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THE TRANSPORT OF ETHACRYNIC ACID IN RENAL TISSUE

BY

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TO THE FOREST WHICH SACRIFICED SO MUCH PAPER

ABSTRACT

Slices of rabbit kidney cortex when incubated in a buffered glucose and salt medium containing ethacrynic acid 7.4 x 10^{-7} M, will accumulate the drug against a concentration gradient. Although accumulation of ethacrynic acid is also observed with slices of rabbit liver, it is not as marked. Therefore, the transport of ethacrynic acid into slices of kidney cortex is interpreted as evidence for the existence of a concentrating mechanism in the kidney for this drug. Some aspects of this phenomenon have been investigated.

The extracellular space as marked by inulin and mannitol has been determined after varying periods of incubation at 37°C, both in the presence and the absence of ethacrynic acid. In conjunction with these studies the morphology of the tissues was examined by electron microscopy. Incubation causes changes in the ultrastructure. The findings from the experiments on the extracellular space and those from the electron microscopic investigation, show no changes that can be attributed to the presence of ethacrynic acid. A notable observation is the unchanged appearance of the basal membranes, even after long periods of incubation.

The accumulation of ethacrynic acid was reduced by the metabolic inhibitors 2,4-dinitrophenol and sodium azide, suggesting that oxidative phosphorylation represents a part of the energy supply for this process.

Ouabain and probenecid which are known to inhibit transport processes were found to inhibit the uptake of ethacrynic acid. Inhibition of ethacrynic acid uptake was not total, irrespective of the agent

that was employed. This finding is discussed in relation to the non-specific binding of ethacrynic acid to tissue slices.

The omission of sodium from the incubation medium reduced the uptake of ethacrynic acid. This sodium dependence is only seen at low concentrations of sodium. Potassium will not replace sodium in the incubation medium. It also causes swelling of the tissue. From this finding the cation gradient as a possible mechanism for the transport of ethacrynic acid is excluded.

Evidence is presented to show that the transport system for ethacrynic acid resembles the organic acid carrier in a number of ways. However, dissimilarities are also reported. Competition could not be demonstrated with p-aminohippuric acid, the classical organic acid. At very high concentrations, however, this organic acid will inhibit the accumulation of ethacrynic acid. The hypothesis that ethacrynic acid is transported by the organic acid carrier is discussed.

Data are presented which support the existence of a dual uptake process for ethacrynic acid into kidney slices. A co-transport mechanism and a process that is similar but not identical to the organic acid transport system, have been suggested as two possible mechanisms. Attempts to demonstrate the existence of both processes are been described. The two processes may act concurrently and attempts to demonstrate this point have also been described. These experiments do not demonstrate unequivocally the concurrent functioning of both uptake processes.

Finally, a model is postulated to explain a number of the _in vitro_ characteristics. The application of this model to an _in vivo_

preparation is discussed. Experiments to test this model have also been suggested.

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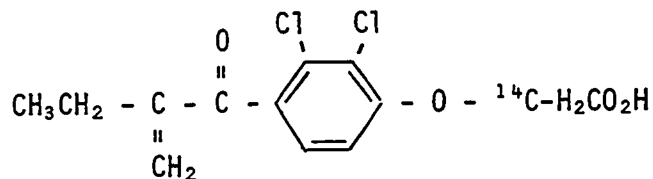
INTRODUCTION

General

About a decade ago Schultz and his co-workers (1962) described a series of α-β-unsaturated carbonyl derivatives of phenoxyacetic acid. These compounds had been developed during a search for non-organometallic inhibitors of sulfhydryl catalyzed enzyme systems. At the time, they believed that administration of such compounds to man, led to inhibition of some sulfhydryl systems which in turn caused diuresis with natriuresis. One of the more potent members of this series, 2,3-dichloro-4-(2-methylene butyryl)-phenoxy acetic acid, was named ethacrynic acid (EA) and later marketed for clinical use. The physico-chemical characteristics of EA have been well documented. The formula of this drug is shown below. It is a white crystalline compound with a melting point of 121-122°C, and is sparingly soluble in water and aqueous acids. It is an organic acid with a pKa of 3.5, and it forms salts with bases quite readily.



Ethacrynic Acid

The market potential of this agent as a clinical diuretic that could replace the organomercurials was immediately recognized, and its pharmacological and physiological effects were quickly investigated. The workers at Merck Sharp & Dohme laboratories in Pennsylvania, were largely responsible for the early studies of the effects of this drug

in experimental animals, and in 1965 Beyer et al. reviewed their findings in some detail. Most of the major pharmacological and physiological parameters, such as the drug clearance, the effective dose, metabolic implications, excretion rates and toxicity had been established from experiments with dogs while other species had also been investigated in some of these studies. Several clinical studies in man (Baer et al., 1962; Baer et al., 1963; Cannon et al., 1963; Cannon et al., 1965; Daley and Evans, 1963; Foltz, 1963; Hagedorn et al., 1965; Melvin et al., 1963) had also been carried out by this time and the effectiveness of EA had been demonstrated in various edematous conditions.

