl & Estensen 1975), which defines two types of control systems. The first type formulates cellular processes activated by cyclic AMP and inhibited cyclic GMP and in the second type of control system cyclic GMP is the activator and cyclic AMP the inhibitor.

Effects of cyclic GMP which are unlike those of cyclic AMP are of interest as these may lead to a possible role for the guanosine nucleotide in hormone action. An example of this is in the action of vasopressin on toad bladder. Vasopressin is a polypeptide hormone which causes an increase in nucleotide levels in both toad bladder (Handler, Butcher, Sutherland & Orloff, 1965) and frog skin (Baba, Smith & Townshend, 1967). In these tissues cyclic AMP appears to mediate the increase in sodium and water permeability which occurs in the presence of the hormone. The observations that cyclic AMP mimics the hydro-osmotic effect of vasopressin in isolated renal tubules (Grantham & Burg, 1966) and that cyclic AMP is also present in the urine support the idea that the physiological effects of vasopressin on the kidney are also mediated by cyclic AMP. To explore the significance of the fact that urine contains cyclic GMP as well as cyclic AMP, Bourgoignie

et al (1969) have studied the effect of the nucleotides on sodium transport and water permeability in toad bladder. They observed that cyclic GMP reproduces the effects of cyclic AMP on scc but is not able to mimic the cyclic AMP induced increase in water permeability. As it has been reported that certain analogues of vasopressin increase permeability to water but do not alter sodium transport (Walter, Rudinger & Schwartz, 1967), the ability to dissociate the effects with analogues on scc and water permeability implies independent and different messengers for the hormone. Differing sensitivity of toad bladder to cyclic AMP and cyclic GMP suggests that vasopressin may give rise to its separate effects via the two nucleotides.

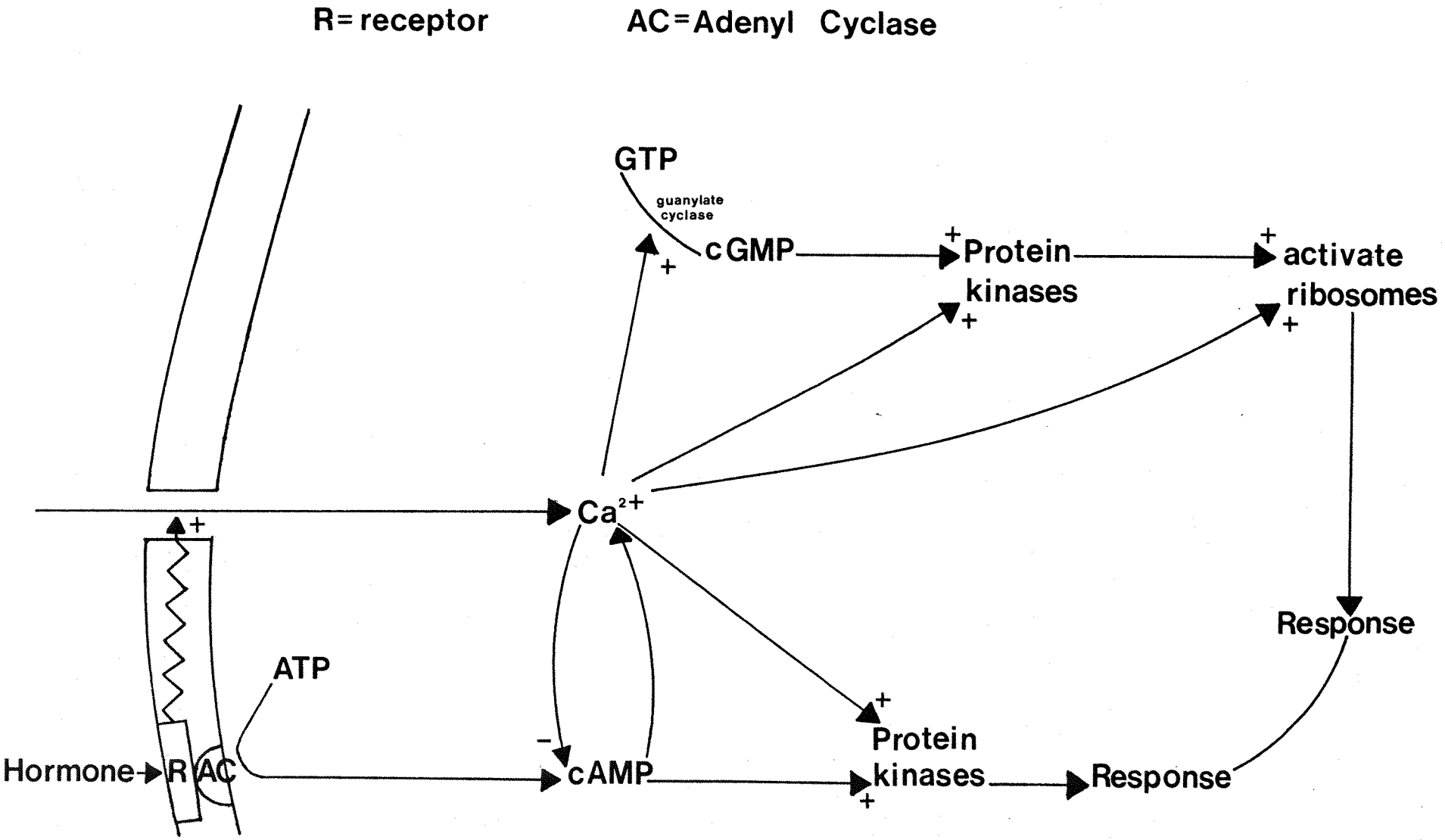
In addition it is now evident that cyclic GMP and calcium ions, as well as cyclic AMP and calcium ions, are interrelated. In several cell types, in which cyclic GMP concentrations increase after interaction with an extracellular messenger, the increase in cyclic GMP concentration may be due to an activation of guanylate cyclase by calcium ions rather than a direct activation of a membrane bound enzyme. This suggests that cyclic GMP may be acting as a tertiary messenger of hormones, calcium ions being the secondary messenger or coupling factor between binding of the hormone at the receptor site and cyclic GMP (reviewed by Rasmussen & Goodman, 1977).

Direct evidence for cyclic GMP's involvement in protein synthesis has been reported by Macchia & Varrone (1971), who during investigations into the role of cyclic AMP and cyclic GMP in the mechanism of thyroid stimulating hormone action, observed that cyclic GMP significantly stimulates protein synthesis in hog thyroid slices in vitro, but does not effect any other metabolic parameter of the gland. Pisarev, De Groot, Wilker & Altschuler, (1971) have also found that cyclic GMP stimulates thyroid protein synthesis when injected into mice. Further work by Varrone et al (1973) shows that cyclic GMP is active in other tissues, being able to stimulate protein synthesis in rat liver and polypeptide synthesis in cell free systems. These observations are consistent with the view that cyclic GMP is a mediator of hormone action and that it has a major effect in regulating ribosomal protein synthesis.

Summary

Sutherland & Rall (1958) demonstrated the role of cyclic AMP in the hyperglycaemic action of adrenaline and glucagon, and from their observations

Fig. 2 A PROPOSED MECHANISM OF POLYPEPTIDE HORMONE ACTION



the secondary messenger hypothesis has been developed to explain hormone action. This hypothesis has dominated thinking in the field of hormonal mechanism of action for some time. However, in many systems in which cyclic AMP has been implicated as a secondary messenger, there is evidence to suggest that there is an equally important role for calcium ions in the hormonal response. The binding of the hormone to its receptor site may cause an influx of calcium into the cell with or without the production of cyclic AMP from ATP. Moreover, cyclic AMP and calcium appear to be interrelated; cyclic AMP causing changes in the concentrations of calcium within the cell; and calcium ions often causing a decrease in cyclic AMP levels by activating its catabolism by phosphodiesterase.

The importance of cyclic GMP as an intracellular messenger is also now being established and it has been shown to have a regulatory effect on ribosomal protein synthesis. Calcium ions have been suggested to increase cyclic GMP concentrations in the target cell by activating the predominantly cytoplasmic enzyme required for its formation. Thus calcium ions and the two cyclic nucleotides are interrelated messengers in the activation of particular cells to polypeptide hormones; cyclic AMP and calcium appear to act as secondary messengers and cyclic GMP as a tertiary messenger. A proposed model of hormone action showing the possible connections between the two nucleotides and calcium ions is shown in Fig. 2.

In addition to the probable roles of calcium, cyclic AMP and cyclic GMP, other messengers have been suggested. These include a range of cyclic nucleotides, sodium ions, potassium ions and magnesium ions. Clearly the mechanisms of action of polypeptide hormones have not been fully elucidated at this time.

Mechanism of Action of Steroid Hormones

There is now a large literature implicating the role of protein synthesis in the mechanism of action of a variety of hormones. Soon after the protein synthesis model of Jacob & Monod (1961) was published, Karlson (1963) proposed that "protein anabolic" hormones act by direct derepression of portions of the genome, so that more mRNA is transcripted to act as a template for the synthesis of more protein. The hypothesis was founded on the results of experiments with the insect hormone, ecydosone, which promotes the induction of dopa decarboxylase. The theory has now been

generalised to apply to many other hormones. In most instances where protein synthesis has been postulated as a primary mechanism of hormonal action, studies have been aided by time course observations of the hormonal effect and suppression of hormone action by protein synthesis inhibitors. The most widely used inhibitors include actinomycin D, which blocks protein synthesis at the transcriptional level, and cycloheximide and puromycin which inhibit the translation of mRNA to protein at the ribosome.

Although exact mechanisms remain obscure, the current hypothesis of steroid hormone action, much favoured by Feldman, Funder & Edelman (1972) includes an initial obligatory step of binding of the hormone to stereo-specific proteins in the cytoplasm of target cell tissues. This concept is also supported by the investigations of Baxter, Rousseau, Benson, Garcea, Ito & Tomkins, (1972), who suggest that the steroid-receptor complex migrates to the nucleus and is capable of binding to a nuclear site on the DNA molecule, initiating transcription of mRNA.

The mode of action of the steroid hormone, aldosterone, which stimulates epithelial sodium transport across toad bladder has been reviewed by Sharp & Leaf (1966). The first demonstrations of an effect of aldosterone on sodium transport in vitro were reported by Crabbé (1961). Aldosterone-induced sodium transport shows a characteristic time lag of 40 - 120 minutes before the action, which is inhibited by cycloheximide, puromycin and actinomycin D, becomes apparent. This evidence suggests that aldosterone acts in the cell to induce mRNA synthesis, resulting in translation of a specific protein. The newly synthesised protein is thought to act as a permease which facilitates entry of sodium through the mucosal surface into the cell.

In addition to steroid hormone action a number of polypeptide hormones are also believed to exert their actions by effecting protein synthesis, generally at the level of translation. Kostyo (1968) has shown that the administration of growth hormone to rat diaphragm muscle in vitro causes increased uptake of amino acids into the intracellular amino acid pools, an effect that is demonstrated after a short latency of 10 minutes. This in vitro effect of growth hormone on protein synthesis is blocked by prior incubation with cycloheximide or puromycin; the effect of the hormone on amino acid uptake being either greatly diminished or abolished. The response is, however, unaffected by actinomycin D. In addition, Wool, Stirewalt, Kurihara, Low, Bailey & Oyer (1968) have demonstrated that the

polypeptide hormone insulin, stimulates protein synthesis in muscle, an action that is independent of the effects of the hormone on glucose and amino acid uptake into the cell. They subsequently showed after experiments with protein synthesis inhibitors that the increase in heart muscle protein synthesis is due to an action at the ribosomal level and concluded that insulin increases the translation of a preformed mRNA into protein.

Mechanism of Action of Angiotensin

Peptide hormones, such as angiotensin 11, are generally thought to act by binding specific receptors on the cell membrane. This means that angiotensin is confined to the extracellular fluid and cannot cross the membrane into the cell. Evidence to support this theory has been supplied by the autoradiographic studies of Richardson & Beaulines (1971), who have shown that angiotensin accumulates on the surface of rabbit aorta endothelial cells and apparently does not enter them. In addition, if angiotensin is coupled to a large molecule, e.g. poly-D-L-alanine, the complex then retains most of the activity of the free peptide (Regoli, Rioux, Park & Choi, 1974). These investigations favour the hypothesis that angiotensin acts on the cell membrane.

It has already been suggested that many polypeptide hormones exert their mechanisms of action by altering intracellular cyclic AMP levels. Angiotensin has been reported to increase intracellular levels of cyclic AMP in a manner analogous to other peptide hormones. Peytrerman, Nicholson, Brown, Liddle & Hardman 1973 compared the effects of angiotensin and ACTH on adrenal cyclic AMP and steroidgenesis in vitro. They observed that levels of cyclic AMP increase within one minute of application of angiotensin. Conversely, Volicer & Hynie (1971) have reported that cyclic AMP levels in vascular smooth muscle of rat tail artery are decreased by angiotensin. The mechanism by which angiotensin exerts its action has therefore proved to be controversial, and the findings that one action of the hormone on one tissue involved the adenyl cyclase system cannot be interpreted to imply that all actions on all tissues occur through the same mechanism.

The Mechanism of Angiotensin Action on Salt and Water Transport

Angiotensin may stimulate adenyl cyclase causing an increase in tissue cyclic AMP levels, and thus stimulate fluid transport in an analogous manner to that suggested for the response of toad bladder to vasopressin (Orloff &

Handler, 1967). Consistent with this theory is the finding that angiotensin stimulation of fluid transport occurs without delay when the hormone is administered to the serosal side of isolated jejunum (Crocker & Munday, 1970) and that stimulation of fluid transport across rat descending colon in vitro by angiotensin has been claimed to be mimicked by the administration of cyclic AMP (Hornych, Meyer & Milliez, 1973). Other evidence has however suggested that the action of angiotensin on salt and water transfer across epithelia cannot be convincingly explained in terms of changes in intracellular cyclic AMP concentrations. This is illustrated in the findings of Davies et al (1972) and Munday et al (1972), who report that neither theophylline, cyclic AMP or dibutyryl cyclic AMP, separately or together, mimic, potentiate or reverse the actions of angiotensin on either rat intestine or rat kidney cortex slices. A comprehensive study by Munday, Parsons, Pout, D'Auriac & Meyer (1976) confirmed the non-involvement of cyclic AMP in the actions of angiotensin II on gut and kidney.

It has now been shown by the ion replacement studies of Munday, Parsons, Poat & Smith (1979) that the stimulation of fluid transported in in vitro rat colon and the enhancement of sodium extrusion by rat kidney cortex slices, after the administration of angiotensin, are dependent upon the presence of calcium ions in the incubation fluid. As calcium ions are only required in the serosal incubatory medium, to obtain an angiotensin response, it has been suggested that they may allow the hormone to interact with its receptor sites on the basolateral membranes of epithelial cells or to link the occupied receptor with some membrane effector. In addition it is possible that angiotensin might stimulate the transfer of calcium ions across the serosal membranes of colonic and kidney epithelia cells as a primary event in response to the hormone. As already stated calcium ions increase cyclic GMP concentrations in target cells by activating soluble guanylate cyclase and cyclic GMP has a regulatory effect on ribosomal protein synthesis. In addition calcium ions will also enhance protein synthesis by ribosomes (Farese, 1971) and the combined effects of calcium and cyclic GMP on ribosomal protein synthesis may be necessary in the intracellular mechanism of action of angiotensin on colonic and kidney epithelial cells.

From studies using protein synthesis inhibitors the actions of a number of polypeptide hormones have been shown to be associated with protein synthesis events. In a similar manner, Davies et al (1972) in a study on rat colon and Munday et al (1972) using rat kidney cortex slices have demonstrated that

the stimulatory effects of angiotensin on salt and water transport are blocked by cycloheximide and puromycin and are unaffected by actinomycin D. Bolton et al (1975) confirmed these observations using rat jejunum in vivo. Consistent with these findings Roth (1972) has also demonstrated that angiotensin-stimulated biosynthesis in guinea pig atria is inhibited by puromycin. All these results indicate that the mechanism of angiotensin in these tissues is mediated through a protein synthesis event at the level of translation. However all attempts to show a stimulation of labelled amino acid incorporation into protein by angiotensin have been unsuccessful, suggesting that the protein involved in transport may well be produced in small quantities. How binding of angiotensin to membrane receptors is linked to increased ribosomal activity cannot be fully explained at this time.

Following the increase in protein biosynthesis, angiotensin can exert its action over salt and water transport in epithelia by either stimulating sodium pump activity or by increasing the permeability of the epithelial cell. Munday et al (1971) have concluded that angiotensin stimulates sodium extrusion from kidney cortex slices by increasing the activity of the second sodium pump (Whittembury, 1968). These observations have been extended by Shaikh (1972) in rat colon in vitro; Bolton, Munday, Parsons & York (1974) in rat jejunum in vivo and Levens et al (1975) in rat colon in vivo, who also suggest that the effect of angiotensin in the intestine involves stimulation of an electroneutral sodium process analogous to the second sodium pump described in kidney tissue. More recently Field, Brasitus, Sheerin & Kimberg (1976) have suggested that noradrenaline may also stimulate intestinal absorption by an electroneutral process. Angiotensin is known to stimulate the release of noradrenaline from nerve terminals and the adrenal medulla (Peach, 1977). Following from this observation, Levens, Munday, Parsons, Poat & Stewart (1979) have suggested that noradrenaline may mediate the action of angiotensin on fluid absorption in intestine. They demonstrated that alpha adrenergic blocking agents abolish both angiotensin and noradrenaline stimulated transport across rat jejunum in vivo, and that following endogenous depletion of noradrenaline stores with reserpine the response of intestine to angiotensin, but not the response to noradrenaline, is lost. Again, both the noradrenaline and the angiotensin stimulated intestinal fluid transfer are blocked with cycloheximide.

Section V

Seasonal Variations

a. Basic concepts and terminology in endogenous rhythms

Throughout recorded history men have observed the changing patterns of behaviour of animals and plants which are easily related to two geophysical phenomena, namely the daily 24 hour rotation of the earth on its axis and the yearly 365¼ day orbit of the earth around the sun. Associated with the former is, of course, day and night and with the latter, the coming and going of the seasons. These are important events in the overall environment of living organisms, and unless adaptations take place, the organism would undoubtedly become extinct. The survival value of such adaptations has been reviewed by Heller & Poulsen (1970).

As a result of the evolutionary process animals exhibit three basic ways of synchronising their physiology to a changing environment. These are:-

a) a direct response to various changing geophysical stimuli
b) an endogenous rhythm which programmes the organism to the exogenous temporal period
and c) a combination of both, which is evident in the majority of cases

In order to demonstrate an endogenous rhythm, or what is also termed an internal clock, precise criteria must always be applied. Firstly, the rhythm must exhibit a frequency which is not exactly synchronous with any known environmental signal such as light or ambient temperature. Secondly, the period of endogenous rhythm must deviate at least slightly from the time being measured. Hence the term 'circadian' has been used for the 24 hour cycle and 'circannual' has been adopted by Pengelley & Asmundson, (1970) to express the yearly rhythm. Finally, the rhythm must be relatively temperature independent. If all these criteria apply then it is reasonably certain that the organism exhibits an endogenous rhythm and the use of the word 'circadian' or 'circannual' implies that the rhythm <u>is</u> endogenous.

Organisms which display endogenous rhythms must have a means of synchronising the rhythm with an exogenous one. Aschoff (1960) has used the word 'zeitgeber' to express the environmental agent which entrains the organisms behaviour to the changing environment. Aschoff postulates that phase control, that is, "the clearly defined and stable phase angle difference between biological oscillation and the zeitgeber" is required for entrainment of the organism. This means that it is necessary for the

organism to undergo a periodically changing sensitivity to the zeitgeber, which corrects the phase of oscillation at least once during each period. In organisms with circadian oscillators, the zeitgeber is usually daily fluctuations in light or temperature. Although similar zeitgebers are presumably evident in an annual situation Pengelley, Bartholomew & Licht (1972) have stressed that the questions surrounding the circannual rhythms are far more complex than those of circadian counterparts.

As the basic concepts determining internal clocks have now been described the remainder of this review will be primarily concerned with seasonal or annual rhythms. In such a rhythm an animal may show characteristic behaviour patterns or there may be changes in metabolism and in hormone secretion. Clearly these three factors are closely interrelated and not mutually exclusive. For example, the drastically reduced intake of food in hibernation is accompanied by a decreased metabolism and almost certainly changes in the rate of secretion of oxycorticosterone, an adrenal hormone which is in some way concerned with the animal's ability to hibernate (reviewed by Smith, 1970).

b. Circannual Variations in Animal Behaviour

From an annual point of view the reproductive activities of animals, as well as the various migrations and behavioural phenomena such as dormancy and hibernation are probably the major physiological events of the year. The timing of these yearly events is so obviously related to the passing seasons that until quite recently it was assumed that environmental changes supplied the direct cues for changes in animal behaviour. However with the increased interest in studying biological phenomena under controlled laboratory conditions it has been observed that many organisms still display cyclic behaviour even when the physical environment is kept constant.

Hibernating animals undergo a dramatic change on a yearly basis between the active and hibernating physiological states, thus making them well suited for the study of long term biological rhythms. Pengelley & Fisher (1957) obtained the first clear indication of an annual clock in the golden-mantled ground squirrel (Citellus lateralis). They observed that the animal maintains its normal cycle of activity and hibernation, corresponding to almost a year, even when environmental temperature, photoperiod, food, water and cage conditions are kept constant. In addition, subsequent experiments by Pengelley & Fisher (1963) have shown

that the animal will hibernate regardless of the ambient temperature of the surroundings, thus fulfilling all the criteria needed for the existence of an endogenous circannual rhythm. Long term experiments have since been carried out to determine potential zeitgebers in hibernation. From the investigations of Pengelley & Asmundson (1970) it seems that light might be a possible zeitgeber for hibernators, although no explanation is offered for the way in which it acts. Other factors influencing circannual rhythmicity in hibernating animals could be reproductive competence or breeding commitments, which must to some extent be dependent on internal neurological, biochemical and physiological cycles, and changing weather conditions (Heller & Poulson, 1970).

The discovery of a circannual clock in hibernators has been followed by investigations of other animals marked by conspicuous annual changes in physiology and behaviour. Pioneering work in the field of bird migration has been carried out by Gwinner (1967), who performed systematic experiments with willow and wood warblers. Migratory urge in birds was studied in terms of night activity and by moult of feathers, which normally occurs during winter after migration to the wintering areas. Gwinner found that imposed differences on environmental conditions did not profoundly effect the birds basic rhythm of migration and moult and concluded that the warblers rhythm is determined primarily by an endogenous timing mechanism. He also noted that the seasonal timing of the bird's rhythm is dependent upon their date of birth. Birds hatched later in the year display a later migratory urge and moult than those which are born earlier. Similar circannual rhythms in birds with different migratory habits have also been reported by Berthold, Gwinner & Klein (1971) and Berthold (1973). Such a mechanism provides two benefits for survival of a species:-

1) A programmed duration of the migratory urge helps to ensure that the migratory birds will arrive at a suitable habitat under any conditions

and 2) the temporal variation of individual birds migratory seasons spread out migration, so that hazards involved in flight will only involve part of the population.

Due to its adaptive importance, reproduction must occur during appropriate seasons, when the ecological conditions are optimum for survival of the young. A number of recurring external stimuli have been

recognised as zeitgebers for reproductive activity in many environments (Marshall, 1970). Among flutuating factors that surround an animal in the wild, seasonal changes in daylength appear to act as a device for timing the reproductive periodicity. A great literature is devoted to an involvement of photoperiodism in both migration and in annual sexual cycles. The photo-gonadol relationship in birds has been reviewed by Farner (1973), although the actual mechanism by which light synchronises the endogenous cycles is not known.

c. Circannual Variations in metabolism and hormone secretion

Endogenous annual rhythms have to date been documented in a small number of species primarily hibernating mammals and migratory birds.. Consequently, the data concerning circannual changes in metabolism and hormone secretion is almost exclusively linked to annual displays of cyclic behaviour. The endocrine glands have long been thought to play an important role in hibernation and it is now generally agreed that most hibernators show an involution of most endocrine systems in preparation for hibernation. Wenberg & Holland (1973) have described circannual variations in hormone secretion in the woodchuck. They have shown that the steroid hormones of this marmot reach very low levels during the winter months when the animal is in deep sleep. This evidence is consistent with the fact that metabolic processes such as protein and lipid turnover are minimal at this time. (Wenberg & Holland, 1972 (1) & (2)). During the arousal period all steroids of the marmot increase as it enters its reproductive cycle, the levels reaching a peak in early summer. The increase is greatest in oestrogen and glucocorticoids with the androgens showing less of a rise. Urinary excretory levels of adrenaline and noradrenaline also show a similar pattern, excretion rates being lowest early in hibernation and highest just prior to arousal and on into the summer months. The investigators suggest that an increase of adrenaline and noradrenaline would have both calorigenic and circulatory effects that would be beneficial to the animal during the arousal and summer breeding periods, a view which is also supported by Feist (1970), from work on the golden hamster.

An extensive study into seasonal variations in the physiology and biochemistry of the european hedgehog has been carried out by Senturia & Johansson (1974). These workers observed that the body temperature of the hedgehog, as for most hibernators, is at a minimum during the winter

months, while body weight reaches its highest peak in autumn prior to hibernation, decreases during the winter to reach its lowest value in the spring. The glycogen content of the liver and heart are high in winter and low in summer, which might indicate a decreased sympathetic nervous system effect upon metabolism. A seasonal variation was also observed in the noradrenaline concentration of the heart and adrenal glands, the highest concentration being evident during the summer months and mating period which supports the findings of Wenberg & Holland (1973). Metabolic changes in the hedgehog are marked by an increased capability for anaerobic and lipid metabolism in winter. There is also a marked reduction of the blood glucose value during hibernation, which may be accounted for by either decreased glycogenolysis or the lack of food intake during this period. Another low point in blood glucose value was also recorded in the summer months during the active non-hibernating time. The workers conclude that those seasonal cycles shown by the hedgehog are the result of synchronisation of an endogenous circannual rhythm by environmental stimuli and suggest that outputs from the hypothalamus may regulate, to a large degree the seasonal variations in physiology and biochemistry of this animal. In addition it is interesting to note that Senturia & Johansson (1974) measured blood glucose and cholesterol levels in man which showed lowered values during the summer, suggesting that seasonal variations are apparent in other non-hibernating species. Investigations by Fischer & Hommel (1970) have shown that the fasting blood sugar level in rabbits and rats kept under constant laboratory conditions and natural light rhythms also follows systematical seasonal variations. Whereas night active rats display a minimum blood sugar level in summer, the day active rats have a maximum value at the same time of year.

It must be emphasised that mammals are not the only creatures found to display seasonal metabolic and hormonal changes. Hermensen & Jorgensen (1969) have recorded the blood sugar concentration of the male toad (Bufo Bufo). They observed that freshly caught toads exhibit a high blood glucose in summer and a low level in the winter. Moreover blood glucose shows a peak at the breeding time being highest in toads in amplexus. The seasonal variation is however abolished if toads are kept in constant laboratory conditions. The authors suggest, therefore, that this rhythm is not endogenous but depends upon the temperature of the environment and

postulate that growth hormone and ACTH may be especially important in determining the blood glucose levels of the toad. In addition to the investigations on the toad seasonal variations in blood glucose concentration of the common frog have been reported by Mizell (1965).

Of importance to this study is the literature concerning seasonal variations in salt and water transport or the hormones which exert a control over this process. McAfee & Locke (1967) have recorded considerable variation in the effects of the hormone, angiotensin, on sodium transport by frog skin. Their investigations showed that the greatest sensitivity of the tissue to angiotensin is displayed from December to March. During April, the egg laying season, frog skin is insensitive to angiotensin action but this is followed by a steady rise in activity throughout the summer months. Similarly, Crocker (1968) demonstrated that the aldosterone produced increase in mucosal water and sodium transfer across isolated rat jejunum is not obtained at certain times and concluded that some seasonal variation in aldosterone or the closely linked renin-angiotensin system might be involved. It appears that in this preparation sensitivity to aldosterone is greater during the early part of the year when possibly endogenous renin-angiotensin levels and hence aldosterone levels are low. Coviello (1970) has also noticed a seasonal variation in the natriuretic effect of angiotensin on toad skins and postulates that moulting may be responsible for interference in this action of the hormone. Furthermore, a study of the effects of vasopressin on sodium transport by frog skin (Hong, Park, Park & Kim, 1968) revealed a rather characteristic seasonal variation in the hormone's effect, greater sensitivity being reported during the cold season when frogs hibernate. As corresponding seasonal changes were also recorded in the glycogen contents of the liver and muscle Hong et al (1968) suggest that alterations in hormone sensitivity and ability to transport sodium more, be closely linked to the energy store of the tissue.

From the evidence presented it seems that seasonal rhythms and circannual clocks are widespread in the animal world and may be almost as universal as circadian ones. The general adaptive nature of a circannual rhythm is that it allows the organism to anticipate and thus prepare for a future annually occuring environmental condition while at the same time providing flexibility in a cyclic environment which varies from year to year. It enables the organism to intergrate a large number of environ-

mental cues and through phasing the rhythm it responds to conserve energy and ensure reproductive success.

Chapter 11

Materials and Methods

Animals

Throughout this study, male albino Wistar rats weighing approximately 300 grams were used. All rats were provided with a sterilised diet of P.R.D. (Labsure Animal Diets, Ltd.) and tap water for drinking. The animals were maintained in a constant environment at a temperature of 22C$\pm$ 1C and under a constant light cycle, 14 hours light and 10 hours dark.

Chemicals

Angiotensin CIBA "Hypertensin", (Val-5 angiotensin 11 amide) supplied in vials containing 0.5mg. Each vial was dissolved in 0.9g/100 ml. saline to give a concentration of 10^{-5} g/ml., sterilised by millipore filtration and stored as 1ml. batches in sealed, sterilised, siliconised ampoules. It was diluted to the required concentration with isotonic saline, immediately prior to use.

(^{3}H) inulin. Specific activity 60μCi/mg. This radioactive chemical was obtained from the Radiochemical Centre, Amersham. A stock solution was prepared by dissolving the solid in 0.9/100ml. saline to give a solution containing 10μCi/ml. A 0.1ml. aliquot of this solution contained approximately 2.28×10^{6} d.p.m.

Ouabain Sigma Chemical Co.

DL - p fluorophenylalanine Sigma Chemical Co.

Guanosine 3'5' cyclic monophosphate acid sodium salt Sigma Chemical Co.

Standard Sodium and Potassium Solutions for Flame Photometry

Sodium 0.634g Analar grade, dry NaCl were dissolved in distilled water and made up to 500 ml.

Potassium 0.477g Analar grade, dry KCL were dissolved in distilled water and made up to 500 ml.

This gives stock solutions containing 50mg/100ml. of sodium and potassium respectively. The stock solutions can be kept at 0 – 4C for several weeks and are diluted (1ml. to 50ml. with distilled water) prior to use to give 1mg/100ml. working standards.

Krebs Bicarbonate Buffer This was prepared according to the method of Krebs & Henseleit (1932). Glucose was added to a concentration of 500mg/100ml.

Modified Krebs Ringer Phosphate

Solutions required

1.	2.41g $CaCl_2 . 6H_2O$	made up to 100ml. with distilled water to give a 0.11M solution
2.	3.82g $MgSO_4 . 7H_2O$	made up to 100ml. with distilled water to give a 0.154M solution
3.	1.78g $Na_2HPO_4 . 2H_2O$	plus 2ml. 1N HCl made up to 100ml. with distilled water to give a 0.1M phosphate buffer pH.7.4.
4.	1.15g KCL	made up to 100ml. with distilled water to give a 0.154M solution
5.	0.9g NaCl	made up to 100ml. with distilled water to give a 0.154M solution

Solution A (loading buffer)

To prepare solution A the following volumes of the above solutions were added together:

Solution	Volume
1	3ml.
2	1ml.
3	4ml.
5	100ml.

The pH of the solution was checked before use and if necessary was adjusted to 7.4 with a 1% solution of tris base (0.1g tris (hydroxymethylaminomethane) in 10ml. distilled water). Glucose was not added

Solution B (unloading buffer, containing potassium)

To prepare solution B the following volumes of the solution were added together:

Solution	Volume
A	48ml.
4	2ml.

Glucose concentration = 10m M.

Methods

Stripped everted sac technique

The everted sac technique of Wilson & Wiseman (1954) as modified by Parsons & Paterson (1965) was used to measure fluid transport across the walls of the colon.

Experimental Procedure

Rats were anaesthetised with intraperitoneal sodium pentobarbitone (Nembutal) at a dose of 100mg./100g body weight. When no "blinking" reflex response was obtained the abdomen was opened by a midline incision and the intestines exposed. A cannula was inserted into the ascending colon near to the caecum colon junction, and oxygenated 0.9% saline perfused through the entire colon in order to remove its contents. The colon was then removed from the animal by cutting across the descending colon close to the anal sphincter and then washed through with more isotonic saline. There was never any evidence of perforation of the intestine as a result of this treatment.

Eversion

A tapered glass rod (30cms. long, 0.6cms. diameter) with a constriction near the tapered end was inserted into the lumen of the gut and a long itudinal incision was made through the muscle, along the whole length of distended colon with a blunt scalpel. The muscle layers were then peeled off gently with the fingers. One end of the colon was tied with a cotton ligature at the constriction in the rod and the rest of the colon was then drawn over the ligature. The ligature was cut and the everted colon placed in oxygenated 0.9% saline.

Lengths of ascending and descending colon (5cms. long) were cut from the everted colon mucosa, the ascending colon being the first 5cms. of tissue measured from the caecum-colon junction and the descending colon being the last 5 cms. adjacent to the anal sphincter.

After gently blotting with a damp piece of filter paper (Whatman No.5), the segments were weighed separately on a torsion balance (W_1). Each segment was tied off at one end by a thread ligature and a second ligature was placed loosely around the other end ready for tying. A blunt needle, attached to a 5ml. syringe was introduced into each "sac" and the loose ligature pulled tight over the needle. 0.5ml. Krebs bicarbonate buffer containing 500mg./100ml. glucose was then injected into each sac (serosal fluid) and as the

needle was withdrawn the ligature was tightly tied. The distended sacs were gently blotted on damp filter paper and weighed. (W_2).

The sacs were placed in separate 100ml. conical flasks containing 10ml. Krebs' bicarbonate buffer with 500mg./100ml. glucose (mucosal fluid). The flasks were stoppered and briefly gassed with 95% O_2/5% CO_2 before incubation in a shaking water bath at 37C and at 80 osc./min for a one hour period. At the end of this time the sacs were removed from the flasks, blotted and reweighed. (W_3).

Control and experimental sacs were prepared alternatively so that experimental conditions would not effect one group more than another.

Calculation of Results

Initial wet weight of colon	W_1
Initial serosal volume (wt.)	$W_2 - W_1$
Increase in serosal volume and gut wall i.e. mucosal transfer (wt.)	$W_3 - W_2$

Expression of Results

Water transfer is expressed as mean mucosal water transfer per hour per gram wet weight of tissue.

Nomenclature

Mucosal Solution.	The solution in the flask bathing the mucosal or luminal surface of the intestine.
Serosal Solution.	The solution inside the sac bathing the serosal surface of the intestine.
Mucosal Fluid Transfer	Volume (wt.) of fluid taken up from the mucosal solution during incubation.
Dry Weight	At the end of the hour's incubation the sac was removed from the Krebs bicarbonate medium, blotted and weighed. The sac was cut open and the serosal fluid drained, the sac was blotted and weighed again. Dry weight of the sac was found by weighing the sac after heating at 105° C to constant weight.

In Vivo Preparation of Jejunum

The method was basically that of Bolton et al (1975).

Operative Procedure

Rats were anaethetised with an intraperitoneal injection of sodium pentobarbitone (100mg./100g body wt.). When no reflex responses were obtained they were tracheotomised and the left common carotid artery and both femoral veins cannulated with PP.25 portex tubing filled with 0.9g/100ml. sodium chloride containing 50 U/ml. heparin (Pularin). The carotid artery cannula was connected to a blood pressure transducer (Bell & Howell, Ltd.) for continous monitoring of the blood pressure on a Servoscribe pen recorder. Isotonic saline, with or without angiotensin, was infused, at a constant rate of 1ml./hr, through the femoral veins, using a Palmer infusion pump.

A mid-line abdominal incision was made through the skin and the muscle layers in order to expose the viscera. The proximal end of the jejunum was located at the ligament of Treitz and ligatured, after which a second ligature was tied approximately 25 cms. distal to the first. The resulting isolated segment of proximal jejunum was cannulated and washed through with isotonic saline at 37C and gently emptied. Finally, two new ligatures were tied around the central region of the isolated segment of the jejunum to give a closed sac, approximately 20 cms. in length. Care was taken with the placing of all ligatures so that the blood supply to the sac of the jejunum was not impaired.

Measurement of Fluid Transport across Rat Jejunum in vivo over Two Consecutive 30 minute periods

A known volume (W_1) (approximately 5ml.) of Krebs' bicarbonate buffer, containing (^3H) inulin (20,000 d.p.m.) as a non-absorbable marker, was injected into the closed sac of the jejunum through a small diameter (26 G) hypodermic needle. The jejunum was gently massaged to mix the contents and at 0 minutes a 0.1 ml. sample (W_2) was withdrawn from the sac through a hypodermic needle. The sac was returned to the abdominal cavity and isotonic saline infused through one of the veins at a rate of 1ml./hr. After 30 minutes, the jejunum sac was again exposed, massaged to mix the contents and a second 0.1ml. sample was removed (W_3). The sac was returned to the abdomen and at the same time the saline infusion was stopped and replaced by an infusion of isotonic saline, with or without angiotensin, at the same rate, through the other femoral vein

for a second 30 minute period.

At the end of 60 minutes, the contents of the sac were mixed and a third 0.1 ml. sample collected (W_4). All the samples removed in this way were weighed to determine their precise volume and then assayed for radioactivity. The sac of jejunum was removed from the animal by cutting the intestine immediately beyond each ligature, damp blotted (Whatman No. 50) and weighed (W_5). The sac was cut longtitudinally, blotted and weighed (W_6) to obtain the volume of the contents. The tissue was stripped of all mesentery, again blotted and reweighed to obtain the weight of the sac. (W_7)

Inulin is a non-absorbable marker so that, following the absorption of fluid from the jejunum sac, there is an increase in inulin concentration and consequently, an increase in concentration of radioactivity. From the volume of fluid in the sac at 0 minutes and the concentration of inulin in the sac at 0, 30 and 60 minutes, fluid absorption over two consecutive 30 minute periods was calculated.

A second method for assessing fluid transport was routinely carried out as a check on the measurement of fluid transport by the inulin method. Fluid transport over the whole 60 minute period was calculated from the volume of the contents of the jejunum sac at 0 and 60 minutes and the total volume of all samples removed from the sac during the experiment.

Assay of (^{3}H) inulin samples

Each 0.1ml. sample of the sac contents was placed in a counting vial together with 10ml. scintillation fluid (1/1 V/V methanol/toluene containing 8g/litre butyl PBD). The samples were counted in a Phillips automatic scintillation counter and corrected for background, quenching and counting efficiency. Results were expressed as disintegrations per minute (d.p.m.).

Calculation of Results

1. Measurement of fluid transport by (^{3}H) inulin method

Since inulin is a non-absorbable marker:

Total d.p.m. injected into sac at 0 min. = Total d.p.m. in sac at 60 min − Total d.p.m. removed from sac in samples taken at 0 and 30 min.

All d.p.m. values were corrected to d.p.m./0.1ml.

Volume (wt.) fluid in sac at 0 mins = $(W_1 - W_2)$

$\therefore$ Fluid transport in first 30 min period

$$= \frac{(W_1 - W_2) - \left((W_1 - W_2) \times \frac{\text{d.p.m. 0 min}}{\text{d.p.m. 30 min}} \right)}{W_7}$$

Let this = a

Results expressed as ml./30min/g wet weight of tissue.

Volume (wt.) fluid in the sac at 30 min = $(W_1 - W_2) - (a + W_3)$

$\therefore$ Fluid transport in second 30 min period

$$= \frac{\left[(W_1 - W_2) - (a + W_3) \right] - \left[\left((W_1 - W_2) - (a + W_3) \right) \times \frac{\text{d.p.m. 30 min}}{\text{d.p.m. 60 min}} \right]}{W_7}$$

Results were expressed as ml./30min/g wet weight of tissue

These calculations were computed by a Hewlett Packard computer

2. Measurement of fluid transport by weight method

Fluid transport over 60 min.

$$= \frac{W_1 - \left((W_2 + W_3 + W_4) + (W_5 - W_6) \right)}{W_7}$$

Results were expressed as ml./60min/g wet weight of tissue.