The dosage for man varies from 50 to 150 mg per day with a maximal permissible dose of 400 mg. The drug will produce a prompt diuresis and natriuresis; a peak effect can be attained in 15 to 30 minutes after intravenous administration, and within 2 hours after oral ingestion. The drug is readily absorbed from the gastro-intestinal tract. In dogs it is not substantially degraded, since less than 1% of a ^{14}C-labelled dose reappears as $^{14}CO_2$, irrespective of the mode of administration. After intravenous administration of the drug, 30% is excreted unchanged in the urine. The remainder undergoes biliary excretion and is not absorbed in the intestine. Other than its renal effect, no significant pharmacological effect has been demonstrated convincingly. Its side effects may include vomiting and diarrhoea, and changes in hepatic function have been reported. With extensive use hyperuricemia is fairly common. Deafness is a more serious side effect that has been reported, with the use of this drug (Pillay et al., 1969). Although the mechanism is not fully understood, it has been shown to be involved with

the loss of hair cells from the organ of Corti (Mathog and Klein, 1969; Meriwether et al., 1971).

Ethacrynic acid shows both similarities and dissimilarities when compared with other diuretic agents. Though it is chemically quite different from furosemide, it displays a marked similarity in its action. Like the organomercurials it is both natriuretic and chloruretic, but unlike the organomercurials it is not inhibited by alkalosis or hypochloremia. Like the thiazides and acetazolamides, diuresis induced by EA is accompanied by elevated potassium excretion, but unlike them the bicarbonate excretion remains unchanged or decreases. It does not inhibit carbonic anhydrase. These few examples are cited to show the unique position the drug occupies among the diuretic agents.

The initial investigations with EA dealt largely with its physiological functions. There is no doubt that the drug acts on the kidney. However, attempts to investigate intrarenal action have been only partly successful. Perhaps the magnitude of the work that has gone into the study of this drug is a measure of the complexity of its function. In describing its renotropic characteristics four years after its introduction, Beyer et al. (1965) cited 43 references. Thus this introduction is not intended to be a review but rather a summary that highlights the areas of conflict and of agreement, particularly those that may contribute to the elucidation of its mechanism of action. It is divided into two major parts, the physiological and the biochemical considerations. Where necessary further subdivision has been made.

Physiological Considerations

Beyer et al. (1965) worked essentially with dogs and employed a number of different techniques to examine the physiological and pharmacological characteristics of this drug. They examined the dose relationships of the natriuretic and diuretic response to this drug and found that in addition to drug concentrations, there existed a time response. Whereas a high dose produced a rapid response, there was a delay before the response was observed with a low dose of the drug. The authors interpreted this delay as evidence for an accumulation of the drug at an active site. The diuretic and natriuretic response to this drug was not influenced by acidosis or alkalosis. The results from stop-flow experiments with EA suggested that sodium reabsorption was depressed in the proximal and distal tubules. By examining total sodium levels in kidneys removed during diuresis, Beyer et al. (1965) also found that EA caused a "washing out" of the sodium gradient. They deduced from this that EA inhibits the mechanism responsible for maintaining the sodium gradient. In a similar approach, Goldberg et al. (1964) treated dogs with EA and removed their kidneys at the peak of the diuresis. The kidneys were immediately frozen, and then sliced. By determining the solute content of these slices Goldberg et al. (1964) demonstrated a complete loss of the sodium gradient in the kidney. He proposed that EA specifically blocks sodium transport in the ascending limb of the loop of Henle, and though other sites of action may exist for this drug, they are of minor importance. This belief is a popular one (Cooke and Lindeman, 1965; Earley and Friedler, 1964; Goldberg et

al., 1964; MacGaffey et al., 1964). From clearance studies Beyer and his associates also showed the effect of EA on hydrated dogs, and dogs made "hydropenic" by salt loading with concurrent administration of anti-diuretic hormone. Diuresis was induced in both situations, with a tendency for the osmolarity of the urine to approach that of plasma. That is to say, in the hydrated state the positive free water production was reduced, and in the dehydrated dog the negative free water production was reduced. They interpret this result as further support of the earlier finding from stop-flow experiments that the distal tubule is involved in the action of this drug. This concept of a second site of action for EA is also shared by Flannigan and Ackerman (1966). These authors suggest that the loss of sodium and water is far too great to be a consequence of inhibition of sodium reabsorption in the ascending limb of Henle's loop alone. Their experimental results showed a non-significant decrease in the clearance of inulin and p-amino-hippuric acid (PAH) in humans treated with EA. Nevertheless Flannigan and Ackerman (1966) postulated that the drug has some effect on the proximal tubule such that an increased load of solute and water is presented to the loop of Henle. More recently, work by Radó et al.(1970) has supported this view. Their investigation was based on their earlier method (Radó et al., 1969) which detects the "latent" proximal tubular effect of diuretics having a dual site of action. In this method, a proximal tubular action is inferred if the diuretic agent induces an increase in free water clearance during angiotensin effect. They worked with healthy volunteers and from their results concluded that in addi-

tion to a major effect in the loop of Henle EA also has a proximal tubular action.

Thus the proximal tubule cannot be excluded as a possible site of action, and with the distal tubule the evidence is even less definite. Potassium and hydrogen ions are secreted in this region of the tubule by exchange with sodium: since EA increases the excretion of potassium and hydrogen ions (Fülgraf et al., 1971; Goldberg et al., 1964), it is unlikely that sodium absorption is totally inhibited in this region. However, the results from the stop-flow experiments suggest that the distal tubule may indeed be another site for the action of EA.