Histological Methods

Samples of jejunum and descending colon were taken from untreated animals. Small portions (5 – 7mm long) were placed immediately into either 10% buffered formalin or Carnoy's rapid fixative. After processing and clearing the samples were impregnated and embedded in wax. Transverse sections, 6 μ thick were cut using a 'Cambridge Rocking Microtome', stained in Ehrlich's haematoxylin and counterstained in eosin.

Nephrectomy and Adrenalectomy

Surgical Procedure

Bilaterally nephrectomised and adrenalectomised rats were used in much of this study.

All instruments were sterilised by immersion in a 0.5% solution of "Hibitane" in ethanol.

The animals were anaesthetised by inhalation of an ether-oxygen mixture using a small face mask and a Boyles apparatus, after which they were placed in a prone position and the fur removed from either side of the spine just below the ribs. The skin was washed with 0.05% "Hibitane", an incision was made on one side and the skin retracted to reveal the muscle of the body wall. The wall was opened by blunt dissection using forceps, and the kidney together with its attached adrenal displayed.

The adrenal and peri-adrenal fat were first removed with forceps (the adrenal blood vessels were not ligatured). The kidney was cleared of fat and a ligature tied around the renal artery, vein and ureter. A cut was made above the ligature and the kidney removed through the hole in the body wall. The muscle wall and skin were closed using linen thread and a curved triangular needle. The contralateral kidney and adrenal were removed through a second incision in a similar manner.

The animals were allowed to recover slowly and the supply of ether reduced while the second incision was being closed. After recovery from the anaesthetic, the animals were placed in large clean cages in a warm room ($22^{\circ}C$), with food and water provided. They were used 24 or 48 hours after the operation as indicated in the text.

Kidney Cortex Slice Technique

This method is described in detail by Poat & Munday (1971).

Male, Wistar rats weighing 300 grams were stunned by a blow on the head and killed by opening the thorax. The abdomen was opened by a midline incision, the kidneys then removed and quickly placed in ice cold isotonic saline.

After removal of the outlying perirenal fat, the kidney was placed upon an inverted glass Petri dish filled with crushed ice, and slices of kidney cortex were cut freehand using a safety razor blade. Outermost slices were rejected and the successful slices, that is, those cut with a single stroke and which curled when lifted with forceps, were rinsed in distilled water, gently blotted on damp tissue and placed in a 50ml. conical flask containing 30ml. of the sodium loading buffer (i.e. modified Krebs' phosphate buffer - solution A). The flask containing the slices was flushed with oxygen-free nitrogen, stoppered with a rubber bung and slowly shaken in a water bath for 12 to 15 minutes at 37C. During this anaerobic phase, sodium pump activity is inhibited and therefore sodium is passively taken up into the slices and potassium lost from the slices. After this first incubation the slices were removed from the medium, washed in distilled water and gently blotted.

Slices selected for ion measurement at this stage were placed in pre-weighed 5ml. conical flasks (approximately 50mg. wet weight of tissue or two slices) and dried to constant weight at 105C. The remaining slices were placed in pairs into 50ml. conical flasks containing 10ml. modified Krebs' phosphate buffer, with or without potassium, according to the experiment carried out, and containing 10mM glucose (solution B). There then followed an incubatory period in a shaking water bath at 25^{o}C and for a period of 10 minutes unless otherwise stated in the text. Under this aerobic phase sodium pump activity is reinstated and sodium is actively removed from the slices and potassium taken up. After the second incubation the slices were removed, washed and blotted. Two slices of the tissue were placed into pre-weighed 5ml. conical flasks and dried to constant weight at 105C.

Analytical Procedure

The slices were digested by warming in the presence of two or three drops of concentrated nitric acid taking care to ensure that spitting did not occur.

The residue was extracted thoroughly with hot, distilled water and made

up to 10ml. in a stoppered graduated test tube.

The sodium and potassium ion contents of the extracts were measured by flame photometry using 1mg/100ml. standards to set full scale deflection.

Calculation of Results

The results are expressed as μ equiv/g dry wt.

$$\underbrace{\frac{\mu g/100ml.}{\text{volume correction}}}_{} \times \underbrace{\frac{1000}{\text{dry wt. in mg.}}}_{} \times \underbrace{\frac{1}{\text{E.W.}}}_{} = \underbrace{\mu \text{ equiv/g dry wt.}}_{}$$

where:-

E.W. is the equivalent weight. $Na^+ = 23$, $K^+ = 39$

μg/100ml. of ion is the flame photometer reading multiplied by 10. Volume correction in this case is 10, as the digested kidney slices were made up to 10ml. with distilled water.

Determination of Extracellular Spaces in Slices during the Sodium Loading and Potassium Depletion Stages

Slices were placed in a 50ml. conical flask containing 28.5ml. of the sodium loading buffer (i.e. solution A) and 1.5ml. of the stock solution of (^{3}H) inulin (0.1ml. stock solution = 2.28 x 10^6 d.p.m.). Duplicate 0.1ml. samples of the starting medium were extracted from the flask. The flask containing the slices was flushed with oxygen-free nitrogen, stoppered with a rubber bung and slowly shaken in a water bath for 12 to 15 minutes. After this incubation the slices were removed from the medium, gently blotted with tissue paper to remove surface moisture and accurately weighed. Samples of 2 or 3 slices, weighing 50 - 60mg. were placed, using a glass rod at the bottom of a glass test tube and o.5ml. of hyamine hydroxide was added to each tube. The tubes were covered with aluminium foil, thoroughly mixed and placed in a 60C water bath with frequent mixing until the tissue was completely digested (3 to 4 hours). The inulin content of the tissue was then measured by liquid scintillation counting.

Determination of Extracellular Spaces in Sodium Loaded, Potassium Depleted Slices during subsequent incubation with or without Angiotensin

Slices of rat kidney cortex were loaded with sodium and depleted of of potassium as described previously. After the first incubation the slices were transferred to 10ml. conical flasks containing 9.5ml. modified Krebs' phosphate buffer, without potassium, but containing glucose (10mM) and 0.5ml. of the stock solution of (^{3}H) inulin. Duplicate 0.1ml. samples of the starting medium were extracted from each flask. One of the flasks remained as a control, while angiotensin was added to the remaining flasks in varying concentrations. Duplicate 0.1ml. samples of the starting medium were extracted from each flask. The flasks were flushed with oxygen, stoppered and incubated for a 10 minute period. After incubation the slices were removed from the medium, gently blotted to remove surface moisture and accurately weighed. Samples of 2 or 3 slices, weighing 50-60mg. were dissolved in hyamine hydroxide and counted.

Estimation of Inulin Content and Counting Technique Used

Each 0.1ml. sample of starting medium was placed in a counting vial with 10ml. scintillation fluid (1/1 V/V methanol) toluene containing 8g/litre butyl PBD). The digested kidney slice extracts were washed into separate

counting vials with 10ml. scintillation fluid. 0.1ml. glacial acetic acid was added to each vial to inhibit phosphorescence. The samples were counted in a Phillips automatic scintillation counter and corrected for background, quenching and counting efficiency. Results were expressed as disintegrations per minute (d.p.m.)

Calculations of Results

As inulin equilibriates with the extracellular spaces, the total d.p.m. in the kidney slice extracts is a direct measurement of the extracellular space of the tissue.

$$\therefore \text{Extracellular space } (ml\text{'}100g \text{ tissue}) = \frac{\dfrac{\text{d.p.m. of kidney slice extract}}{\text{wt. of kidney slice sample (g)}}}{\text{d.p.m./ml. starting medium}} \times 100$$

Statistical Evaluation of the Results

a. Unless otherwise stated, 't' was obtained from the Students' Unpaired 't' test.

The standard error of the mean of each set of results was calculated from the following:-

$$s^2 = \frac{\bar{D}^2}{n-1}$$

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

where D^2 is the sum of the squares of the deviation from the mean, n is a number of observations, S.E.M. is the standard error of the mean and s is the standard deviation of the mean.

The value of the Students 't' is then given as:-

$$t = \frac{(x_1 - x_2)}{\sqrt{(S.E.M._1)^2 + (S.E.M._2)^2}}$$

Where x_1 and x_2 are the means of the two groups and $S.E.M._1$ and $S.E.M._2$ the respective standard errors of these means.

The value for the probability, p, was obtained from the appropriate tables.

The mean, S.E.M. and Students 't' were computed by a Hewlett Packard computer.

b. In investigations into the stimulation of sodium extrusion from rat kidney cortex slices by cyclic GMP, it was found that although the compound always gave an effect, the absolute value of the results varied from day to day and from animal to animal and masked response, when analysed by a Students 't' test. Therefore a second test based upon the analysis of variance was used to determine the significance of these results. In such a test, the total variance in a set of experiments is reduced to individual components, such as variance from animal to animal etc. and their relative

importances to the effect on the experimental condition are assessed. In an analysis of variance the sum of the squares, the number of degrees of freedom and the variance ratio must be computed for each source of variability in turn.

The analysis can be illustrated by the following hypothetical example in which the total variation, the variation due to the experimental condition, the variation with time and the variation within each group (error) may be ascertained.

Example

	Control (C)	Experiment (E)
Day 1	120	145
	110	155
	122	148
Day 2	77	109
	86	116
	81	112

The Total Variation

From the observation table $\leq x = 1381$ where x is each observation. A correction factor (CF) is calculated for, in this one does not work with mean values.

$$CF = \frac{(\leq x)^2}{N} = \frac{1907161}{12} = 158930$$

when N = total number of observations

Total sum of squares $= \leq x^2 - CF = 166105 - 158930$

$= 7175$

Variation due to Experimental Condition

Σx for columns C and E is found

Group C $\Sigma x = 596$

Group E $\Sigma x = 785$

$$\text{Total sum of squares} = \frac{y^2}{n} - CF = 2977$$

where y is the Σx of this group and n is the number of observations used to obtain each value of y.

Variation with time

Σx for Day 1 and Day 2 is found

Day 1 $\Sigma x = 800$

Day 2 $\Sigma x = 581$

$$\text{Total sum of squares} = \frac{y^2}{n} - CF = 3997$$

where y is the Σx in this group and n is the number of observations used to obtain each value of y.

From the above data the following table can be set up :-

Component	Total Sum of Squares (ss)	N	Variance (ss/N)	Variance Ratio (f ratio)
Experimental Condition	2977	1	2977	133.3
Time	3997	1	3977	178.1
Error	201	9	22.33	1
Total	7175	11		

The experimental error (within sample sum of squares) together with N, the number of degrees of freedom for the error, are calculated by difference.

For other components N = number of observations − 1

The variance ratio 'f' is the variance estimate of the component divided by the smallest variance.

Hence the variance ratio due to the experimental condition is 133.3 ($^{N_1}/_{N_2} = 1/9$). The probability, p, can be obtained from the appropriate tables.

In cases where an uneven number of observations are made in each group the test can be modified (Kirk,1971).

The results obtained by this test were completed by a Hewlett Packard computer.

Chapter 111

Results

Section 1

The Actions of Angiotensin on the Colon of the Rat

Fluid Transport Across Sacs of Stripped Colon taken from Untreated Animals

Experiments were carried out to determine the rate of fluid tranfer across the rat colon. Angiotensin is a potent constrictor of intestinal musculature so that it is advisable to remove the colonic muscle before investigating the effects of the hormone on fluid transfer and thus eliminate possible angiotensin induced pressure changes which may influence fluid transport. Hence everted sacs of stripped and descending colon were prepared as described in the previous chapter and incubated in Krebs' bicarbonate buffer for a one hour period at 37C. The results in Table 1 show the rates of mucosal fluid transfer obtained in sacs of stripped ascending and descending colon. Ascending colon gives a rate of 0.68 — 0.04 and descending colon gives a rate of 1.20 $\pm$ 0.04 ml./hr./g wet wt. tissue. These values are similar to those obtained by Davies et al (1970) using a similar preparation.

The effect of Angiotensin on Stripped Colonic Sacs taken from Untreated and Pretreated Animals

Crocker & Munday (1969) sensitised rats to angiotensin by adrenalectomy and nephrectomy 48 hours previously while Davies et al (1970) used rats which were adrenalectomised and nephrectomised 24 hours prior to experimentation. The following experiments were carried out to determine the more suitable pre-treatment of animals, which will give a large and reproducible response to angiotensin.

Initially everted sacs of stripped ascending and descending colon were prepared from untreated rats and incubated with angiotensin (10^{-11}M) in the serosal compartment only. The results in Table 2 show that there is no significant difference in the rate of mucosal fluid transfer in ascending colon incubated with or without angiotensin. There is however a small significant increase in fluid transport across sacs of stripped descending colon incubated with the hormone (27% increase over the control value).

Although 24 hour nephrectomy and adrenalectomy appeared to slightly lower the basal fluid transport rate in stripped everted sacs of descending colon, this decrease was non-significant (Table 3). Angiotensin at a concentration of 10^{-11}M significantly stimulated fluid transport by stripped descending colonic mucosa when applied to the serosal compartment of everted sacs taken from such animals (Table 3), a percentage increase of 47% being recorded.

However, when nephrectomy and adrenalectomy were carried out 48 hours

Table 1

Fluid transport across everted sacs of stripped ascending and descending colon taken from untreated animals

	Fluid Transport (ml./hr./g wet weight of tissue)		
Ascending colon	0.68 ± 0.04	(26)	
			$P < 0.001$
Descending colon	1.20 ± 0.04	(26)	

Results expressed as Means $\pm$ S.E.M.

Number of animals in parentheses.

Table 2

The effect of angiotensin (10^{-11}M) added to the serosal compartment only, on fluid transfer across everted sacs of stripped ascending and descending colon taken from untreated animals.

Sacs of ascending colon	Mucosal Fluid Transfer (ml./hr./g wet wt. tissue)		
Control	0.52 ± 0.02	(5)	N.5
Angiotensin	0.53 ± 0.08	(5)	

Sacs of descending colon	Mucosal Fluid Transfer (ml./hr./g wet wt. tissue)		
Control	1.11 ± 0.10	(9)	$P < 0.05$
Angiotensin	1.41 ± 0.16	(8)	

Results expressed as Means ± S.E.M.

Numbers of animals in parentheses.

Table 3

The effect of angiotensin (10^{-11}M) added to the serosal compartment only, on fluid transfer across everted sacs of stripped descending colon taken from animals which had been nephrectomised and adrenalectomised 24 hours prior to the experiment

	Mucosal Fluid Transfer (ml./hr./ g wet wt. tissue)		
Control	1.11 ± 0.10	(9)	
			N.S.
NephX/AdrX	0.83 ± 0.12	(9)	
			P < 0.02
NephX/AdrX + angiotensin	1.22 ± 0.09	(8)	

Results expressed as Means ± S.E.M.

Numbers of animals in parentheses.

prior to the experiment basal fluid transport was significantly decreased across sacs of both ascending and descending colon, 58% and 42% decreases being recorded respectively (Table 4). These figures compare favourably with the percentage reduction in fluid transport across descending colon taken from 48 hour adrenalectomised and nephrectomised recorded by Davies (1973). As the wet weight of the everted sacs was unaltered by this surgical procedure, this could not account for the reduction in fluid transport measured. Angiotensin (10^{-11}M) added to the serosal compartment of everted sacs of descending colon taken from rats which had been pretreated 48 hours previously was found to stimulate fluid transport by 106% above the control nephrectomised and adrenalectomised value. These results are in good agreement with those of Davies et al (1970), who also recorded an angiotensin stimulation of fluid transport in descending colon under similar conditions. Although an increase in fluid transport (59%) was also recorded when angiotensin was added to the serosal compartment of sacs of ascending colon, this increase was not significant. Thus in this series of experiments the descending colon appears to be more sensitive to angiotensin than the ascending colon of the rat and sensitivity increases with adrenalectomy and nephrectomy. The duration of the period following nephrectomy and adrenalectomy is also important since it has been demonstrated that the response to angiotensin is greater after 48 hours than after 24 hours following the surgical procedure. Furthermore, the rat descending colon appears to be sensitive to angiotensin in agreement with the observations of Davies et al (1970) while the ascending colon does not respond which contrasts with the findings of Hornych et al (1973)

Table 4

The effect of angiotensin (10^{-11}M) added to the serosal compartment only, on fluid transfer across everted sacs of stripped ascending and descending colon taken from animals which had been nephrectomised and adrenalectomised 48 hours prior to the experiment

Sacs of ascending colon	Mucosal Fluid Transfer (ml./hr./g wet wt. tissue)		
Control	0.52 ± 0.02	(5)	
			$P < 0.001$
NephX/AdrX 48 hours	0.22 ± 0.05	(6)	
			N.S.
NephX/AdrX 48 hours + angiotensin	0.35 ± 0.18	(6)	

Sacs of descending colon	Mucosal Fluid Transfer (ml./hr./g wet wt. tissue)		
Control	1.12 ± 0.10	(9)	
			$P < 0.01$
NephX/AdrX 48 hours	0.65 ± 0.10	(6)	
			$P < 0.001$
NephX/AdrX 48 hours + angiotensin	1.34 ± 0.04	(6)	

Results expressed as Means $\pm$ S.E.M.

Numbers of animals in parentheses.

Section 11

Seasonal Variation in Fluid Transport and Sensitivity to Angiotensin

In physiological concentrations angiotensin has been shown to stimulate fluid transfer across the rat descending colon *in vitro* (Davies et al, 1970) and the rat jejunum *in vivo* (Bolton *et al*, 1974), although the magnitude of the angiotensin stimulation in these two intestinal preparations appears to be variable. In view of these observations and the findings of McAfee & Locke (1967) on frog skin that the angiotensin response might be susceptible to seasonal variation, it was decided to investigate the rate of fluid transport by both the descending colon *in vitro* and the jejunum *in vivo* and to monitor the sensitivity of these tissues to angiotensin. During this set of experiments the rats were kept under constant conditions of temperature and in a predominated cycle of 14 hours light and 10 hours darkness.

Seasonal Variation on the Rate of Fluid Absorption across Rat Descending Colon *in vitro* and Rat Jejunum *in vivo*

To obtain rates of basal fluid transport in the *in vitro* colon, everted sacs of stripped descending colon were prepared from untreated rats and transport measured over a 1 hour period. Control transport in the *in vivo* rat jejunum was measured over two consecutive 30 minute periods.

Measurements of fluid transport in the *in vitro* colon are depicted on a monthly basis in Figure 3a, and the mean and standard error of the mean for each month are shown in Table 5. For most of the year fluid transfer across the descending colon was relatively high, a mean of 1.30ml./hr./g wet weight tissue being representative of this. However during August and September a significant fall in colonic transport rate was recorded. A low mean 0.78ml./hr./g wet weight of tissue being measured (40% decrease).

Table 5 and figure 3B depict fluid transport over 12 months in the *in vivo* jejunum. Once again the rate for a large part of the year was fairly high and constant. A mean value of 1.65 ml/hr./g wet weight of tissue was recorded. In the jejunum *in vivo* a stepwise decrease in the rate of fluid transport was observed. Fluid transport began to fall in August and by November had reached a significantly low value of 0.64 ml./hr./g wet weight of tissue (61% decrease).

This this study shows that the rate of fluid transport in the colon and the jejunum fluctuates in a similar manner throughout the year. Identical traits might not be expected since the *in vivo* studies were carried out in 1973/74 and the *in vitro* studies 1974/75.

Table 5 Fluid transport measured across the rat jejunum in vivo and the descending colon in vitro over a period of a year.