Finally the work of Barger (1966) is cited. He has shown that changes in blood flow within the kidney can influence sodium reabsorption. If the blood flow is distributed largely to the inner cortical and outer medullary regions, then sodium retention is observed. Since EA is known to induce changes in blood flow in the kidney (Fülgraf et al., 1971), it introduces further complexity in the interpretation of the results from stop-flow and clearance studies.

With the use of a ^{14}C-labelled preparation of EA, Beyer et al. (1965) were able to show that this drug is filtered, secreted and reabsorbed. In this work they also studied the effects of probenecid, on the ratio of excreted drug to creatinine. Probenecid, an organic acid, inhibits the renal excretion of PAH (Beyer, 1954) and creatinine (Swanson and Hakim, 1962), and this inhibition is generally seen as evidence of secretion by the organic acid secretory mechanism. Their results indicate that EA is transported in the manner of the organic acids. Furthermore, stop-flow experiments with PAH confirmed this finding.

The excretion pattern of ^{14}C-EA was similar to that of PAH in the presence and absence of probenecid.

From this brief discussion of some physiological investigations it is clear that diuresis by EA occurs through a complex mechanism, and the observations that have been made can be listed as follows:

(1) Ethacrynic acid appears to act on the ascending limb of the loop of Henle.

(2) It definitely decreases the sodium gradient in the kidney.

(3) The loss of sodium gradient is believed to occur by inhibition of active sodium reabsorption in the loop.

(4) Sodium reabsorption in the proximal tubule may also be inhibited by EA.

(5) The distal tubule is a less likely site of action.

(6) Probenecid inhibits the excretion of EA and in this respect it is thought to be similar to PAH.

(7) The excretion of EA is possibly effected by the organic acid carrier.

If these findings are considered at the cellular level, they suggest that the drug action of EA is involved with metabolic and membrane phenomena and these aspects are dealt with in the following section.

Biochemical Considerations

The biochemical investigation into the mechanism of action of EA is immense and four subdivisions have been used to discuss this area.

(1) Na-K-ATPase involvement

(2) Permeability changes

(3) A secondary sodium pump

(4) Metabolic effects

Na-K-ATPase involvement

By far the most work has gone into the investigation of Na-K-ATPase as a possible "receptor" for EA.

Na-K-ATPase was first isolated by Skou in 1957, and shown to be an enzyme that was implicated in the active transport of sodium ions in many systems. It is a membrane bound ATP hydrolysing system which, *in vitro*, uniquely requires the cooperative effect of both sodium and potassium ions for full activation; this enzyme system has vectorial properties, and can be specifically inhibited by low concentrations of cardiac glycosides. It has been found in almost all animal tissues examined, and in particular the kidney is well endowed with this enzyme. Association of this fact with the reabsorption of sodium by the tubules seemed obvious (Katz and Epstein, 1967). Changes in tubular transport of sodium can be correlated with activity of Na-K-ATPase. Katz and Epstein (1967) found that after unilateral nephrectomy in rats, both the tubular transport of sodium and Na-K-ATPase activity increased in

the remaining kidney. Potter et al. (1969) re-examined this finding. After unilateral nephrectomy on rats, they studied the remaining kidney at short intervals. For the first two hours the glomerular filtration rate and the tubular transport of sodium showed comparable increases of about 10 to 15%. A further increase in these two parameters was noticed 18 hours after surgery. Yet they were unable to detect any change in Na-K-ATPase activity at these intervals. This questions the involvement of Na-K-ATPase in the tubular transport of sodium. However, the kidney is capable of varying its intra-renal function (Barger, 1966; Gertz et al., 1969; Horster and Thurau, 1968) and the results of Potter and co-workers could be due to changes in hemodynamics resulting from surgical trauma. The important point in this work is the finding that factors other than Na-K-ATPase could influence sodium transport. Some additional support for this idea came from Fanestil (1968) who showed that an increase in load to the kidney was not the stimulus for an increase in Na-K-ATPase activity. In his research he could only find correlation of increase in Na-K-ATPase activity with increase in kidney mass.

Na-K-ATPase is susceptible to inhibition by cardiac glycosides like ouabain, which has been extensively used to probe the involvement of Na-K-ATPase with transport of ions across biological membranes. Furthermore it is well known that the cardiac glycosides can act on renal tubules to produce a natriuresis (Farber et al., 1951; Hyman et al., 1956; Orloff and Burg, 1960). These glycosides can also be used in in vitro experiments to inhibit the Na- and K-activated portion of renal ATPase. A large amount of information has been built up on the comparison of these two situations. There is good correlation between natri-

uresis induced by ouabain, and the decrease in kidney microsomal Na-K-ATPase activity (Allen _et al_., 1969; Hook, 1965; Nelson and Nechay, 1969). The transition from inhibition of Na-K-ATPase activity to characterization of this enzyme as a receptor for natriuretic agents has not been quite so straightforward. A number of diuretic agents such as EA, furosemide, the mercurials, and amiloride will inhibit Na-K-ATPase _in vitro_ (Duggan and Noll, 1965; Ebel _et al_., 1969; Jones _et al_., 1965; Landon and Norris, 1963; Nechay _et al_., 1967; Rendi and Uhr, 1964; Taylor, 1963); however, other natriuretic agents such as the thiazides, the xanthines, spironolactone, and acetazolamide have no effect on the isolated enzyme preparation (Duggan and Noll, 1965; Frazer, 1963; Nechay _et al_., 1967; Taylor, 1963). In addition, Na-K-ATPase can be inhbited _in vitro_ by compounds which do not possess natriuretic activity in man or experimental animals (Frazer, 1963; Jones _et al_., 1965; Nechay _et al_., 1967; Bendi and Uhr, 1964; Taylor, 1963). Among these, p-chloromercuribenzoate (PCMB) and p-chloromercurisulphonate (PCBS) are potent inhibitors of Na-K-ATPase comparable to the mercurial diuretics (Nechay _et al_., 1967). Generally then, agents may cause natriuresis _in vivo_ and inhibit Na-K-ATPase _in vitro_, or they may be capable of one function and not the other. Ethacrynic acid will cause natriuresis _in vivo_ and will inhibit Na-K-ATPase _in vitro_, but a causal relationship has not been established.