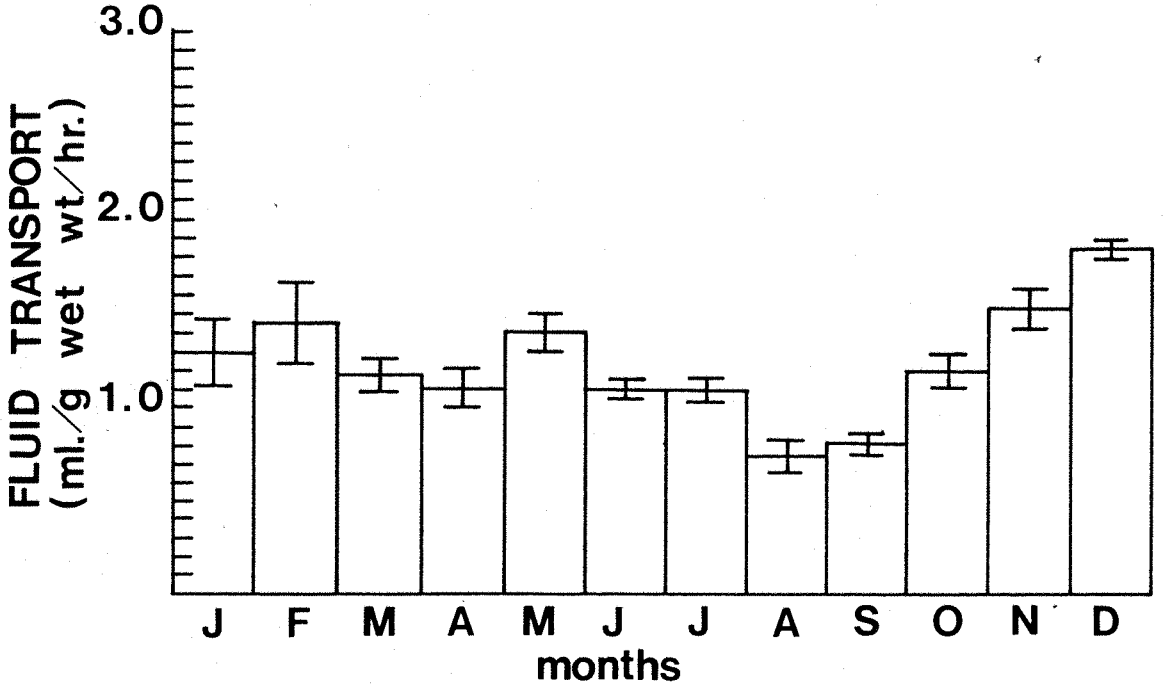
Jejunum (transport measured in ml./hr./g wet wt. of tissue)

Month	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Means	1.68	1.66	2.24	1.53	1.64	1.58	1.40	1.16	1.08	0.72	0.64	1.48
S.E.M.	0.06	0.21	0.12	0.14	0.20	0.18	0.05	0.11	0.11	0.21	0.10	0,12
No.	2	4	8	7	11	11	12	16	14	6	8	4

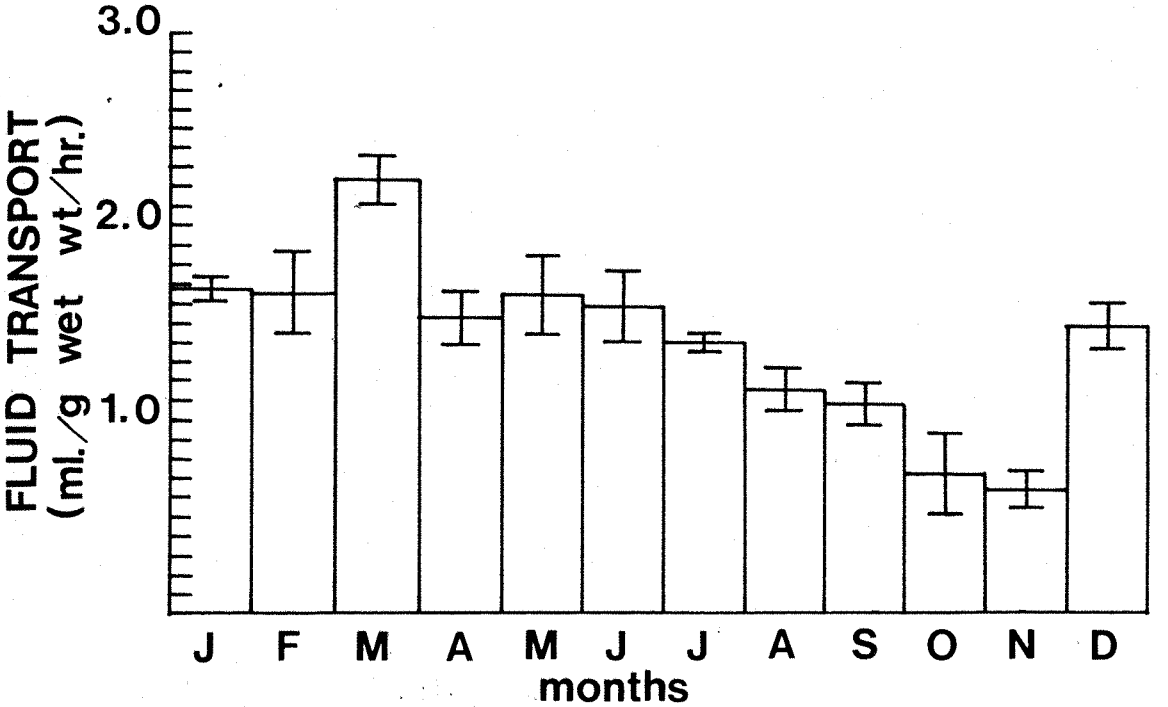
Descending Colon (transport measured in ml./hr./g wet wt. of tissue)

Month	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Means	1.29	1.44	1.17	1.10	1.40	1.10	1.10	0.74	0.81	1.20	1.52	1.84
S.E.M.	0.17	0.22	0.09	0.10	0.10	0.05	0.06	0.09	0.06	0.09	0.11	0.05
No.	4	5	8	9	6	6	5	6	13	12	5	4

Fig. 3 RATE OF FLUID ABSORPTION THROUGHOUT A YEAR



A. DISTAL COLON IN VITRO



B. JEJUNUM IN VIVO

The Effect of Season on the Fluid Transport Response of Isolated Colon and In Situ Jejunum to Angiotensin

Table 6 and Figure 4 show the results of fluid transport recorded in descending colonic sacs, taken from animals which had been sensitised by 48 hour nephrectomy and adrenalectomy, and incubated either with or without angiotensin. Nephrectomy and adrenalectomy reduces the rate of fluid transport in the descending colon to approximately 50% of the value recorded in untreated animals. Angiotensin (10^{-11}M) increases transport rate back towards a normal untreated value.

For the major part of the year, angiotensin stimulated the rate of fluid transport by a mean value of 99 $\pm$ 9% above the control nephrectomised and adrenalectomised value. But during August and September the stimulation produced by the hormone fell to only 44 $\pm$ 11%, a significant decrease. Thus at the time of year when the rate of fluid transport in untreated animals is low, the response to angiotensin is impaired.

Table 7 and Figure 5 show the monthly results of fluid absorption measured in the in vivo jejunum during two consecutive 30 minute periods. During the first 30 minutes of the experiment isotonic saline was infused at a rate of 1ml./hr. into one of the femoral veins. In the second 30 minutes this infusion was stopped and isotonic saline plus angiotensin was then infused at the same rate into the other femoral vein of the rat. The first period thus acts as a control for the second test period. As in the in vitro colon, angiotensin stimulated fluid absorption in the in vivo jejunum by 71 $\pm$ 8%, when infused at a dose of 0.59 ng/kg/min into the anaesthetised animal. This response, however, was significantly decreased in August and by October there was no detectable stimulation of fluid transfer by the hormone. Thus, in both intestinal preparations sensitivity to angiotensin is reduced or lost in those months in which basal transport is low.

During this comprehensive 7 month study histological techniques were used to investigate the possibility that changing colonic and jejunal structure might explain the low transport phenomenon. However, prepared slides showed no visible alterations in structure during the high or low transporting periods and there was no change in the wet weight of the tissue. These results do not preclude the possibility that structural changes below the resoltion of light microscopy may be responsible for the seasonal changes in intestinal transport, but clearly there are no gross changes in structure.

Table 6

The effect of Season on the fluid transport response of isolated colon to angiotensin (10^{-11}M)

Control Transport (in ml./hr./g wet wt. tissue)

Month	March	April	June	July	Aug.	Sept.
Means	0.64	0.64	0.52	0.46	0.41	0.44
S.E.M.	0.04	0.15	0.10	0.11	0.06	0.02
No. of animals	6	4	5	5	5	4

Angiotensin Stimulated Transport (in ml./hr./g wet wt. tissue)

Month	March	April	June	July	Aug.	Sept.
Means	1.11	1.28	1.11	0.97	0.67	0.64
S.E.M.	0.15	0.05	0.07	0.08	0.09	0.10
No. of animals	6	4	4	5	5	4

Fig. 4 THE EFFECT OF SEASON ON THE RESPONSE OF ISOLATED COLON TO ANGIOTENSIN

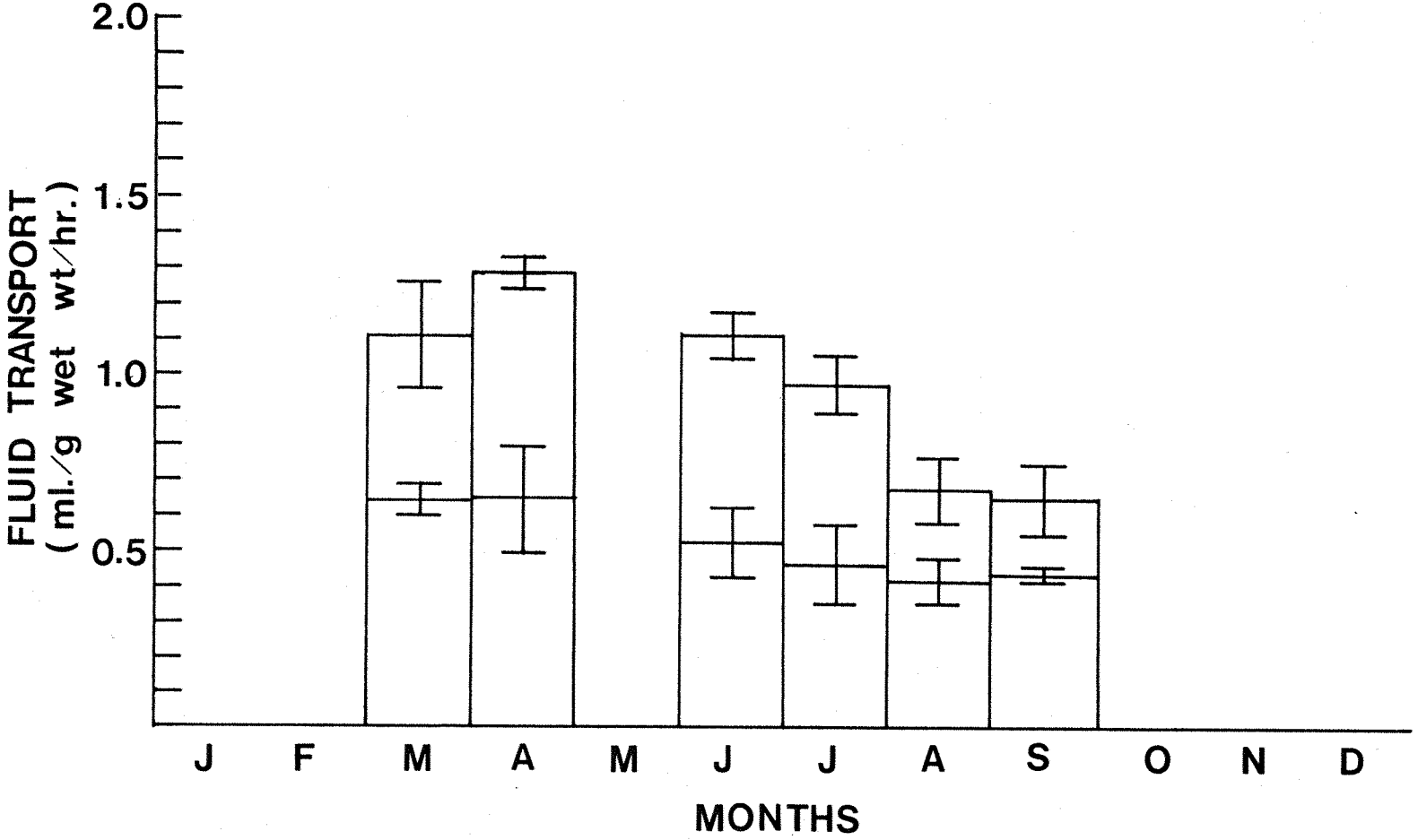


Table 7

The effect of Season on the fluid transport response of *in situ* jejunum to angiotensin

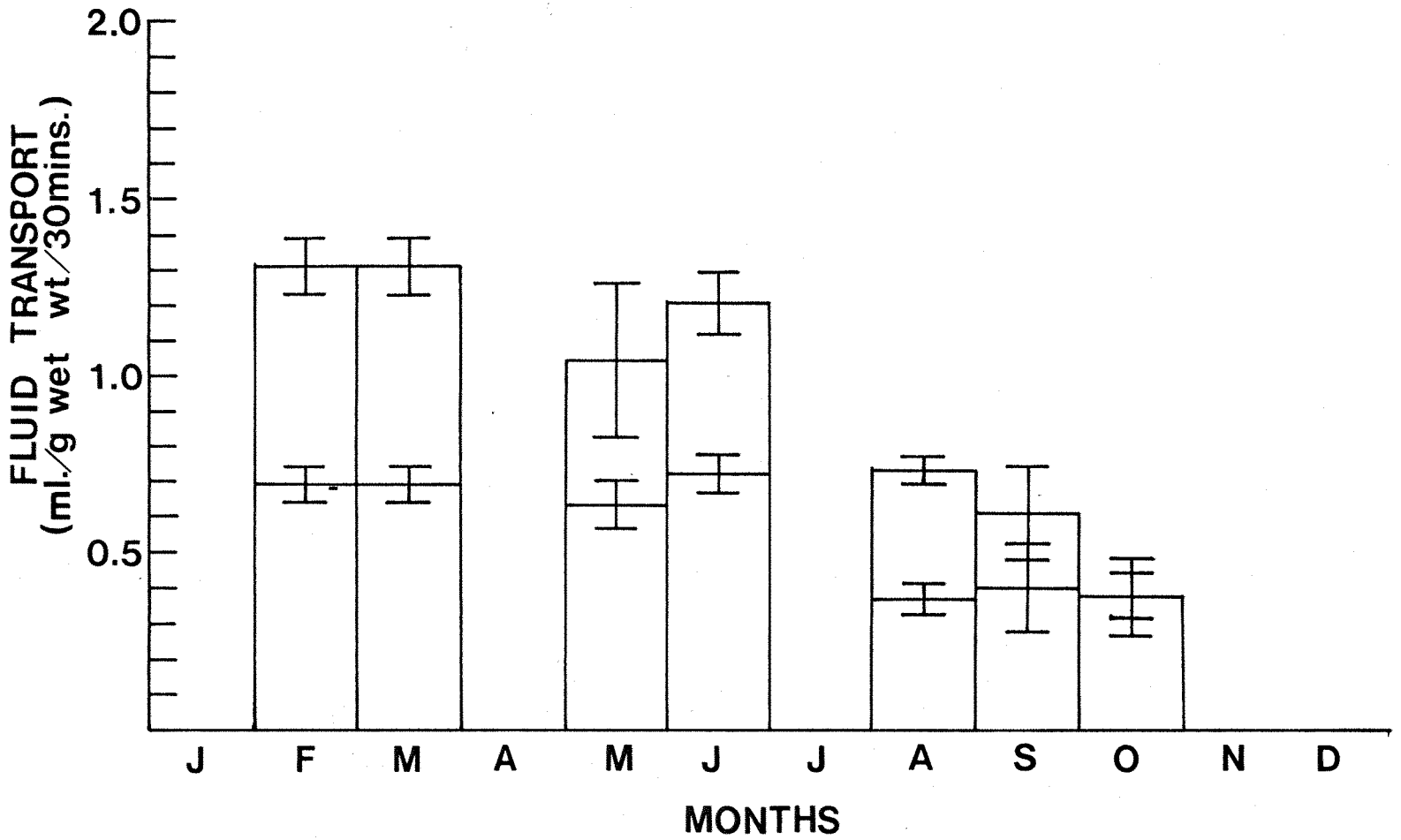
Control Transport (in ml./g wet wt./30 mins)

Month	Feb. - March	May	June	Aug.	Sept.	Oct.
Means	0.69	0.63	0.72	0.37	0.40	0.38
S.E.M.	0.05	0.07	0.06	0.04	0.12	0.07
No. of animals	5	4	3	2	4	4

Angiotensin Stimulated Transport (in ml./g wet wt./30 mins)

Month	Feb. - March	May	June	Aug.	Sept.	Oct.
Means	1.31	1.04	1.21	0.73	0.61	0.37
S.E.M.	0.08	0.22	0.09	0.04	0.13	0.11
No. of animals	5	4	3	2	4	4

Fig. 5 THE EFFECT OF SEASON ON THE RESPONSE OF IN SITU JEJUNUM TO ANGIOTENSIN



Hence concomitant with the reduction in fluid transport during the autumn is a significant decrease in the sensitivity of the two intestinal preparations to physiological concentrations of angiotensin. Considerable variation in the effects of angiotensin stimulated sodium transport across frog skin has been recorded by McAfee & Locke (1967) and Crocker (1968) has also demonstrated a seasonal variation in the aldosterone stimulation of water and sodium transport across *in vitro* rat jejunum, which might also possibly be linked with the renin-angiotensin status of the animal.

As already stated, sexually mature, male 300g rats were used throughout this study. It was evident, however, that the age of the rats ranged from 7 to 10 weeks, some rats maturing to this weight faster than others. Recently, Levens (personal communication) has carried out a series of experiments in which a comparison was made bwteen the rate of fluid transport across the *in vivo* jejunum of 250 → 300g and 400 → 450g male rats. It was found that there was no significant difference in either basal fluid transfer rate across the jejunum or the sensitivity of the intestine to angiotensin in these two groups.

For the reason that age difference might affect the rate of fluid absorption and hence account for a seasonal variation, measurements of fluid transport were made in both the younger 7 week old rats and the older 10 week old rats. Table 8 shows the results obtained. It is clear from the measurements made that there is no significant difference between the transport rate in these two groups. It is evident from these results and the results of Levens (personal communication) that neither the age difference or any slight differences in weight which may be apparent, can account for the seasonal phenomenon.

Table 8

A comparison of basal fluid transport across everted sacs of descending colon taken from 7 and 10 week old rats (all weighing 300g)

	Mucosal Fluid Transport (ml./hr./g wet wt. tissue)		
Sacs taken from 7 week old rats	1.48 ± 0.11	(6)	
			N.S.
Sacs taken from 10 week old rats	1.57 ± 0.11	(8)	

Results expressed as Means $\pm$ S.E.M.

Number of animals in parenthesis

Section III

Metabolic Requirements for Fluid Transport and the Angiotensin Stimulated Response

Barry, Matthews, Smyth & Wright (1962) have shown that the potential which exists across the intestine depends not on the mechanism responsible for fluid movement but on the presence of sugars and other solutes which can be transferred against a concentration gradient. Barry, Smyth & Wright (1965) postulate two separate processes concerned in sodium and hexose transport.

1. a hexose pump, which is electrogenic and is concerned with the active transport of sugars. In this situation the measured s.c.c. exceeds the net sodium transfer.

and 2. a neutral pump, which is promoted by hexoses with hexokinase specificity and results in the net sodium transfer being greater than the s.c.c.

The hexose sugar, glucose, has been shown to stimulate both electrogenic and neutral processes, while fructose appears to operate the neutral pump causing net sodium transfer to be greater than s.c.c. (Barry et al, 1965); Binder, Powell & Curran, 1972). With these results and postulations in mind, a series of experiments was carried out to investigate the sugar requirements needed for fluid transport across the descending colon.

The Importance of Glucose for Fluid Transport across the Descending Colon

Everted sacs of stripped descending colon taken from untreated animals were incubated for a one hour period in Krebs' bicarbonate buffer with or without glucose (500mg/100ml.). As shown in Table 9 there is a significant decrease in the rate of fluid absorption across the sacs incubated in the absence of glucose (64% decrease). These results are in keeping with those of many other in vitro workers, who also add glucose to the incubatory medium to sustain an adequate transport rate.

Fructose promotes a neutral pump in the intestine (Binder et al, 1972) and angiotensin has been shown to stimulate fluid transport in the absence of changes in transmural p.d. in the in vivo jejunum (Bolton, Munday, Parsons & York, 1974), the in vivo rat colon (Levens et al, 1975) and the in vitro rat colon (Shaikh, 1972), suggesting the involvement of an electroneutral process. In view of these findings, it was decided to replace glucose with

Table 9

The effect on fluid transport of incubating everted sacs of stripped descending colon in Krebs' bicarbonate buffer either with or without glucose (500mg/100ml.)