One approach to relating these phenomena would be to characterize structure-activity relationships for the inhibitors of Na-K-ATPase and then to demonstrate presence of these groups on the EA molecule.

Na-K-ATPase from several tissues has been shown to contain sulfhydryl groups which are necessary for enzyme activity (Beyer et al., 1965; Glynn, 1963; Skou, 1963). Conversely, a number of reagents that combine with sulfhydryl groups such as N-ethyl-maleimide will inhibit Na-K-ATPase (Skou, 1965). Moreover, as pointed out earlier, sulfhydryl inhibition was one of the reasons for which EA was designed as an aryloxy acetic acid derivative. The sulfhydryl binding characteristics had been demonstrated very early on by Beyer et al. (1965). They showed that dimercaprol (BAL) would combine with EA in vitro; however, they were unable to antagonize its natriuretic effect with a dimercaptan. Duggan and Noll (1965) took a more systematic approach to this problem. Working with ATPase from guinea-pig renal cortex, they found that the methylene group of the EA molecule was essential for inhibition of Na-K-ATPase and was also necessary for sulfhydryl binding activity. By putting substituents on the aromatic nucleus they showed the existence of a steric relationship as well. Increased substitution of the aromatic ring caused a decrease in both sulfhydryl binding activity as well as Na-K-ATPase inhibition. However, they felt their data were inadequate to prove that the sulfhydryl groups on the enzyme were specifically the loci for inhibition by EA. This concept was presented again some years later by Davis (1970). He reasoned that if sulfhydryl groups are the receptors for the inhibition of ATPase by EA, then cysteine, which binds to sulfhydryl groups should show competitive antagonism of EA. His results supported this concept. However, he recognized that the presence of cysteine in the system could result in a decrease in the concentration of EA that was available for inhibition. Therefore

he did not interpret his data as evidence for direct association between enzyme inhibition and sulfhydryl groups. So the overall evidence from structure-activity relationships does not deny, but does little to support the hypothesis that Na-K-ATPase and natriuresis induced by EA, are related.

It is known that the rat has a high tolerance for cardiac glycosides (Reiter, 1953) and it is possible that this tolerance extends to EA. High doses of EA have been shown to cause diuresis in this species (Deetjen et al., 1969; Zins et al., 1968). Zins et al. (1968) used the equivalent of 250-times the average human dose to demonstrate diuresis, which perhaps may be interpreted as high tolerance. It is generally accepted that EA is not effective as a natriuretic agent in the rat (Beyer et al., 1965; Cole et al., 1969). With this in mind, Hook and Williamson (1965) showed an incongruity in the natriuretic effect of EA and its ability to inhibit Na-K-ATPase. Ethacrynic acid will inhibit Na-K-ATPase from rat kidneys in vitro despite its inability to cause natriuresis in vivo. By way of comparison they showed that furosemide has both actions; it will inhibit the ATPase in vitro and cause natriuresis in vivo in this species. A disparity was also demonstrated with the plasma membrane fractions of rat kidneys by Ebel et al. (1969). In this preparation they found that furosemide did not affect Na-K-ATPase activity while EA actually increased the activity of this enzyme. Cole et al. (1969) precluded the uniqueness of species as a possible explanation. Using an analogue of EA, 2,3-dichloro-4-(2-ethylidine butyryl)phenoxy acetic acid (L 589-420) they showed it to be an agent with potent natriuretic activity in dogs, yet

it increased the activity of microsomal Na-K-ATPase from homogenates of dog kidney. They also found that intravenous administration of L 589-420 to dogs did not influence the activity of microsomal Na-K-ATPase subsequently isolated from the cortex or medulla of the kidneys from these animals.

Nechay et al. (1967) drew attention to another obstacle in the supposition that Na-K-ATPase and EA induced natriuresis are related. They examined the levels of drug in the kidney during diuresis and the drug concentrations necessary for inhibition of Na-K-ATPase in vitro. At a concentration of 10^{-3} M, EA caused a 30% inhibition of Na-K-ATPase activity of isolated dog kidney microsomes. This was 1000 to 2000 times the figure they obtained for EA in a similar fraction prepared from a kidney removed at the peak of the natriuretic response. They concluded, that unless a drug concentrating mechanism is shown to exist in the kidney, the hypothesis that Na-K-ATPase is a receptor for EA is an unlikely one. This view is held by other workers (Beyer et al., 1965; Charnock, 1970).

On the other hand some indirect evidence in favour of the relationship came from Charnock et al. (1970). Using a microsomal preparation from guinea pig cortex, they showed that the inhibition of Na-K-ATPase by EA could be enhanced by lowering the potassium concentration in the medium, a finding similar to that of Binder et al.(1966) who used EA to inhibit the transport of sodium into sacs made from everted guinea pig intestines. By lowering the potassium concentration in the medium they were able to increase this inhibition. This potassium dependence is pertinent because it has long been known that ouabain inhibition of

Na-K-ATPase *in vitro* can also be prevented by increasing the concentration of this ion in the reaction mixture (Dunham and Glynn, 1961). Furthermore, ouabain induced natriuresis, can also be prevented or reversed by increasing the potassium levels in renal plasma (Cade *et al.*, 1961; Heidenreich *et al.*,1966; Nechay and Pardee, 1965; Palmer and Nechay, 1964).

Thus the overall position is unclear. The evidence in favour of the hypothesis that EA inhibits Na-K-ATPase to produce natriuresis, is inconclusive, but it may not be ignored. If it is assumed that Na-K-ATPase is not the "receptor" for EA, then three other mechanisms can be called upon to explain natriuresis. Ethacrynic acid may induce a change in membrane permeability to ions, or it may interfere with the metabolic production of energy and lastly, the drug may inhibit some other pump mechanisms. The evidence from the literature does not exclude any one of these possibilities.

Permeability changes

Fülgraf (1969) has cited an extensive list of references in support of the correlation between renal venous oxygen (VO_2) and sodium reabsorption (T_{Na}). Since active transport requires both substrate and O_2, any change in the transport efficiency should be reflected in the VO_2/T_{Na} ratio. Or, if the efficiency is constant the VO_2/T_{Na} should be constant. This test system can be used to probe the action of diuretic agents, provided the drugs do not uncouple sodium transport and oxygen consumption. Having satisfied himself that this condition was met

(Fujimoto et al., 1964; Senft et al., 1966) Fülgraf examined EA and furosemide by this test and found results which were very similar for both drugs. Urine volume increased and sodium reabsorption was reduced to about 70% of the filtered load; but oxygen consumption remained constant. That is, the quotient T_{Na}/VO_2 was significantly decreased by about 30%. He also tested ouabain and found that the reabsorbed sodium was reduced to about 83% of the filtered load but in this experiment the oxygen consumption was also reduced. That is to say, the T_{Na}/VO_2 remained constant. This latter result was similar to the findings of Deetjen and Kramer (1961) and Kessler et al. (1965) for the mercurial agents, and to those of Thurau (1961) with hydrochlorothiazide. The obvious conclusion from these experiments is that ouabain, the mercurials, and hydrochlorothiazide act by inhibition of active sodium transport, but some other mechanism must exist for EA and furosemide.

Based on the assumption that the utilization of oxygen has not been impaired, two interpretations have been offered to explain the results of the experiments described above. The first postulates a change in permeability of the peritubular membrane. This could cause a back diffusion of sodium ions which in turn could lower the net efficiency of the sodium pumping mechanism. Further support for this hypothesis was provided by Holzgreve et al. (1965). With micropuncture studies they showed that the unidirectional sodium influx in a sodium-free tubular perfusion solution is increased by furosemide. A similar increased permeability of frog skin (Herms and Hoffman, 1965) to sodium has been shown after treatment with EA and furosemide.

An alternative explanation suggests that a change in electri-

cal properties of the cell could be the cause of increased oxygen consumption. The internal resistance of the cell must be overcome by the transport mechanism if sodium is to move into the cell. If the drug increases the internal resistance of the cell, then the energy requirement and consequently the oxygen requirement would increase for the same amount of sodium transported. Both effects imply a change in permeability of the membrane.

The possibility that membrane permeability to anions could vary, was demonstrated by Eigler _et al_. (1966). They found that EA enhances the short circuit current and the potential difference of toad skin. This effect was more rapid when the drug was applied to the outer surface of the skin. The authors offer the explanation that EA increases the permability of the toad skin to sodium ions which results in an increased short circuit current. Moreover, this increased permeability is not as marked for chloride ions and this would explain the increase in potential difference. Here again, the point is made that a change in membrane permeability can result from the application of EA.

A secondary sodium pump

The idea of a second pump, as opposed to the Na-K-ATPase pump began in 1964 with the work of Kleinzeller and Knotkova. They caused slices of rabbit kidney cortex to swell and lose potassium in a K-free medium containing lithium, at 0°C. These slices when warmed to 25°C in a K-free medium or a medium containing ouabain, lost sodium and chloride ions, some lithium, and also some potassium. As the experi-

mental conditions under which these ions are extruded (K-free or ouabain) preclude activation of the classical Na-K-pump, it seems reasonable to suppose that some other mechanism must be in operation. Furthermore, they found that 0.3 mM ouabain neither affected this extrusion mechanism nor inhibited the respiration of the slice. Alternatively 0.1 mM DNP completely inhibited the water loss and the electrolyte extrusion. They saw this as evidence of a new mechanism for water and sodium chloride movement; possibly a volume control mechanism. Their inability to locate a similar mechanism in slices of rabbit liver increased their confidence in the existence of a second pump. The kidney is after all an organ of water control.

Two years later Hoffman and Kregenow (1966) specifically referred to a second Na pump for the red blood cell. This pump differed from the Na-K-ATPase driven pump in a number of important ways. It was insensitive to ouabain, it did not require extracellular K and it did not use ATP. However, it was sensitive to EA, and had a requirement for extracellular sodium. Measured in output they estimated this second pump would eliminate as much as one third of the sodium pumped by the Na-K-ATPase pump. To make this study these workers also preloaded their cells with sodium and then observed the rate of efflux of this ion. The loading was carried out by shaking for several hours at 37°C or in some instances by pre-incubation for 10 days at 0°C.