	Mucosal fluid transport (ml./hr/g wet wt. tissue)		
Sacs incubated with glucose	1.40 ± 0.10	(6)	
			$P < 0.001$
Sacs incubated without glucose	0.50 ± 0.08	(6)	

Results expressed as Means $\pm$ S.E.M.

Number of animals in parentheses.

fructose in the incubatory medium surrounding everted sacs of rat jejunum and to examine the effect of angiotensin under these conditions, in the expectation that as fructose stimulates a neutral transport mechanism, sensitisation of the animal by prior nephrectomy and adrenalectomy might not be necessary to obtain an angiotensin stimulation of fluid absorption.

The effect on fluid transport of Incubating Everted Sacs of Jejunum with or without Angiotensin in Krebs' Bicarbonate Buffer containing Fructose

In the experiments of Barry et al (1962, 1965) and Binder et al (1972), involving fructose and the neutral transport process, in vitro preparations of small intestine were used. For this reason it was decided to incorporate everted jejunal sacs into this set of experiments. These sacs were prepared in a similar manner to the everted sacs of stripped descending colon, except that the sacs were 15cm long rather than the 5cms length of the everted colonic sac. The jejunal sacs were filled with 1.5ml. Krebs' bicarbonate buffer containing fructose. The prepared everted sacs of jejunum were incubated with or without angiotensin (10^{-11}M) in Krebs' bicarbonate buffer containing fructose (28mM).

The results obtained are depicted in Table 10. From the table it is clear that there is no significant difference between sacs incubated with or without angiotensin. These results show that even in the presence of adequate energy for non-electrogenic sodium transfer, angiotensin is ineffective in in vitro tissue prepared from untreated animals. Consequently, this approach to avoiding the unsatisfactory sensitisation procedure of adrenalectomy and nephrectomy is unsuccessful.

Table 10

The effect on fluid transport of incubating everted sacs of jejunum with or without angiotensin (10^{-11}M) in Krebs' bicarbonate buffer containing fructose (28mM)

	Mucosal fluid transport (ml./hr./g wet wt. tissue)		
Jejunal sac incubated without angiotensin	1.10 ± 0.05	(6)	
			N.S.
Jejunal sac incubated with angiotensin	1.15 ± 0.06	(6)	

Results expressed as Means ± S.E.M.

Number of animals in parentheses.

Section 1V

The Importance of Chloride in Angiotensin Stimulated Transport

Munday et al (1971) have shown that in the kidney cortex slice, angiotensin stimulates loss of sodium and water through a second sodium pump. This pump is refractory to ouabain and is potassium independent (Whittembury, 1968) Work by Bolton, Munday, Parsons & York (1974) and Levens et al (1975) suggests that angiotensin might possibly stimulate fluid movement across the intestine via a second non-electrogenic pump in which sodium and anion movement is tightly coupled, or by stimulating independent sodium and anion transport mechanisms. There is now great interest to the exact nature of the ion linked to the second sodium pump. Whittembury & Proverbio (1970) have presented evidence to suggest that in the kidney cortex slice this might be chloride and have been supported by the replacement studies of Smith (1975). Smith has also indicated that the angiotensin stimulation of the extrusion of sodium from preloaded kidney cortex slices is dependent upon the presence of chloride in the incubatory medium. Experiments have therefore been carried out to determine if chloride is linked to sodium transport in the intestine and to investigate whether or not this anion is required for the angiotensin stimulation of fluid transport across the rat descending colon.

The Importance of Chloride Ions in the Angiotensin Stimulation of Fluid Transport across Descending Colon taken from 48 hour nephrectomised and adrenalectomised rats

Initial experiments were carried out using sacs of descending colon taken from untreated rats. Chloride ions in the Krebs' bicarbonate buffer were replaced completely with sulphate ions and the buffer corrected to its usual osmolarity (300m Osmoles). Everted sacs of descending colon, filled with 0.5ml. sulphate buffer, were incubated in the modified medium for a one hour period at 37Cand fluid transport measured by weight difference. The results are expressed in Table 11. The measurements made show no significant difference in the rate of fluid absorption between sacs incubated in the conventional bicarbonate buffer and the modified buffer containing sulphate. Thus the absence of chloride ions does not under these conditions significantly alter basal transport rate.

The angiotensin stimulation of fluid transport across everted sacs of descending colon, taken from 48 hour nephrectomised and adrenalectomised rats was then checked (Table 12a). A stimulation of 110% was recorded above the control nephrectomised and adrenalectomised value, a significant increase.

Table 11

Fluid transport measurements across everted sacs of stripped descending colon incubated in either Krebs' bicarbonate or a modified Krebs' sulphate buffer

	Mucosal fluid transport (ml./hr./g wet wt. tissue)		
Sacs incubated in bicarbonate buffer	0.97 ± 0.07	(5)	
			N S.
Sacs incubated in sulphate buffer	0.97 ± 0.08	(5)	

Results expressed as Means $\pm$ S.E.M.

Number of animals in parentheses.

Table 12a

The effect on fluid transport of incubating everted sacs stripped descending colon from rats, which had been nephrectomised and adrenalectomised 48 hours previously in Krebs' bicarbonate buffer and either with or without angiotensin (10^{-11}M)

	Mucosal fluid transport (ml./hr./g wet wt. tissue)		
Sacs incubated without angiotensin	0.46 ± 0.12	(5)	
			$P < 0.01$
Sacs incubated with angiotensin	0.97 ± 0.08	(5)	

Results expressed as Means ± S.E.M.

Number of animals in parentheses.

The normal Krebs' bicarbonate buffer was then replaced with the modified sulphate buffer. Everted sacs of descending colon from pretreated animals were filled with 0.5ml. of the buffer with or without angiotensin (10^{-11}M) and were incubated for one hour in the sulphate medium. The results depicted in Table 12b show that the angiotensin stimulation of fluid transport did not occur under these conditions and indicate that the angiotensin stimulated process is dependent upon the presence of chloride in the buffer medium and is consistent with the view that angiotensin stimulates the activity of a sodium chloride linked transport process.

Table 12b

The effect on fluid transport of incubating everted sacs of stripped descending colon from rats which had been nephrectomised and adrenalectomised 48 hours previously in a modified Krebs' sulphate buffer and either with or without angiotensin (10^{-11}M)

	Mucosal fluid transport (ml./hr./g wet wt. tissue)		
Sacs incubated without angiotensin	0.45 ± 0.08	(5)	N.S.
Sacs incubated with angiotensin	0.42 ± 0.04	(6)	

Results expressed as Means ± S.E.M.

Number of animals in parentheses.

Section V

The Action of Angiotensin on the Rat Kidney Cortex Slice

In the in vivo kidney, large infusions of angiotensin have been shown to cause sodium diuresis while low infusions of the hormone cause sodium retention (Barraclough et al, 1967; Bonjour & Malvin, 1969). Using the sodium loaded kidney cortex slice preparation Munday et al (1971) have shown that physiological concentrations of angiotensin can stimulate the activity of a potassium independent, ouabain insensitive sodium pump similar to that proposed by Whittembury (1968).

Time Course of Potassium Uptake and Sodium Extrusion by Rat Kidney Cortex Slices

Munday et al (1971) have also shown that the hormone does not effect the maximum amount of sodium extruded and potassium accumulated by kidney slices, but it alters the rate at which these processes take place. For this reason it is important to establish the time course of potassium uptake and sodium extrusion by the tissue before undertaking any experiments with the hormone, angiotensin.

Prepared kidney slices were loaded with sodium and depleted of potassium by a 12 minute incubation in a glucose-free, potassium-free buffer at 37C under anaerobic conditions. The sodium loaded slices were subsequently incubated aerobically in phosphate buffer, containing both glucose and potassium at 25C. As measurements of the rate of potassium accumulation and sodium extrusion were being made, this second incubation was limited to either 3, 6, 10 or 30 minutes. From Table 13 and Figures 6 and 7 it would appear that in order to observe an effect of angiotensin on the processes of sodium extrusion and potassium uptake, experiments must be carried out for periods of less than 30 minutes. For practical reasons 10 minutes was used as the incubation time in most of the following experiments. It must be noted that in this particular set of time course experiments the amount of sodium extruded was far greater in any subsequent experiments. This has to be explained in terms of unfamiliarity with a new technique.

Whittembury & Proverbio (1970) have proposed the presence of two sodium pumps in the kidney. One pump exchanges sodium for potassium and is inhibited by ouabain and the second pump is insensitive to ouabain and is independent of potassium. The above suggestion has been supported by the work of

Table 13

The effect of time on the amount of potassium taken up and sodium extruded by sodium loaded rat kidney cortex slices incubated at 25C in a medium containing glucose and potassium under aerobic conditions

Time (mins.)	Potassium uptake (μ - equiv/g dry wt.)		Sodium loss (μ-equiv/g dry wt.)	
3	36 ± 3	(5)	110 ± 10	(5)
6	53 ± 4	(5)	234 ± 15	(5)
10	65 ± 6	(5)	295 ± 15	(5)
30	100 ± 9	(5)	320 ± 8	(5)

Results expressed as Means ± S.E.M.

Number of animals in parentheses.

Fig. 6 SODIUM EXTRUSION IN RAT KIDNEY CORTEX SLICES

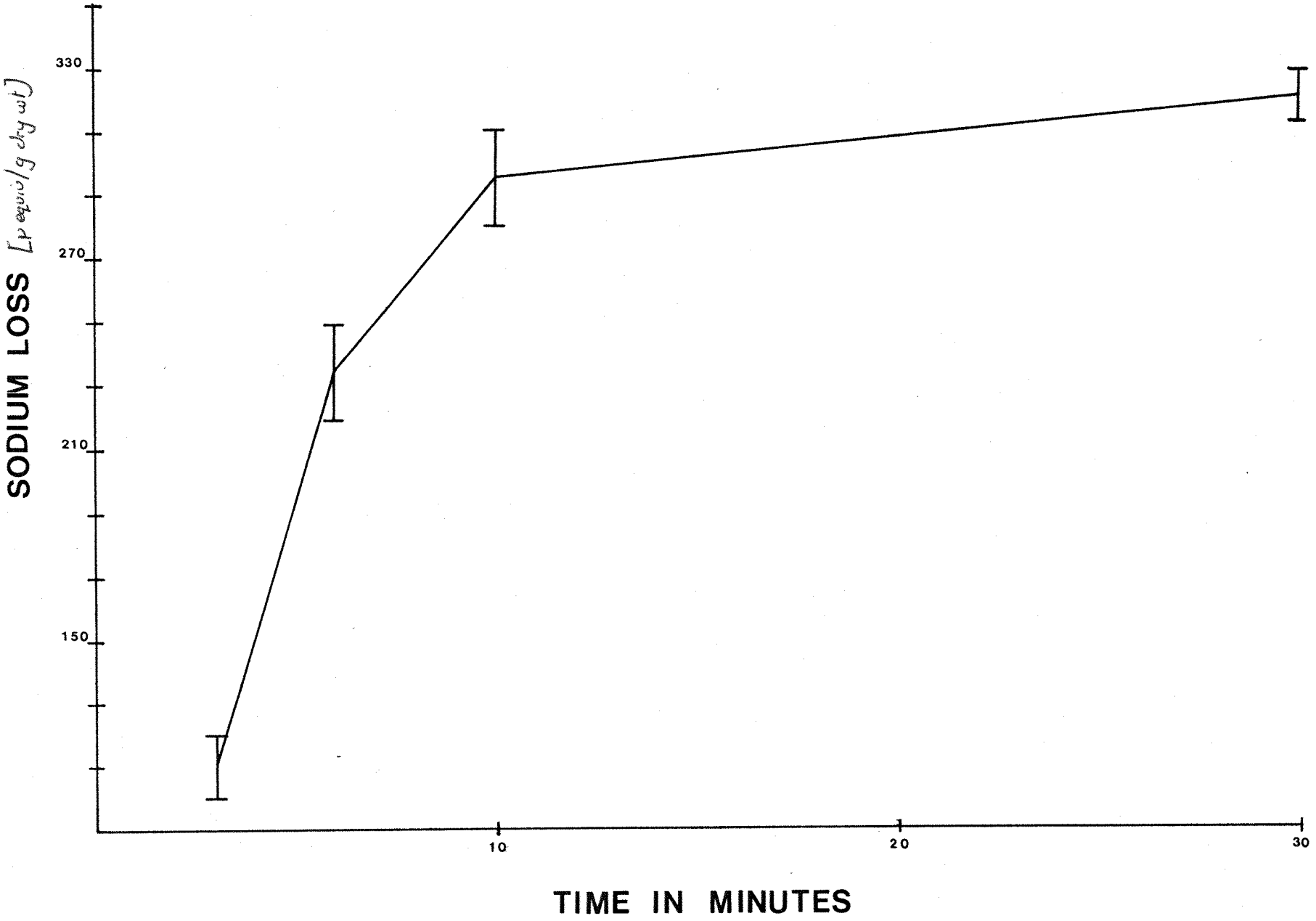
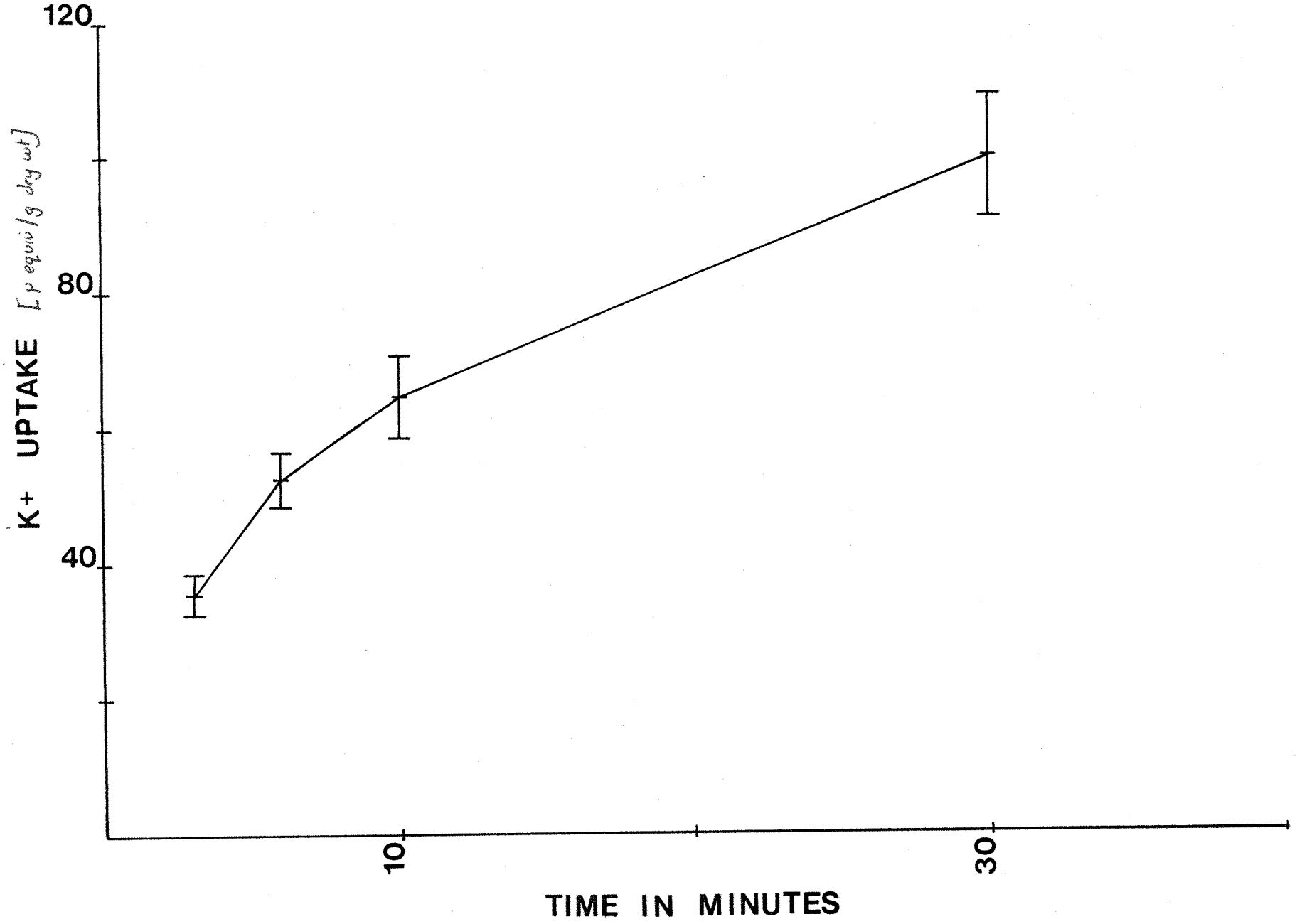


Fig. 7 POTASSIUM UPTAKE IN RAT KIDNEY CORTEX SLICES



Munday et al (1971). Experiments have also been carried out in this thesis to investigate the effect of ouabain on potassium uptake and sodium extrusion by sodium loaded rat kidney cortex slices incubated with potassium and glucose at 25C for 10 minutes under aerobic conditions.

From Table 14 it is clear that potassium uptake and therefore the sodium-potassium exchange pump was completely blocked by the addition of 7mM ouabain to the incubatory medium. Although sodium loss against an electrochemical gradient was reduced from 234 ± 6 to 93 ± 12 µ/equiv/g dry weight tissue, a very significant decrease, it was not completely inhibited by ouabain. This suggests that a second mode of sodium transport is present in the kidney and supports the view of previous investigators.

The effect of Angiotensin on Potassium Uptake and Sodium Loss by Sodium Loaded Rat Kidney Cortex Slices incubated with Potassium in the Medium

From the observations of Munday et al (1971) it is evident, that when sodium loaded rat kidney cortex slices are incubated in the presence of angiotensin (10^{-12} g/ml.) glucose and potassium under aerobic conditions at 25C, there is a stimulation of sodium extrusion and an inhibition of potassium uptake by the slices. This can be explained if the hormone stimulates loss of sodium from the slices by a route which is independent of potassium uptake. Stimulation of this route could inhibit loss of sodium through the sodium/potassium pump and therefore would be seen as an inhibition of the potassium accumulation by the slices. The experiments of Munday et al (1971) were repeated. Sodium loaded slices were incubated aerobically in the presence of glucose, potassium and varying concentrations of angiotensin at 25C for 1o minutes. The results are expressed in Table 15.

Concentrations of 10^{-8} and 10^{-6} g/ml. angiotensin were found to have no significant effect on potassium uptake or sodium loss from the slices. There was no stimulation or inhibition in either of these two processes. These results are difficult to interpret, since a consistent inhibitory action of angiotensin on salt and water transport has been reported in many transporting epithelia, when large doses of the hormone are applied. Leysac, Lassen & Thayson (1961) using a kidney cortex slice preparation reported that high concentrations of angiotensin have a potent inhibitory action on sodium transport by this tissue and in the kidney in vivo large doses of the hormone exert natriuretic/diuretic actions (Barraclough et al, 1967, Bonjour & Malvin, 1969). In addition high concentrations inhibit fluid transport across

Table 14

The effect of ouabain (7mM) on potassium uptake and sodium loss by sodium loaded rat kidney cortex slices incubated with potassium and glucose under aerobic conditions

	Potassium uptake (μ - equiv/g dry wt./10 mins.)	Sodium loss (μ - equiv/g dry wt/10 mins.)
Control	107 ± 11 (4)	234 ± 6 (4)
Ouabain	-21 ± 5 (4)	93 ± 12 (4)
	$P < 0.001$	$P < 0.001$

Numbers of observations in parentheses.

Significance is expressed as difference between the control and experimental condition.

Results expressed as Means ± S.E.M.

Table 15

The effect of different concentrations of angiotensin on potassium uptake and sodium loss by sodium loaded rat kidney cortex slices incubated at 25C for 10 mins in the presence of glucose and potassium under aerobic conditions

Angiotensin concentration (g/ml.)	Potassium uptake (μ -equiv/g dry wt.)	Sodium loss (μ - equiv/g dry wt.)
None	67 ± 5 (4)	58 ± 14 (4)
10^{-12}	40 ± 3 (4)	154 ± 20 (4)
	P < 0.01	P < 0.01
10^{-8}	76 ± 6 (4)	75 ± 7 (4)
	N.S.	N.S.
10^{-6}	62 ± 6 (4)	70 ± 14 (4)
	N.S.	N.S.