Whittembury and Proverbio (1970) working with slices from guinea pig kidney cortex, were also convinced of the existence of a second mechanism for the extrusion of sodium, from cells loaded with sodium at 0.6°C. One component is extruded on warming from 0.6°C to

25°C without potassium present which they designated mode A and another when potassium is present in the bathing medium during the warming period,which they called mode B. Mode A extrusion of sodium was accompanied by outward movement of chloride ions and mode B was accompanied by movement of potassium ions inwards. They also studied the effects of inhibitors on these two modes of sodium extrusion. Ouabain inhibition was directed mainly at mode B with some effect on A, whilst 2 mM EA inhibited mainly mode A with some effect on B. They claimed from their findings that the two pumps could be completely independent or superimposable. They also believed that the pumps had independent sites of action which displayed some interaction, and could not be separated. Since mode A had the extrusion of chloride and water coupled to it, it would be important for maintaining tubular sodium reabsorption. Proverbio and his co-workers (1970) subsequently claimed that the source of energy for pump II (which is responsible for mode B extrusion) was quite independent of the energy supply for pump I and that it was really a volume pump.

Quite obviously the information that is currently available on these 'secondary' mechanisms of sodium extrusion is far too scant to attempt to relate them to natriuresis or diuresis. However, they must be considered and the following observations have been made. Firstly, the arguments postulating a permeability effect of EA have not been completely excluded. Secondly, although it may not have been known then it is generally accepted now that a change in temperature like that involved in the experiments of the workers cited (Hoffman and Kregenow, 1966; Kleinzeller and Knotkova, 1964; Whittembury and Proverbio, 1970) can cause conformational and functional changes in membrane lipids and

proteins. Charnock *et al*. (1971) and Raison *et al*. (1971) have discussed these effects and both these papers cite numerous references pertaining to these phenomena. It now seems possible that a conformational change due to lowered temperature could produce a membrane that loses its elasticity. Warming the membrane could perhaps restore its elastic properties making the change in volume possible. If low temperatures cause conformational changes in the membrane, then changes in its other dimensions would not be unusual. Perhaps a potassium-free medium also causes changes in membrane properties. The permeability of the membrane and its electrical properties could well be different, which in turn would lead to new dimensions for ionic equilibria. Perhaps these views can be summed up in a single question: can it be that the vision of the apostles of the latter day pump is really a transfiguration of the membrane that can be explained thermodynamically?

Metabolic effects

Gordon and de Hartog (1969) inhibited glycolysis in Erlich ascites tumor cells and red cells in the absence of potassium transport. With ghost-free hemolysates and cell free extracts of Erlich ascites tumour cells these workers showed that EA inhibited lactate formation. The authors felt that this energy source could be the fuel for the second pump. This idea, that EA interferes with cellular metabolism rather than direct pump incapacitation was shared by MacKnight (1969). He showed that 1 mM EA prevented the volume recovery of swollen tissue slices from rat kidney cortex and caused a further increase in volume. With a slightly

lower concentration of EA, 0.75 mM, there was a tendency towards recovery, and at 0.5 mM drug concentration, the ability of the slices to restore their volume was fully operational. He also found that 0.1 mM DNP exhibited a time effect. The slices gradually lost their ability to recover their volume. From these results he inferred that EA inhibition was at the metabolic rather than the membrane level of cellular function. The author offers no comment on the concentrating effect that can be claimed from his results. There is a definite suggestion that should the drug action of EA be a consequence of metabolic inhibition, then a drug concentrating mechanism for this drug would still be necessary. However, it must be noted that even the lowest concentration of EA used by McKnight (1969), 0.5 mM is about eight times the concentration that would be expected from complete dispersion of a high dose of the drug in the total blood volume of an average man.

Daniel et al. (1971) working with rat uteri were able to duplicate the water and sodium accumulation that Whittembury and Proverbio (1970) achieved with slices from guinea pig kidney cortex. Like the latter workers they showed this accumulation could be reversed in the presence of ouabain but not in the presence of EA. However, in contrast to Whittembury and Proverbio (1970), Daniel et al. (1971) do not see this selective action on cell volume as inhibition of a sodium pump that controls cell volume. This is based on their finding that depletion of ATP by treatment of the tissue with a combination of IAA and DNP showed similar inhibition of recovery. Also, if the utilization of ATP is prevented by incubation at low temperature (5°C) then again the tissues fail to recover. This favours the idea of metabolic blockade

rather than a direct pump incapacitation, as the mechanism by which EA produces its effects.

Daniel et al. (1971) then examined the effect of EA on nucleotide levels, with a comparative study of electrolyte movements and substrate effects. Their evidence supports the idea that the mechanism of action of EA is through inhibition of metabolism. They observed that sodium gain and potassium loss can be correlated in time with depletion of ATP suggesting a run down of the energy supply; in this respect, ouabain did not reduce ATP levels. With either glucose or pyruvate as the substrate, the reduction in ATP levels due to EA could still be demonstrated suggesting inhibition of anaerobic metabolism. Studies with DNP showed a possible interference by EA on energy production from oxidative phosphorylation. Finally, the work demonstrates complete inhibition of mitochondrial succinic dehydrogenase activity and partial inhibition of lactate production. The authors feel that their data supports the hypothesis that EA completely inhibits ATP production from oxidative phosphorylation with partial inhibition of glycolysis. It is debatable as to whether or not this mechanism should be extrapolated to explain renotropic phenomena, but the evidence is certainly very strong in this work and it agrees with the findings of other workers engaged with this aspect of the problem. Perhaps its major weakness is the high drug concentrations that have been necessary to demonstrate these effects.