Numbers of observations in parentheses.

Significance is expressed as difference between the control and each experimental condition.

Results expressed as Means ± S.E.M.

the in vitro rat colon. (Davies et al, 1970) and the in vivo rat jejunum (Bolton et al, 1974).

In the present study when the kidney cortex slice experiment was carried out in the presence of angiotensin, 10^{-12} g/ml., in the incubatory medium, a significant decrease in potassium uptake, from 67 ± 5 to 40 ± 3 μ equiv/g dry weight of tissue was recorded. Conversely a significant stimulation of sodium loss from 58 ± 4 to 154 ± 20 μ equiv/g dry weight of tissue was measured. The results at this physiological concentration of angiotensin agree favourably with the observations and suggestions of Munday et al (1971).

The effect of Angiotensin on Potassium uptake and Sodium loss by Sodium Loaded Rat Kidney Cortex Slices incubated in the absence of Potassium

As Whittembury (1968) has demonstrated a second mechanism for sodium extrusion in guinea pig kidney cortex tissue and Munday et al (1971) have shown that angiotensin stimulates sodium extrusion through this second sodium pump, experiments were carried out to investigate the effect of angiotensin on sodium loss from rat kidney cortex slices in conditions where the sodium-potassium exchange pump was repressed. The sodium-potassium pump has been shown to be sensitive to ouabain and dependent upon potassium. Therefore it is possible to inhibit the mechanism by the addition of ouabain to, or the removal of potassium from the incubation medium.

Sodium-loaded potassium depleted slices were incubated with varying concentrations of angiotensin in a medium containing glucose but in the absence of potassium, The results depicted in Table 16 show that angiotensin 10^{-8} and 10^{-6} g/ml., once again have no significant effect on either potassium uptake or sodium extrusion. However angiotensin 10^{-12} g/ml. when added to the incubation medium, greatly stimulates the rate of sodium loss from the kidney slices. Although no potassium is present in the medium there is a significantly increased loss of potassium from the slices in the presence of this dose of angiotensin. This cannot be explained at present but may be due to a change in the passive permeability of the membrane, and the possible changes in passive permeability following the administration of angiotensin might be well worthwhile investigating.

Extracellular Space

The effects of angiotensin on potassium uptake and sodium loss may have been due to a redistribution of fluid between extracellular and intracellular

Table 16

The effect of different concentrations of angiotensin on potassium uptake and sodium loss by sodium loaded rat kidney cortex slices incubated at 25C for 10 mins in the presence of glucose and absence of potassium under aerobic conditions

Angiotensin concentration (g/ml.)	Potassium uptake (μ - equiv/g dry wt.)	Sodium loss (μ - equiv/g dry wt.)
None	-8 ± 4 (12)	88 ± 6 (12)
10^{-12}	-22 ± 3 (13)	153 ± 11 (13)
	$P < 0.01$	$P < 0.001$
10^{-8}	-4 ± 3 (10)	70 ± 9 (10)
	N.S.	N.S.
10^{-6}	-12 ± 4 (3)	113 ± 19 (13)
	N.S.	N.S.

Number of observations in parentheses.

Significance is expressed as difference between the control and each experimental condition.

Results expressed as Means $\pm$ S.E.M.

compartments. Consequently the extracellular space was measured in the sodium load, control and angiotensin conditions. Results are expressed in Table 17. Under conditions where varying concentrations of angiotensin were added to the incubatory medium, there was no significant difference between the control and experimental states, suggesting that angiotensin does not in any way effect the distribution of fluid and agreeing with results obtained by Whittembury & Proverbio (1970) and Munday et al (1972). However, under conditions of sodium load, the extracellular space was significantly decreased. This indicates sodium enters the cell accompanied by large amounts of fluid, which leads to an increase in the intracellular volume.

Table 17

The effect of sodium load and different concentrations of angiotensin on the extracellular space of slices of rat kidney cortex measured with (^{3}H) inulin. The slices (except in the case of sodium load) were incubated for 10 mins. at 25C under aerobic conditions.

	Extracellular space (ml/100g tissue)	
Control	21.0 ± 0.08	(10)
Na load	11.7 ± 1.07	(12)
	$P < 0.001$	
Angiotensin 10^{-12} g/ml.	23. 1 ± 1.12	(6)
	N.S.	
10^{-8} g/ml.	20.4 ± 0.74	(6)
	N.S.	
10^{-6} g/ml.	23.1 ± 0.98	(5)
	N.S.	

Number of observations in parentheses.

Significance expressed as difference between control and experimental conditions.

Results expressed as Means ± S.E.M.

Section VI

The Mechanism of Action of Angiotensin

Hormone Action and Protein Synthesis Inhibitors

Studies on the mechanism of action of angiotensin have shown that the protein synthesis inhibitors, puromycin and cycloheximide, inhibit the stimulatory response of the hormone in the kidney cortex slice (Munday et al, 1972), the rat colon (Davies et al, 1972) and the rat jejunum (Bolton et al, 1975). However in the presence of actinomycin D, angiotensin is fully effective suggesting that a protein synthesis event at the translational level is, in some way, necessary for a response. If angiotensin is stimulating the synthesis of a new protein, then it should be possible to show the incorporation of labelled amino acids into protein. All attempts to do this have, however, failed.

In the following experiment fluorophenylalanine, a substituted amino acid, was used. If fluorophenylalanine is incorporated into the protein which is synthesised following angiotensin stimulation, then a nonsense protein is produced, which might lead, in turn, to a repression of the angiotensin stimulated sodium transport response. Fluorophenylalanine has been shown to abolish the aldosterone stimulation of sodium transport by toad bladder in an analagous way (Fanestil & Edelman, 1966).

Sodium loaded rat kidney cortex slices were incubated with or without angiotensin (10^{-12} g/ml.) and fluorophenylalanine (10^{-3}M) in the absence of potassium. The results are shown in Table 18. Angiotensin (10^{-12} g/ml.) greatly stimulated sodium extrusion from the slices and also increased the loss of potassium, although this ion was not present in the medium.

Fluorophenylalanine (10^{-3}M) did not effect control potassium uptake and sodium extrusion by the slices. The substituted amino acid failed to inhibit the angiotensin stimulation of sodium extrusion from the cortex, indicating the fluorophenylalanine is probably not incorporated into the protein synthesised by angiotensin action.

Cyclic GMP and Hormone Action

The mechanism of action of angiotensin has not been fully elucidated but Munday et al (1972) demonstrated that the intracellular secondary messenger cyclic AMP is not involved and that a protein synthesis event, at a stage later than transcription, follows the application of the hormone to the tissue and precedes the subsequent induced changes in ion transport. Cyclic GMP is present in most tissues and its cellular concentration has been shown to vary in the presence of a number of hormones suggesting that this cyclic

Table 18

The effect of fluorophenylalanine, a protein synthesis inhibitor, on potassium uptake and sodiumloss by rat kidney cortex slices incubated with or without angiotensin (10^{-12} g/ml.) for 10 mins. at 25C in the absence of potassium under aerobic conditions.

	Potassium uptake (μ - equiv/g dry wt.)		Sodium loss (μ - equiv/g dry wt.)	
Control	-7 ± 1	(6)	61 ± 13	(6)
Angiotensin (10^{-12} g/ml)	-15 ± 3	(7)	108 ± 14	(7)
	$P < 0.05$		$P < 0.05$	
Control	-7 ± 1	(6)	61 ± 13	(6)
Fluorophenylalanine (10^{-3}M)	-3 ± 2	(6)	77 ± 8	(6)
	N.S.		N.S.	
Angiotensin + Fluorophenylalanine (10-3M)	-15 ± 6	(7)	107 ± 14	(7)
Angiotensin (10^{-12} g/ml)	-15 ± 3	(7)	108 ± 14	(7)
	N.S.		N.S.	

Number of observations in parentheses.

Results expressed as Means ± S.E.M.

nucleotide, like cyclic AMP, may be an intracellular messenger (McMahon, 1974) Moreover, liver ribosomal protein synthesis is enhanced by low concentrations of cyclic GMP (Varrone et al, 1973) in a way analgous to the action of angiotensin on rat kidney cortex in vitro. Consequently the following experiments were carried out in an attempt to mimic the actions of angiotensin by adding cyclic GMP to the incubation fluid of kidney cortex slices and thus implicate cyclic GMP as an intermediary in the transport control process.

The effect of Cyclic GMP on Potassium uptake and Sodium extrusion by Sodium Loaded Rat Kidney Cortex Slices

Sodium loaded rat kidney cortex slices were initially incubated with 10^{-3}M, 10^{-4}M and 10^{-5}M cyclic GMP, in the absence of potassium under aerobic conditions. The results obtained are expressed in Table 19. These concentrations of the compound had no effect on potassium uptake by the tissue. The 10^{-3}M concentration of cyclic GMP had, however, a very significant inhibitory action on sodium extrusion and the addition of 10^{-4}M cyclic GMP to the incubatory medium also resulted in decreased sodium loss by the tissue. Although angiotensin in high concentrations inhibits fluid transport across the in vivo jejunum (Bolton et al, 1974) this action has not been reproduced in the kidney cortex slice preparation. Thus this inhibitory action of cyclic GMP on sodium and potassium transport by cortex slices does not mimic angiotensin action on the cortex slice.

The effect of Cyclic GMP (10^{-6}M) on potassium uptake and Sodium extrusion by Sodium Loaded Rat Kidney Cortex Slices

The work of Varrone et al (1973) indicates that the nucleotide, cyclic GMP, is a mediator of hormone action regulating protein synthesis at the ribosomal level. As angiotensin stimulation of sodium transport in kidney slices has also been suggested to be secondary to protein synthesis at the ribosome (Munday et al, 1972), it was decided to carry out experiments using physiological concentrations of cyclic GMP, to investigate the possibility that this compound might act as a secondary messenger to angiotensin.

Sodium loaded slices were incubated with cyclic GMP (10^{-6}M) in the presence of glucose and absence of potassium for 10 minutes at 25C. The results obtained are expressed in Table 20. During the series of experiments, it was noticed that cyclic GMP stimulated extrusion from the slices in each experiment carried out, but due to variation from day to day this stimu-

Table 19

The effect of cyclic GMP (10^{-3}, 10^{-4}, 10^{5}M) on potassium uptake and sodium extrusion by sodium loaded rat kidney cortex slices incubated for 10 mins. at 25C in the absence of potassium under aerobic conditions.

	Potassium uptake (μ - equiv/g dry wt.)		Sodium loss (μ - equiv/g dry wt.)	
Control	− 20 ± 4	(5)	88 ± 9	(5)
Cyclic GMP 10^{-3}M	− 13 ± 3	(6)	19 ± 10	(6)
	N.S.		P < 0.001	
10^{-4}M	− 7 ± 6	(5)	57 ± 7	(5)
	N.S.		P < 0.05	
10^{-5}M	− 12 ± 2	(7)	105 ± 12	(7)
	N.S.		N.S.	

Results expressed as Means ± S.E.M.

Numbers of observations in parentheses.

Significance expressed as difference between control and each experimental condition.

Table 20

The effect of cyclic GMP (10^{-6}M) on potassium uptake and sodium extrusion by sodium loaded rat kidney cortex slices incubated for 10 mins. at 25C in the absence of potassium under aerobic conditions

	Potassium uptake (μ - equiv/g dry wt.)	Sodium loss (μ - equiv/g dry wt.)
Control	-15 ± 3 (12)	105 ± 7 (12)
Cyclic GMP	-19 ± 4 (16)	116 ± 8 (16)
	N.S.	$P < 0.05$

Results expressed as Means $\pm$ S.E.M.

Number of observations in parentheses.

An Unbalanced Analysis of Variance was used to obtain the P value for sodium loss.

lation was not apparent when the results were analysed by the Students' 't' test. An Unbalanced Analysis of Variance was therefore used to calculate the results. Using this test it was found that the nucleotide did indeed significantly stimulate the rate of sodium loss from the tissue in a similar manner to that of the hormone angiotensin, although it must be noted that this stimulation was not as great as that with angiotensin (10^{-12} g/ml.) (see Table 16).

The effect of cyclic GMP (10^{-6}M) on potassium uptake and Sodium extrusion by Sodium Loaded Rat Kidney Cortex Slices incubated in the presence of Potassium

Angiotensin has been found to decrease the potassium uptake by rat kidney cortex slices, when the slices are incubated in the presence of potassium, glucose and the hormone (see Table 15). With the view that the nucleotide, cyclic GMP, might mimic the action of angiotensin in inhibiting potassium uptake, a further experiment was carried out. Sodium loaded rat kidney cortex slices were incubated in the presence of potassium and cyclic GMP (10^{-6}M) for 20 minutes at 25C. Potassium uptake and sodium extrusion were measured under these conditions. The results are expressed in Table 21. Although a sodium loss indeed seemed to be stimulated by the nucleotide, a 78 ± 5 to 103 ± 13 μ equiv/g dry weight being recorded, a Students' 't' test failed to signify the result. However, using this concentration of cyclic GMP a very significant inhibition in potassium uptake was measured. A similar result was obtained using angiotensin at a dose of 10^{-12} g/ml. (see Table 15) and in this second respect cyclic GMP mimicked this action of angiotensin.

The effect of cyclic GMP (10^{-7} and 10^{-8}M) on Potassium uptake and extrusion of Sodium by Rat Kidney Cortex Slices incubated in the absence of Potassium

Owing to the difficulty of obtaining a cyclic GMP stimulation of sodium extrusion from rat kidney cortex slices, the concentration of cyclic GMP was altered in the hope of improving the produced increase in sodium loss. Sodium loaded, potassium depleted slices were incubated in the presence of glucose and cyclic GMP (10^{-7} or 10^{-8}M) and in the absence of potassium. Potassium uptake and sodium extrusion by the tissue was measured. The results are expressed in Table 22. As seen from the table, these concentrations of the nucleotide failed to significantly effect either potassium uptake or sodium extrusion by the tissue.

Table 21

The effect of cyclic GMP (10^{-6}M) on potassium uptake and sodium extrusion by sodium loaded rat kidney cortex slices incubated for 10 mins. at 25C in the presence of potassium under aerobic conditions.

	Potassium uptake (μ - equiv/g dry wt.)		Sodium loss (μ - equiv/g dry wt.)	
Control	62 ± 3	(10)	78 ± 5	(10)
Cyclic GMP	47 ± 4	(12)	105 ± 13	(12)
	P < 0.01		N.S.	

Results expressed as Means ± S.E.M.

Number of observations in parentheses.

Significance expressed as the difference between the control and experimental conditions.

Table 22

The effect of cyclic GMP (10^{-7} and 10^{-8}M) on potassium uptake and sodium extrusion by sodium loaded rat kidney cortex slices incubated for 10 mins. at 25C in the absence of potassium under aerobic conditions.

	Potassium uptake (μ - equiv/g dry wt.)		Sodium loss (μ - equiv/g dry wt.)	
Control	-14 ± 3	(8)	103 ± 7	(8)
Cyclic GMP 10^{-7}M	-14 ± 6	(6)	117 ± 18	(6)
	N.S.		N.S.	
10^{-8}M	-8 ± 3	(8)	100 ± 15	(8)

Results expressed as Means $\pm$ S.E.M.

Number of observations in parentheses.

Significance expressed as difference between the control and the experimental conditions.

The effect of cyclic GMP (10^{-6}M) on potassium uptake and sodium extrusion by Rat Kidney Cortex Slices incubated for 15 minutes in the absence of Potassium

Following the previous experiment, the incubation time was altered and the original concentration of cyclic GMP, 10^{-6}M was used. Sodium loaded potassium depleted slices were incubated in the presence of cyclic GMP (10^{-6}M) and the absence of potassium for 15 minutes at 25C, under aerobic conditions. Sodium extrusion and potassium uptake were measured and the results are expressed in Table 23. Increasing the incubation time to 15 minutes did not significantly alter transport of either ion under these experimental conditions.

From the results of the cyclic GMP experiments, it can be suggested that the nucleotide might indeed be a secondary messenger in hormone action. When used in a 10^{-6}M concentration, cyclic GMP does mimic two of the actions of angiotensin, namely inhibition of potassium uptake and stimulation of sodium extrusion by the rat kidney cortex slice. Clearly further investigation of this possible involvement of cyclic GMP in the action of angiotensin is necessary. In particular, conditions giving a larger response to added cyclic GMP should be found and direct measurements of tissue concentration of cyclic GMP should be made.

Table 23

The effect of cyclic GMP (10^{-6}M) on potassium uptake and sodium extrusion by sodium loaded rat kidney cortex slices incubated for 15 mins. at 25C in the absence of potassium under aerobic conditions

	Potassium uptake (μ - equiv/g dry wt.)		Sodium Loss (μ - equiv/g dry wt.)	
Control	-4 ± 7	(6)	162 ± 5	(6)
Cyclic GMP 15 mins.	-12 ± 6	(10)	169 ± 8	(10)
	N.S.		N.S.	

Results expressed as Means $\pm$ S.E.M.

Number of observations in parentheses.

Significance expressed as the difference between the control and experimental conditions.

Chapter 1V

General Discussion

Section 1

The Actions of Angiotensin on the Colon of the Rat

The Absorption of Water and Electrolytes Across the Intestine

A considerable quantity of fluid and electrolytes enters the gut every day. This is derived from oral intake and from secretions of the stomach, pancreas, liver and small intestine. Almost all this fluid must be re-absorbed if the animal is to survive. Edmonds (1974) has reviewed the re-absorption of fluid and electrolytes across the intestines. Over most of the small intestine fluid transport takes place so that the volume passing the terminal ileum is only 10-30% of that passing through the duodenum. In the colon fluid volume is further reduced, for example in man about 500 ml./day enters from the ileum, while daily faecal fluid loss is only 50 to 200 ml.

Observations of the electrolyte composition of intestinal fluid also suggest that the epithelium is not uniform in its transport characteristics. Early investigations by Kramer, Kearney & Ingelfinger (1962) on healthy subjects with well established ileostomies have shown that 60.3 mEq/day of sodium is excreted in the ileal dejecta. As the average total sodium load presented to the adult human intestine by dietary intake and ion secretion is in the order of 800 mEq/day, this indicates that the small intestine is the major site for sodium absorption.

Further studies (Kramer, 1966) have, however, shown that the small intestine has a limited capacity to absorb sodium, the sodium excretion from ileostomies far exceeding the amount being ingested, suggesting that the colon can also be considered as an important site of sodium absorption.

Ross & Spencer (1954) have found that in the absence of adrenal secretions, sodium absorption by the rat colon *in vivo* is impaired, while Edmonds (1967) has demonstrated enhanced sodium transport by the colon of sodium-depleted rats. In addition the administration of aldosterone and other mineralcorticoids, while having a variable effect on salt and water absorption by the small intestine, consistently result in increased salt and water absorption by the colon (reviewed by Edmonds, 1974). These investigations together with those of Vogel, Meyering & Stoeckert, (1969), who found that the absorbing capacity of the colon for sodium exceeds that of the jejunum, emphasise the importance of the ability of the colon to respond to altered sodium balance by changes in sodium absorption function and indicate the role of this tissue in the maintenance of fluid and sodium balance. The importance of the colon in limiting sodium loss, its response to such conditions as adrenalectomy and dietary sodium depletion, together with its consistent response to mineralcorticoids suggest that this tissue must pro-

vide an appropriate system for the study of the actions of hormones, involved in homeostasis, such as angiotensin, on electrolyte and fluid balance in the rat.

The In Vitro Intestinal Preparation

As angiotensin is known to stimulate smooth muscle (reviewed by Regoli et al, 1974) and adrenergic ganglia (reviewed by Zimmerman et al, 1972), it was thought that a muscle free preparation, in which the complication of contributions made by muscle and nerve in the transport response could be reduced, would be most suitable for an investigation of the action of angiotensin on colonic fluid transfer. For this reason the in vitro stripped colonic sac preparation described by Parsons & Paterson (1965) was used in this study. The stripped sac has been shown to actively transport fluid against an osmotic gradient and to remain viable for periods up to three to four hours. This preparation has been used by Shaikh (1972) to demonstrate that both aldosterone and angiotensin increase sodium and water absorption by segments of colon; and by Davies et al (1970), who established a dose-dependent dual action of angiotensin on the stripped intestine and showed that the stripping procedure increases the effective hormone concentration at the mucosal epithelium by a factor of ten. This group of workers also claim that as the stripped sacs of intestine contain no ganglia, the inhibitory and stimulatory effects of angiotensin on fluid transfer by this preparation, are the results of a direct action of angiotensin on the epithelial cells. The results of this present study also support the use of the stripped everted colon as a tool for the investigation of hormonal action on fluid transport across the intestine.