In summary, the introduction to this point has considered possible mechanisms of action for natriuresis and diuresis by EA at the cellular level. Some physiological and biochemical investigations have been assessed with emphasis on the latter. Four mechanisms are consid-

ered possible for the pharmacological action of EA. Thus:

(a) Na-K-ATPase is inhibited by EA.

(b) EA induces direct permeability changes in the cell membrane.

(c) EA inhibits a secondary sodium pump.

(d) EA inhibits metabolic pathways leading to loss of cellular transport processes.

Rationale

The first question that might be asked is whether or not the kidney is capable of concentrating EA. The concept of a concentrating mechanism is supported by considerable circumstantial evidence in much of the work that has been discussed. This is especially true of the experiments that have been done to show involvement of the Na-K-ATPase enzyme. In the works that have been cited the smallest concentration that demonstrated an inhibition of the enzyme was 1 mM which is about 16 times the plasma concentration that might be expected from an average dose given to man. This disparity was best demonstrated by Nechay *et al*. (1967) who estimated the drug concentration in the dog kidney removed at the peak of a diuretic episode. They found that the concentration of drug necessary for a 50% inhibition of the *in vitro* activity of kidney microsomes, was 1000 to 2000 times greater than the concentration of drug bound to kidney microsomes isolated from the *in vivo* experiment. The actual figures used, 29 μmoles for the *in vitro* and 15 ηmoles for the *in vivo* kidney microsomes, might be questioned on grounds of accuracy and precision. Although this evidence fails to demonstrate the absence of a concentrating mechanism it does suggest the need for one if the

differences in drug levels are to be reconciled.

Another observation supporting the concept of a concentrating mechanism for EA, was made by Beyer et al. (1965). They noted that after administration of a low dose of drug there was a time lag before the diuretic response was seen, whereas with a high dose the response was immediate. Not unreasonably they speculated that this delay could represent an accumulation process prior to the drug having its effect. One explanation for this discrepancy could be that the drug was excreted before the kidneys were removed. This is possible because EA is excreted very rapidly. However, an alternative explanation could be that the 'binding' phenomenon is greater with broken cells. Citing unpublished results, Beyer et al. (1965) describe the uptake of EA by slices of kidney cortex as qualitatively reproducible but quantitatively unreliable because of 'considerable binding of EA to the slice'. We felt that the error due to adsorption could be reduced by comparing uptake of the drug during standard and anoxic conditions (Despopoulos and Callahan, 1962). We chose to use tissue slices in an attempt to further minimize the problem of binding, as the ratio of whole or unbroken cells to damaged cells is much greater than is the case in homogenates or fractured membrane (that is, microsomal) preparations. The slice system would also overcome possible effects due to hemodynamic changes which could occur with whole animal experiments.

If a concentrating mechanism were established, two lines of research could be pursued: investigation of the involvement of ATP, or a study of the parameters of the transport of EA. It must, however, be emphasized at the outset, that neither one of these approaches is en-

tirely independent of the other.

To investigate ATP involvement, experiments could be designed to examine the correlation between concentrating activity of the slice with its ATP hydrolysing activity. This would depend on ATP being the source of energy for the uptake. A large amount of information is available on the ATP-hydrolysing enzymes and this could be applied to the uptake system of EA. However, the problem of distinguishing between the hydrolysis of ATP by cut or damaged cell surfaces from that occurring by intact cells is a major technical difficulty in this approach.

The appeal of the transport approach is greater in that it encompasses the ATP involvement. In many cases the energy that drives the transport mechanism is derived from ATP. In the words of Whittam and Wheeler (1970), 'the feature of transport which has become best established is the nature of the ATPase reaction catalysed by cell membranes'.

In planning such a study it is necessary to consider the criteria for transport, which are both numerous and varied. They differ with the organ or system in which transport is being studied, and also with the solute being transported. No list of criteria is universally acceptable. The headings listed below, are some of the investigative features chosen for this study, followed by the questions asked:

Active transport: Is EA accumulated in the kidney against a concentration gradient? Is this concentrating mechanism peculiar to kidney tissue?

Morphological effects: Does EA effect tissue morphology? Is the morphology changed by the experimental procedure? Is the extracellular

space changed by EA?

Metabolic process: Is glucose necessary for the uptake? Is oxygen necessary for uptake? Is the uptake process sensitive to temperature?

Metabolic energy: Is the uptake influenced by metabolic inhibitors such as 2,4-dinitrophenol and sodium azide?

Transport inhibition: Is the uptake diminished or retarded by the classical inhibitors of transport mechanisms, ouabain and probenecid?

Characterization of transport: Is the transport of EA a sodium dependent process or is it of the organic acid type movement, or does it have an exclusive carrier?

In general, this scheme was adhered to except where it was necessary to heed the advice of the poet Burns (1876), concerning '...the best laid schemes of mice and men...'.

MATERIALS AND METHODS

1. Practical Aspects

(a) Ethacrynic acid.