Angiotensin (10^{-11}M) was found to increase the rate of fluid transport across sacs of descending colon, which had been stripped of major nerve plexuses. This suggests that the stimulatory effect of angiotensin on this tissue is the result of a direct action of the hormone on the mucosal epithelium. These results are consistent with those of McAfee & Locke (1967) who concluded that the stimulation of active sodium transport across frog skin by angiotensin does not involve adrenergic or cholinergic mechanisms.

Pretreatment of Animals

Crocker & Munday (1970) have described conditions under which the isolated rat jejunum responds to angiotensin administration. Angiotensin is only

effective in altering the rate of fluid transport if the sources of endogenous aldosterone and angiotensin are removed by prior nephrectomy and adrenalectomy or the levels of these hormones are reduced by sodium loading the animal. Furthermore, it has been shown, using the everted sac preparation (Davies et al, 1970) that all areas of the rat intestine respond to angiotensin, providing that the animals are sensitised to the hormone by nephrectomy and adrenalectomy, and that the hormone is administered to the serosal compartment of the sac. In addition, Barraclough et al (1967) found the need for an initial sodium loading period to demonstrate the antinatriuretic effect of angiotensin on the in vivo rat kidney, while Coviello (1970) has shown that pretreatment of toads in 0.6% saline, in order to reduce endogenous angiotensin levels, is essential for the demonstration of the hydro-osmotic action of the hormone on toad skin.

These observations are in contrast with the findings of Hornych et al (1973) who recorded angiotensin stimulated fluid transport across sacs of ascending colon taken from normal rats. Bolton et al (1974) have shown that no pretreatment of the animal is necessary to obtain a dose-dependent dual effect of angiotensin on fluid transport by the rat jejunum in vivo and Levens et al (1975) have demonstrated angiotensin stimulated colonic fluid transfer in untreated rats in vivo. In addition Munday et al (1971), using kidney cortex slices, have found that angiotensin (10^{-12}M) stimulates sodium extrusion from sodium loaded slices in the absence of any sensitisation treatment to the rat from which the kidneys were obtained; and Bonjour & Malvin (1969) have also demonstrated that infusions of angiotensin (50 or 250 ng/kg/min) produce either an antinatriuretic or a natriuretic effect in the in vivo rat kidney without any presentisation of the animal being obligatory.

In view of these conflicting observations an initial study was carried out to determine the exact conditions under which isolated rat colon responds to angiotensin. The results obtained in this present thesis agree with the investigations of Davies et al (1970). It was found that 48 hour adrenalectomy and nephrectomy significantly lowers the basal transport rate across both ascending and descending colon. Angiotensin, at a concetration which is comparable to circulating levels in the blood (10^{-11}M), when added to the serosal compartment of sacs of descending colon stimulates the transport level back towards a normal basal rate, although it must be emphasised that sacs of ascending colon do not appear to be sensitive to angiotensin under

similar conditions. In this initial series of experiments angiotensin was also found to increase fluid transport rate across stripped everted sacs of colon taken from untreated and 24 hour nephrectomised and adrenalectomised animals, where basal fluid transport had not been significantly lowered by the surgical pretreatment. This suggests that sensitisation of the animals to angiotensin by removal of the endogenous source of the hormone is not strictly necessary for a stimulatory response of fluid transport to occur. However the results show that the duration of the post-operative period is important, a greater and more reproducible stimulation of fluid transport by angiotensin being recorded after nephrectomy and adrenalectomy had been carried out 48 hours prior to the experiment.

This study therefore indicates that angiotensin can significantly modify the transport of fluid in the rat descending colon and also demonstrates that the descending colon is more sensitive to the action of the hormone than is the ascending colon of the rat. The insensitivity of the ascending colon to angiotensin contrasts with the observations of Hornych et al (1973). This group of workers showed that net transfer of sodium and water across everted sacs of ascending colon taken from normal animals, is increased by angiotensin, whereas the descending colon of untreated animals all doses of the hormone inhibited net transfer, which they suggest could be secondary to the inhibition of active transport. However, the sensitivity of the descending colon to angiotensin recorded in this thesis is in agreement with the observations of Davies et al (1970), who also recorded a stimulation of fluid transport to a dose of 10^{-11}M angiotensin in the descending colon.

In conclusion, the study has established that pretreatment of the animals by adrenalectomy and nephrectomy is not necessary to demonstrate an angiotensin stimulation of fluid transport in the descending colon of the rat, as increases in absorption were obtained by the administration of physiological concentrations of the hormone to sacs of descending colon taken from untreated animals. The angiotensin response was, however, enhanced by 48 hour nephrectomy and adrenalectomy, where the surgical procedure had significantly reduced basal fluid transport. Surprisingly, the response to angiotensin was not enhanced 24 hours after nephrectomy and adrenalectomy. The half-lives of renin and aldosterone are relatively short and the circulating concentrations of both hormones would be expected to be very low at this time. Consequently the increased sensitivity to angiotensin

following nephrectomy and adrenalectomy must involve more than merely low plasma levels of the hormones. Differences in the sensitivity of the ascending and descending colon were observed. Hornych et al (1973) have also reported dissimilarities in the sensitivity of the rat colon to angiotensin, fluid transport being stimulated across the ascending colon and inhibited across the descending colon by the hormone. The present study differs with their findings in that angiotensin, administered at a dose within the limits of the physiological range of hormone concentration was found to stimulate fluid absorption in the descending colon, while being ineffective in the ascending colon of the rat.

The results show that a variety of tissues which are involved in the control of salt and water homeostasis respond to angiotensin. The preliminary treatment which is required in order to obtain angiotensin responses varies from tissue to tissue and is also technique dependent. Consequently the preliminary treatment does not appear to be necessary for angiotensin action but is required but some techniques so that the hormone response may be unmasked.

Apart from the central actions of angiotensin, to secrete vasopressin (Mouw et al, 1971), to induce drinking (Fitzsimons & Simons, 1969), to induce salt appetite (Chiaraviglio, 1976) and its indirect action through the release of aldosterone (Davis, 1971), which all serve to control the extracellular fluid volume of the body, there is now a large literature to suggest that the hormone alters sodium and water homeostasis, both in in vitro and in vivo preparations, by direct action on the intestinal epithelial cells. The evidence in this section supports the view that angiotensin has a physiological role as a salt and water retaining hormone and suggests that the descending colon is a sensitive and important site for action by angiotensin.

Section 11

Seasonal Variation in Fluid Transport and Sensitivity to Angiotensin

Investigations into the actions of angiotensin on sodium and water transport across a variety of epithelia have yielded conflicting results. The hormone has been found to stimulate sodium pump activity in rat kidney cortex slices (Munday et al, 1971) and both stimulate and inhibit sodium transport, depending on the dose, in the rat kidney in vivo (Barraclough et al, 1967; Bonjour & Malvin, 1969). In contrast to the results of Munday et al (1971), Leysac, Lassen & Thaysen (1961) have reported that angiotensin inhibits transport across rat kidney cortex slices in a qualitatively similar way to the action of angiotensin on kidney in vivo. The action of the hormone on intestinal preparations also appears to be inconsistent. Hornych et al (1973) reported that although angiotensin increases net fluid transport across the rat descending colon in vitro, all doses inhibit salt and water transport across the descending colon. This present thesis reports that angiotensin can significantly increase transport of fluid in the rat descending colon in vitro, but has emphasised that sacs of ascending colon do not appear to be sensitive to the hormone. In addition, McAfee & Locke (1967) have shown that angiotensin stimulates mucosal to serosal sodium flux across an in vitro preparation of frog skin, whereas Coviello & Crabbe (1965) using electrical methods to measure sodium transport could demonstrate no action of the hormone on toad bladder or toad skin. The inability of scc measurements to detect a response to the hormone may be due to the non-electrogenic nature of the processes affected by angiotensin as described previously. Nevertheless, inconsistencies exist which are not fully explained and other factors must influence the responses to the hormone.

In some cases tissue or species differences may explain why such inconsistency in the action of angiotensin on transporting epithelia exists. Another alternative is the possibility of seasonal variation in the sensitivity of tissues to angiotensin, which has been reported by a number of workers. McAfee & Locke (1967) have recorded considerable variation in the effects of angiotensin on sodium transport across frog skin, which displays greatest sensitivity to the hormone between December and March. During the egg laying season in April, this tissue becomes insensitive to the action of the hormone. Such a seasonal cycle could possibly reflect an overall hormone and metabolic change in the amphibian. Crocker (1968) has also noted a variation in the aldosterone stimulation of salt and water transport across rat jejunum, which displays a greater sensitivity to this hormone during the

winter months. As aldosterone is closely linked to the renin-angiotensin system, this heightened sensitivity could presumably occur when endogenous angiotensin and hence aldosterone levels are low.

Other variations in the sensitivity of tissues to salt and water retaining hormones have been reported by Coviello (1970), who noticed a seasonal change in the response of frog skin to angiotensin and Hong et al (1968) whose study of the action of vasopressin on sodium transport by frog skin showed an increased sensitivity to vasopressin during the cold season.

During this present study a seasonal variation was noted in fluid transport and sensitivity to angiotensin in both the in vivo rat jejunum and the in vitro rat colon. The results showed that the rate of fluid transport fluctuated throughout the year in a similar manner in both preparations. It was low during August and November (colon 0.78; jejunum 0.9ml./hr./g wet weight of tissue) and high for the remainder of the year (colon 1.32; jejunum 1.65 ml./hr./g wet weight of tissue). Concomitant with the reduction of fluid transport in the early autumn, was a significant decrease in the response of both tissues to angiotensin. In the colon in vitro, angiotensin stimulation of fluid transport was 99 $\pm$ 9% above control transport except during the late summer/early autumn when the stimulation by the hormone was only 44 $\pm$ 11%. Similarly in the in vivo jejunum infusion of angiotensin normally caused a 71 $\pm$ 8% increase in fluid transfer. This response however was significantly decreased in August and during October there was no detectable stimulation of fluid transport by the hormone. These findings are consistent with those of Hong et al (1968) who showed that frog skin varies in its sensitivity to vasopressin on a seasonal basis and the findings of McAfee & Locke (1967), who also recorded a greater sensitivity of frog skin to angiotensin during the winter months.

The rats used in this study were maintained in constant environmental conditions, at a temperature of 22 $\pm$ 1C and under a constant light rhythm. In this way it was hoped that any environmental stimulus such as the onset of the changing seasons would be eliminated or at least attenuated. Although all animals weighed 300g and were sexually mature, they ranged from 7 to 10 weeks in age. A subsequent study measuring fluid transport in the young 7 week old rats and the older 10 week old rats has shown no significant difference between the two groups. Furthermore Levens (personal communication) has shown that fluid transport and angiotensin sensitivity in the in vivo jejunum is not significantly different in male rats weighing 300 and 450g. The age

difference in the rats of the same weight cannot therefore account for the observed circannual variation in fluid transport or hormone sensitivity.

During the period of slow fluid transport and decreased sensitivity to angiotensin it was noticed that both the litter size and the number of successful matings of the rats were reduced. This could possibly be attributed to a change in the reproductive hormone levels or an altered metabolism of the rats at that time. Fischer & Hommel (1970) have shown that rats, kept under constant laboratory conditions and light rhythms, display a minimum blood sugar level during the summer and this may be a consequence of reduced carbohydrate turnover in these animals. Many workers have now shown that the metabolic state of the animal is closely linked to the levels of circulating hormones. For example, hibernating animals such as the woodchuck show an involution of endocrine systems and decreased protein and lipid turnover during the winter months when the animal is asleep (Wenberg & Holland, 1973; 1 & 2). During the arousal period steroid levels increase as the animal enters its reproductive cycle and this is accompanied by increases in catecholamines which have calorigenic and circulatory effects beneficial to the animal at this time. Hong et al (1968), who have recorded a seasonal variation in the sensitivity of frog skin to vasopressin, have also observed corresponding variations in liver and muscle glycogen and have suggested that the alteration in hormone sensitivity could be linked to the energy store of the tissue. From the above and many similar studies it can be concluded that the circannual changes in animal behaviour, such as breeding and hibernation and seasonal variations in hormone secretion and in metabolism are closely linked and probably mutually dependent on one another.

The results in this study clearly demonstrate a seasonal variation in fluid transport in two intestinal preparations, namely the in vivo jejunum and the in vitro colon. Furthermore during the period when fluid transport is decreased there is also a reduced sensitivity to angiotensin. Such variation could reflect changes in the levels of a number of hormones in the blood, which contribute to sodium balance, and/or an alteration in the overall metabolic state of the animal. However whether this present annual variation in fluid transport and sensitivity to angiotensin can be attributed to an endogenous , biological rhythm is not certain at this stage. A further

study, where environmental conditions are kept constant, food intake is monitored and the endogenous hormone and metabolic state of the animal is determined, is needed to demonstrate the existence of a circannual clock. The present results explain, at least in part, some of the inconsistencies regarding the responses of transporting epithelia to angiotensin and should be taken into account by any worker in this field.

Section 111

Metabolic Requirements for Fluid Transport and the Angiotensin Stimulation of Fluid Absorption

Metabolic Requirements for Optimal Fluid Transfer

Individual regions of the intestine show very different metabolic requirements (Barry, Matthews & Smyth, 1961; Parsons & Paterson, 1965) and as already described different transport characteristics. In this present study it was observed that the addition of glucose to both the incubatory medium and the serosal compartment was necessary to promote basal fluid transport across sacs of rat descending colon. If this sugar was removed from the bicarbonate buffer, fluid transfer was not abolished, although a large decrease in net transport was measured. This result is in keeping with the observations of many in vitro workers, who also use exogenous glucose to sustain an adequate transport rate across epithelial tissues. Barry et al (1961) have shown the importance of sugars in maintaining optimum water transport across the intestine. They postulate that water transport can be divided into two fractions, one glucose-dependent and the other glucose-independent. The former appears to be more important in the upper jejunum, whereas the latter is more apparent in the lowest part of the ileum. This present study confirms that glucose is essential for rapid transport across an in vitro gut preparation. However, it cannot uphold the view that glucose-dependent transport is not important in the lower intestine, since a 64% reduction in the rate of fluid absorption was recorded when sacs of descending colon were incubated in the absence of this sugar. Moreover, Parsons & Paterson (1965) using a similar preparation to that used in this present thesis, have also recorded a large reduction in fluid transport across rat colon (approximately 50%) when glucose substrate is removed from the mucosal and serosal mediums. Thus it seems that in vitro rat colon, where limited substrate is available, requires glucose for optimal fluid transfer, although appreciable transport is still observed in the absence of any exogenous substrate.

The Use of Fructose as a Substrate and its Effect on the Angiotensin Stimulated Response

Angiotensin has been shown to stimulate fluid absorption in the absence of any change in transmural pd or scc in the in vivo jejunum (Bolton, Munday, Parsons & York, 1974) in the in vivo colon (Levens et al, 1975) and in the in vitro colon (Shaikh, 1972), which suggests that the hormone is exerting this effect by action on a non-electrogenic process. Barry et al (1965) have postulated, after demonstrating that the potential across rat small

intestine depends upon the presence of sugars, two intestinal pump processes concerned with sodium and hexose transport. The first, a hexose pump, is electrogenic and is concerned with the active transport of hexose sugars, while the second neutral pump is similar to the sodium pump proposed by Diamond (1962, 1) in gall bladder. The former workers suggest that the neutral pump can be operated by the substrate fructose. Furthermore, Binder et al (1972) have shown that glucose stimulates a sodium absorptive system in guinea pig ileum and postulate that fructose supports an electrically neutral system, which transports sodium and chloride from mucosa to serosa in the same tissue.

In view of the accumulated evidence that angiotensin stimulates an electroneutral transport process, leading to increased absorption, across the in vitro ratjejenum and that fructose specifically enhances, non-electrogenic sodium transport, it was hoped that in the presence of fructose angiotensin might exert a large effect on absorption, in the absence of any prior treatment to the animals. However, from the results of this study, it is evident that even when adequate energy for non-electrogenic sodium transfer is supplied, angiotensin does not stimulate fluid transfer across this in vitro jejunal preparation. It can therefore be concluded that the addition of fructose does not increase the sensitivity of this preparation to angiotensin.

Section 1V

The Importance of Chloride in Angiotensin Stimulated Transport

A study in this present thesis, where chloride ions were replaced by sulphate ions in the incubatory buffer has shown that the characteristic angiotensin stimulation of fluid absorption, in sacs of descending colon taken from 48 hour nephrectomised and adrenalectomised rats, is completely lost under these conditions. The lack of response cannot be explained by a toxic action of sulphate, since basal fluid absorption in untreated animals remained at a normal level when sacs of descending colon were incubated in the modified sulphate buffer. From these results it is suggested that angiotensin stimulates sodium and water transfer in the descending colon via a chloride-dependent process.

A sodium-chloride pump has been reported to operate in the rat colon in vitro by Binder & Rawlins (1973) who propose that this pump is non-electrogenic in nature. They have suggested that sodium is absorbed by two transport processes; an electrogenic system, which is measurable by scc, and a neutral or coupled system which is identifiable by the component of net flux of sodium that is greater than scc and by the net flux of chloride ions. In addition an electroneutral pump has been shown to be present in the rabbit gall bladder by Diamond (1964), who also suggests that the electroneutral nature of the pump is a result of the movement of sodium and chloride ions in a tightly coupled, one for one ratio. Frizzell, Dugas & Schultz (1975) have proposed that the electroneutral sodium-chloride transport mechanism is located on the mucosal border of rabbit gall bladder and a similar process has been suggested to occur on the brush border of rabbit ileum (Nellans, Frizzell & Schultz, 1973).

The existence of an angiotensin sensitive sodium-chloride transport process in the intestine would explain the observations of Bolton, Munday, Parsons & York (1974) and Levens et al (1975) that sodium transport across in vivo rat jejunum and colon respectively, is not accompanied by any change in the transmural potential difference or scc. These workers have suggested that angiotensin stimulation of fluid absorption across these tissues is through a non-electrogenic sodium-anion linked mechanism. Similarly the sodium-chloride transport mechanism could explain the observations of Shaikh (1972) who found that angiotensin stimulates colon sodium and fluid transfer by an increase in the activity of an electrically neutral, potassium-independent, sodium transport process.

The present evidence for the existence of a sodium-chloride linked process also supports the conclusions drawn by Whittembury (1968) and

Whittembury & Proverbio (1970), who have postulated the existence of two sodium pumps in the kidney cortex tissue. The first pump exchanges sodium for potassium, whereas the second, ouabain-refractory pump extrudes sodium accompanied by chloride and water from the cells. In addition the results of this study are in agreement with Smith (1975), who has shown that the addition of angiotensin, at a concentration which normally stimulates sodium extrusion, to a chloride-free buffer, has no effect on sodium loss from pre-loaded rat kidney cortex slices. This evidence indicates that the angiotensin stimulated process in the kidney is dependent upon the presence of the anion chloride in the incubation medium.

It is concluded that angiotensin enhances the activity of a sodium-chloride linked transport mechanism in rat colonic mucosa in vitro. This conclusion is consistent with the observations that angiotensin stimulated sodium transport in the intestine is an electroneutral process and is also in agreement with the findings that angiotensin stimulates a potassium independent and chloride independent pump in rat kidney cortex. The ability of angiotensin to specifically stimulate this mode of sodium transport suggests that the hormone could be an important tool in the elucidation of the mechanism of the non-electrogenic sodium absorption process.

Section V

The Action of Angiotensin on the Rat Kidney Cortex Slice

Whittembury (1968) and Whittembury & Proverbio (1970) have suggested that there are two sodium pumps in kidney cells, one involving Na/K exchange and the second involving neutral Na/Cl transport. This second process is independent of potassium, is refractory to ouabain and has been referred to as the second sodium pump. It may not, however, be necessary to postulate a separate primary sodium pump in transporting epithelia. There could be a single sodium pump at the lateral and serosal surface of the cell, but its apparent mode of operation could vary depending upon the way in which sodium enters the cell at the mucosal side. Support for the existence of the second sodium pump has been put forward by Bolton, Munday, Parsons & York (1974), who demonstrated the presence of a non-electrogenic sodium pump in rat jejunum, in which sodium and anion movement is apparently tightly coupled, and by Binder & Rawlins (1973) who postulate that rat colonic mucosa transports sodium by two separate mechanisms, an electrogenic mechanism and a "neutral" or coupled Na/Cl transport system. They propose that the coupled Na/Cl absorption could in fact represent two related ion changes; Na/H and Cl/HCO_3.

From the experiments in this present thesis it is evident that when preloaded rat kidney cortex slices are incubated in the presence of ouabain, sodium extrusion from the tissue is significantly decreased but is not completely inhibited. This suggests that although sodium transport can and does take place through the Na/K exchange pump, a second mode of sodium extrusion is apparent in the kidney. This additional evidence supports the conclusions reached by Whittembury (1968) and Munday et al (1971) that there are two processes for the extrusion of sodium in this tissue.

It has been shown by Munday et al (1971) using a sodium loaded rat kidney cortex slice preparation that the hormone, angiotensin, in physiological concentrations, stimulates the rate of sodium extrusion via the second sodium pump. In the kidney in vivo low concentrations of angiotensin have also been found to exert an action, resulting in an antinatriuretic/antidiuretic response, although it is not certain whether this action is potentiated through a direct stimulation of fluid transport by the hormone or is secondary to an alteration in renal blood flow (Barraclough et al, 1967; Bonjour & Malvin, 1969).

In the present investigation, using the sodium loaded kidney slice preparation and a short incubation time of 10 minutes, it was observed that angiotensin at a physiological concentration (10^{-12} g/ml) stimulates the

loss of sodium from kidney cortex and inhibits the uptake of potassium by the slices. If the hormone stimulates sodium extrusion from the slices by a route independent of potassium uptake, then excitation of this route might well reduce the sodium available to the sodium/potassium exchange pump and this would be observed as an inhibition of potassium uptake by the slices. An additional study, where preloaded kidney cortex slices were incubated with a similar concentration of angiotensin but absence of potassium, showed that under these conditions angiotensin greatly stimulates the rate of sodium extrusion from the slices. It was also noticed that although potassium was not present in the medium, this ion was lost from the tissue in the presence of the hormone, which suggests that there may have been a change in the passive permeability of the membrane or that reuptake of potassium was reduced. The observations that angiotensin (10^{-12}g/ml) does stimulate sodium extrusion from kidney cortex slices in both of the conditions described (with and without potassium) and that the hormone does not effect the distribution of fluid in the tissue, supports the suggestion of Munday et al (1971) that angiotensin exerts its action in this tissue via Whittembury's second sodium pump.

In this series of investigations the sodium-loaded kidney slices were also incubated with much higher concentrations of angiotensin (10^{-6} and 10^{-8} g/ml), both in the presence and the absence of potassium in the medium. The high doses of angiotensin used did not stimulate or inhibit either sodium loss or potassium uptake by the kidney slices. These observations are very difficult to interpret since a consistent dose dependent dual action of angiotensin has been established in many transporting epithelia, low doses stimulating and high doses seemingly inhibiting the rate of sodium and water transport across these tissues. Davies et al (1970) have demonstrated dose dependent opposite effects by the hormone on rat colon in vitro and Bolton et al (1974) have also observed angiotensin dual action in the in vivo rat jejunum. Similarly both antidiuretic and diuretic effects of the hormone have been found in the rat kidney in vivo (Barraclough et al, 1967; Bonjour & Malvin, 1969). Of interest to this study are the experiments of Leysac et al (1961) who used a similar kidney preparation to the one used in the present investigations. This group of workers preloaded rat and rabbit kidney cortex slices with ^{22}Na and found that the efflux of radioactive sodium was significantly reduced in the presence of differing concentrations of angiotensin. The hormone was found to have a potent inhibitory action on

sodium transport in concentrations ranging from 5×10^{-8} to 5×10^{-3}M. The present findings do not agree with the observations of Leysac et al (1961) since no inhibitory action was seen and low concentrations of angiotensin stimulated sodium transport by kidney cortex slices. Leysac (Bojesen & Leysac, 1965) has since criticised the technique used in the earlier work and hence the initial results of Leysac et al (1961) should be examined thoughtfully.

In the preparation used for this present study the lumen of the tissue is collapsed so that the effect of angiotensin on pump mechanisms only is being investigated. Any effect of the hormone on ion entry from the lumen or transepithelial transport is excluded by this preparation. It is apparent that these conditions are not suitable for the demonstration of an inhibitory action of angiotensin on sodium extrusion from this tissue. It may even be possible that angiotensin does not exert its inhibitory actions via the second sodium pump but perhaps through a more generalised mechanism, as indeed the natriuretic effect observed using in vivo kidney may be a consequence of altered blood flow or hydrodynamic changes.

Angiotensin (10^{-12} g/ml) was found to stimulate sodium extrusion and inhibit potassium uptake by rat kidney cortex through the second sodium pump. This observation supports the work of Munday et al (1971) and is further evidence in favour of the second mode of cellular sodium transport proposed by Whittembury (1968). Although angiotensin has been shown to exert a dose dependent dual effect in many transporting epithelial tissues, no inhibitory effect of the hormone was found in this in vitro kidney preparation. Whether the inability to obtain an inhibitory effect of angiotensin is due to inherent limitations of this preparation or is indicative that the natriuretic effect of the hormone is not caused by a direct action on the tubule cells cannot be determined from these experiments. Clearly, the stimulatory effect and the inhibitory effect of angiotensin take place through fundamentally different mechanisms in agreement with the findings in the intestine in vivo, in which Bolton et al (1975) observed that the stimulatory but not the inhibitory action of the hormone is blocked by cycloheximide and Levens et al (1976) found that the half life of the inhibitory response is much longer than that of the stimulatory effect.

Section VI

Intracellular Mechanism of Action of Angiotensin

In this present thesis the present investigations concerning the action of angiotensin on an *in vitro* rat colon preparation support studies cited in the literature, suggesting that the hormone alters epithelial transfer by acting directly on the transporting cells. Recently however Levens *et al* (1979) have suggested that noradrenaline may mediate the actions of angiotensin upon intestinal fluid transport in the rat. This group of workers have shown that alpha-adrenergic antagonists abolish both the angiotensin and noradrenaline stimulations of fluid transport and hypothesise that angiotensin might act by releasing noradrenaline from nerve endings and that noradrenaline, in turn, stimulates fluid transport by intestinal epithelial cells.

There also remains controversy over the intracellular changes, which take place following angiotensin administration and which subsequently lead to the alteration in electrolyte and water transport across the tissue. It has been suggested that the changes in intracellular processes caused by the hormone could in some way either alter the activity of ion pump mechanisms, or change the permeability of the mucosal membrane to sodium, or modify energy production in the cell. The study, carried out in this thesis, on the action of angiotensin on the rat kidney cortex slice agrees with the work of Munday *et al* (1972) and supports their suggestion that the hormone exerts its effect at least in part by an action on the sodium-chloride linked pump, although the data is inadequate to exclude other actions of the hormone. Nevertheless, it is hoped that the following discussion will clarify the present situation concerning the intracellular changes which lead to angiotensin stimulated increase of sodium extrusion via the second sodium pump in the rat kidney cortex.

An effect on protein synthesis has been proposed to explain the mechanism of action of a number of hormones. The evidence to support this suggestion is mainly derived from studies which demonstrate that the actions of these hormones can be blocked by either protein synthesis inhibitors or by compounds which alter the structure of the synthesised protein. In a limited number of cases the induced protein(s) have been identified.

In an investigation to elucidate the mechanism of aldosterone action on sodium transport by toad bladder, Faniestil & Edelman (1966) have used the

substituted amino acid, fluorophenylalanine. If a hormone is acting by altering the synthesis of a protein into which phenylalanine is incorporated, introduction of the substituted amino acid will result in the production of a "nonsense protein" leading to the loss of the hormone effect. In their study Fanestil & Edelman showed that the addition of fluorophenylalanine inhibited the effect of aldosterone and hence concluded that the action of this hormone on sodium transport processes in toad bladder was mediated through a protein synthesis event.

Similarly in this present study, fluorophenylalanine was also used to obtain further information concerning the mechanism of action of the polypeptide hormone, angiotensin, on sodium pump processes in the rat kidney cortex. It was found, however, that the characteristic angiotensin stimulation of sodium extrusion and inhibition of potassium uptake was not effected by the presence of the substituted amino acid in the incubatory medium. In view of evidence which proposes that the effects of angiotensin on transport mechanisms can be explained by a primary action on protein synthesis, as can some of the effects or other polypeptide hormones including insulin (Wool et al, 1968) and growth hormone (Kostyo, 1968), these present results are at first sight contradictory. The observations of Davies et al (1972) that puromycin and cycloheximide, inhibitors at the stage of translation block the increase in fluid transport caused by angiotensin across in vitro colonic sacs, suggest that the action of angiotensin could be mediated through an effect on protein synthesis. Moreover this group of workers reported that actinomycin D, an inhibitor of protein synthesis at the transcriptional stage, failed to inhibit angiotensin-stimulated fluid absorption, indicating that the angiotensin action on protein synthesis is at the ribosome on the process of translation. In a similar manner Bolton et al (1975) have also indicated that the angiotensin stimulation of fluid transport across the in vivo jejunum is also mediated through a protein synthesis event at the level of translation and Munday et al (1972) have reached the similar conclusions for the mechanism of angiotensin action on sodium transport in the rat kidney cortex slice. In a study made by these latter workers the protein synthesis inhibitors, puromycin and cycloheximide were shown to inhibit angiotensin stimulated efflux of sodium, whilst actinomycin D was totally without effect on the hormone stimulation of transport mechanisms in the kidney cortex. Seemingly angiotensin in some way activates the ribosome leading to the translation of preformed mRNA into a protein(s) necessary for sodium and water

transport.

Although the substituted amino acid used in this present study did not inhibit the angiotensin stimulation of sodium extrusion from rat kidney cortex slices, this does not necessarily mean that angiotensin action is not mediated via a protein synthesis event. It is suggested that either fluorophenylalanine is not incorporated into the protein synthesised by angiotensin or alternatively that owing to the very short latent period prior to the onset of the angiotensin response reported both in the kidney cortex (Munday et al, 1972) and in the rat jejunum (Levens et al, 1976) endogenous intracellular phenylalanine is incorporated into the newly translated protein before the inhibitor can have its effect. It must also be noted that attempts by Davies et al (1972) to demonstrate a stimulation of incorporation of labelled alanine into protein by angiotensin was also unsuccessful. This suggests that the protein involved in the transport process may be produced in very small quantities compared with the total synthetic capacity of the tissue, and emphasises the difficulty of success in incorporation-inhibitor studies. Negative observations are extremely difficult to interpret but the findings that fluorophenylalanine inhibits the response to aldosterone yet has no effect on the action of angiotensin is consistent with the view that these two hormones which stimulate sodium transport by a mechanism presumably mediated through protein synthesis, do so through different mechanisms possibly involving the induction of different proteins.

The Involvement of Cyclic Nucleotides and Calcium in the Angiotensin Stimulation of Ion and Water Transport

The possibility that the secondary messenger, cyclic AMP, might be involved in the change of electrolyte and water transfer by transporting epithelia,following the administration of angiotensin, has been tested by many workers and contradictory findings have been reported. Coviello (1971) observed that the angiotensin stimulation of fluid transport across toad bladder is potentiated by cyclic AMP and theophylline, a finding which is in agreement with the observations of Hornych et al (1973), who also suggest a possible mediatory role for cyclic AMP in the fluid transport response of rat colon to angiotensin. It must be noted, however, that both these preparations are also sensitive to vasopressin and the increase in fluid transport following the administration of the cyclic nucleotide could be attributed to a vasopressin-like rather than an angiotensin-like process. In contrast

to these reports is a large literature which concludes that cyclic AMP is not involved in the action of angiotensin on transporting tissues. Examples include the work of Munday et al (1972) who found that additions of cyclic AMP and dibutyryl cyclic AMP to the incubation medium fail to mimic the action of angiotensin on sodium pump activity in the rat kidney cortex; and Davies (1973) who reported that neither cyclic AMP nor theophylline added separately or together are able to mimic the angiotensin stimulation of intestinal fluid transfer in either the rat jejunum or rat colon in vitro. Furthermore Munday et al (1976) observed that adenyl cyclase activity is neither stimulated nor inhibited in the rat kidney cortex or the rat colon after administration of angiotensin.

This literature does not however preclude the involvement of an alternative messenger in the mechanism of action of angiotensin on transporting epithelia. Thus in the present study experiments were carried out to attempt to mimic the actions of angiotensin by adding cyclic GMP to the incubation fluid of rat kidney cortex slices and thus implicate this cyclic nucleotide as an intermediary in the transport responses.

In initial experiments, using high concentrations of cyclic GMP, it was observed that sodium extrusion by kidney cortex slices is severely inhibited under these conditions. This result was indeed unexpected as unphysiologically high concentrations of angiotensin do not effect sodium loss from the tissue. A dose-dependent action of angiotensin has been reported in many transporting epithelia and the observations of Bolton et al (1975) and Levens et al (1976) suggest that the inhibition of fluid transfer by high doses of the hormone in the in vivo rat jejunum take place through fundamentally different mechanisms. Thus although cyclic GMP is capable of producing an inhibitory response in the rat kidney cortex, this effect would not appear to be relevant to the mechanism of action of angiotensin in these tissues. In subsequent experiments it was found that low concentrations of cyclic GMP enhance the rate of sodium extrusion and inhibit the rate of potassium accumulation by kidney cortex slices, a result which is analogous to the unique action of physiological concentrations of angiotensin on the same preparation. Therefore it is true to say that this result is consistent with the view that the stimulatory effect of angiotensin on sodium transport mechanisms in the rat kidney may be mediated by cyclic GMP.

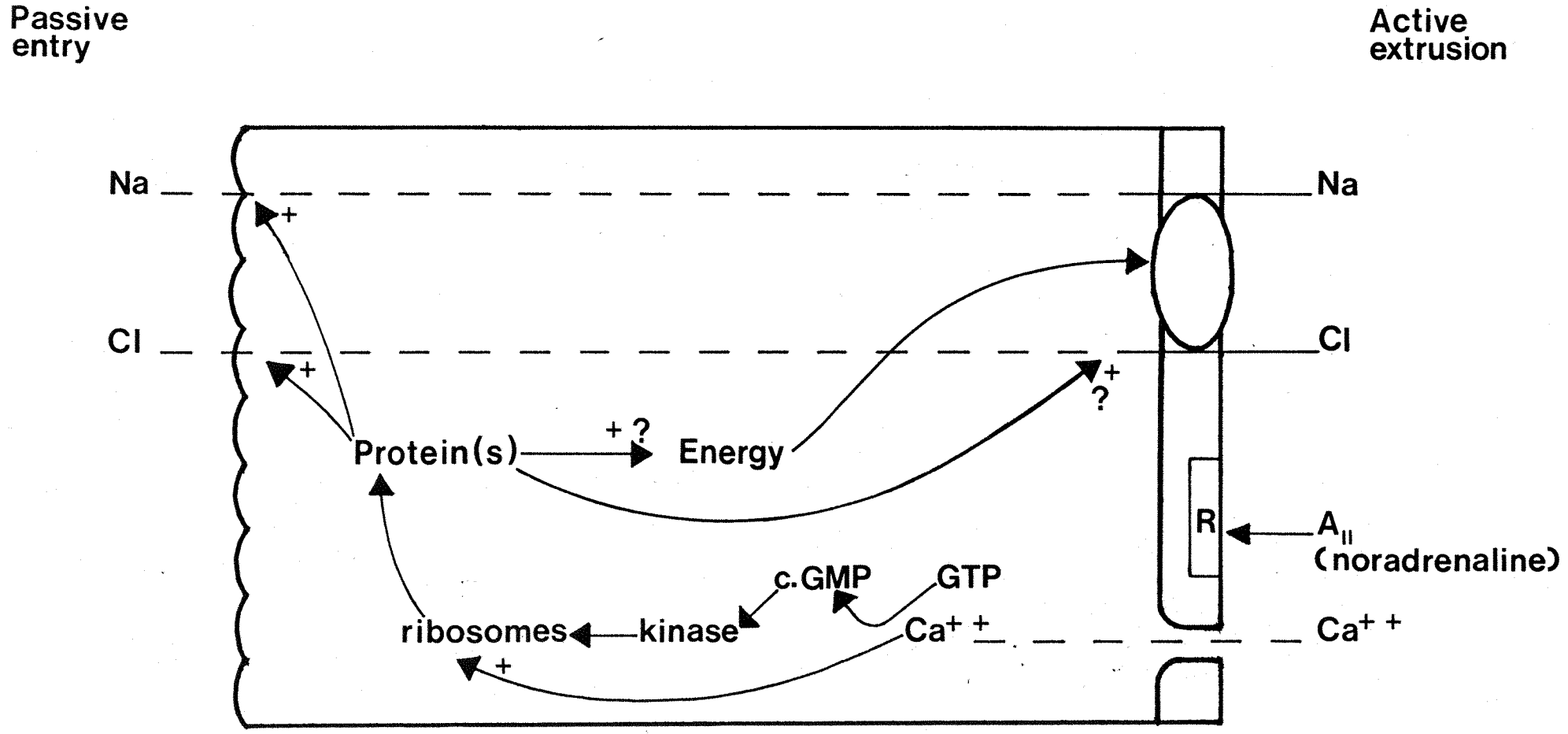
Other groups of workers have also implicated a role for cyclic GMP in hormone action. McMahon (1974) has reviewed the existence of cyclic GMP in

most tissues and its cellular concentration has been found to vary in the presence of a number of hormones, suggesting that this cyclic nucleotide, like cyclic AMP, may be an intracellular messenger. In addition, liver ribosomal protein synthesis is enhanced by low concentrations of cyclic GMP (Varrone et al, 1973) in an analogous manner to the action of angiotensin on rat kidney in vitro. Although from this study it cannot yet be confirmed that cyclic GMP is an intracellular messenger for the action of angiotensin on the kidney cortex slice, the immediate evidence does form a basis for further studies concerning the involvement of this cyclic nucleotide in the hormone's action. Subsequent studies, using intermediate concentrations of cyclic GMP and differing incubation time have demonstrated the importance of determining strict experimental conditions for the effect of the nucleotide on the kidney to occur, and measurements of intracellular concentrations of cyclic GMP in the normal and angiotensin stimulated conditions might well be valuable. The reports of McMahon (1974) and Varrone et al (1973), the observation that a translational protein synthesis event precedes the changes in ion transport induced by angiotensin (Munday et al, 1972), together with the finding that cyclic GMP appears to mimic the effects of low concentrations of angiotensin in the kidney cortex, encourage the interesting speculation that cyclic GMP might be an intracellular messenger of angiotensin stimulated changes in ion transport in this tissue.

In addition to the cited nucleotides, it is becoming evident that calcium ions play an important role in the intracellular mechanism of action of some hormones. Calcium has been shown, by Munday et al (1979) to be obligatory for the angiotensin stimulation of fluid transport across the rat colon in vitro. This group of workers suggest that calcium may allow the hormone to interact with its receptor site, and/or that angiotensin may stimulate the transfer of calcium ions across the serosal membrane as a primary event in response to the hormone. This latter suggestion implicates calcium itself as a secondary messenger of hormone action. Furthermore, in a number of tissues, calcium ions have been found to activate cytoplasmic guanylate cyclase, giving rise to increased concentrations of cyclic GMP within the cell (reviewed by Rasmussen & Goodman, 1977). As cyclic GMP and calcium ions activate ribosomes (Farese, 1971), hormone induced changes in their concentrations may lead to alterations in ribosomal function and translational protein synthesis and thus ultimately to changes in cell function.

Hence from the information in this section a model can be postulated

Fig. 8 A PROPOSED MECHANISM OF ACTION OF ANGIOTENSIN ON KIDNEY CORTEX CELLS



for the mechanism of action of angiotensin in the kidney cortex slice. (Illustrated in Fig. 8). Angiotensin, the primary messenger, may cause intracellular concentrations of calcium ions to increase. Calcium, acting as a secondary messenger, may then increase concentrations of cyclic GMP. This cyclic nucleotide (the tertiary messenger) and calcium may activate ribosomes leading to changes in protein synthesis at the level of translation. The production of a specific protein(s) could then effect the activity of the sodium-chloride linked pump either directly or indirectly (Whittembury, 1968), resulting in the angiotensin stimulated extrusion of sodium from the kidney cortex cells.

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