(b) Extracellular markers.

(c) Radioactive counting.

(d) Solutions: wash solution and incubation solution.

(e) Preparation of tissue slices.

(f) Electron microscopy and autoradiography.

(g) Drug concentrations.

2. Theoretical Aspects

(a) Expression of results.

(b) Sources of error and variation.

Ethacrynic Acid

The radiochemical drug labelled with ^{14}C at the 2-C position, (see page 1) was obtained as a gift from Merck Sharp & Dohme Laboratories, Rahway, N.J., U.S.A. It was supplied as a solid in a vial labelled as 50 mg and about 100 μc. By addition to the vial of a buffer solution of 2.0 M Tris base (Mann Research Laboratories), the drug was dissolved in a minimal volume, of about 0.5 ml. The pH of this solution was then adjusted to about 7.5 with 1.0 M hydrochloric acid, as determined by pH paper (Whatman-BDH). The volume was made up to 1.0 ml with water. Hereafter this solution is referred to as stock EA solution.

Stock EA solution was diluted a thousand fold (0.01 to 10 ml) and this solution was examined for chemical and radiochemical purity.

The purity of the labelled EA was assessed chromatographically. An aliquot of the diluted stock solution was spotted on Whatman #1 chromatography paper, and run in a butanol:acetic acid:water (12:3:5) system according to the method of Bourke et al. (1966). After development of the chromatogram, the EA could be seen as a single spot under a short wavelength ultraviolet lamp. One chromatogram was cut into centimetre square pieces and counted in a Beckman LS-100 liquid scintillation system. By this method the radioactivity was also located in one spot; it represented a recovery of 87%. On a second chromatogram the EA was aminated by standing it in a vapour of ammonia for one hour. The excess ammonia was blown off and the chromatogram was sprayed with ninhydrin and developed. The single stained spot which was detected

was again counted and represented a recovered fraction of 84% which is similar to the previous value.

The purity of the radiochemical was also assessed spectrophotometrically. Ethacrynic acid has an absorbance peak at 278 mμ. With water in the reference cuvette, the diluted stock EA was scanned over the range 200 mμ to 300 mμ (see Appendix A). The spectrum of the radiochemical drug compared favourably with the spectrum of a standard solution of EA made from unlabelled drug of certified purity (also obtained as a gift from Merck, Sharp & Dohme Laboratories). The absorbance at 278 mμ was also recorded and assayed against a known standard. The concentration of the EA in the stock solution was calculated to be 0.147 M. This is 89% of that on the label.

The activity of the radiochemical was also determined by counting 50 λ aliquots of the diluted stock solution. A mean value of nine aliquots was 10034 dpm per aliquot and this figure represented 90% of the label claim. Based on these investigations a specific activity of 1.33×10^6 dpm/μmole EA was assigned to the stock solution. These experiments confirm the purity of the radiolabelled drug as established spectrally.

Extracellular Markers

Three radiolabelled compounds for use as extracellular markers were obtained from New England Nuclear, Boston, Massachusetts.

[1-^{3}H]-mannitol had a specific activity of 3.82×10^9 dpm/mg. It was used at a concentration of about 50,000 dpm/ml of incubation med-

ium, to study the effect of EA on the extracellular space of tissue slices.

$[^{14}C]$-D-mannitol with a specific activity of 6.13×10^8 dpm/mg and [carboxyl-^{14}C]-inulin (molecular weight 5000 to 5500) with a specific activity of 7.6×10^6 dpm/mg were both used at a concentration of about 100,000 dpm/ml of incubation medium, to mark the extracellular spaces of tissue slices incubated for various time intervals.

Radioactive Counting

Aqueous samples of carbon-14 or tritium solutions, were counted in a toluene fluor containing 5% BBS-3 (Beckman Biosolve) and 0.5% PPO (2,5-diphenyloxazole). Ten ml of this fluor could accomodate up to 0.5 ml of water or aqueous solution with no detectable increase in quench.

Tissue samples containing carbon-14 were dissolved in NCS tissue solubilizer (obtained from Amersham/Searle). NCS is supplied as a 0.6 N solution in toluene. 0.5 ml of this solution was added to the tissue slice in a counting vial, and it was left to stand overnight at room temperature. Subsequently the NCS was reduced and the toluene increased, by using 1.0 ml of 0.12 N solution of NCS in toluene. After the tissue had dissolved, 10 ml of a toluene fluor containing 5% BBS-2 (Beckman Biosolve) and 0.5% PPO were added to the vial, followed by two drops (one drop when the NCS was reduced) of freshly prepared 4% stannous chloride in 0.1 N hydrochloric acid. The samples were usually dark adapted overnight and then counted. The minimum period of dark

adaptation was found to be four hours.

Tissue samples containing tritium were dried overnight at room temperature, on 2 cm squares of filter paper. They were combusted in the tritium oxidizer (Packard tri-carb) and counted as aqueous samples.

To determine the background radioactivity, a slice of tissue without any prior treatment was dissolved in NCS. It was subsequently treated in a manner similar to the experimental slices and counted. The radioactivity in cpm (counts per minute) was subtracted from the raw counts. Such a sample was prepared for every experiment.

The Beckman LS-100 liquid scintillation system is designed to determine the degree of quench in a sample by the external standard method. It uses caesium-137 as the external standard, and the gate settings are calibrated at installation.

To examine the accuracy and reproducibility of the instrument, quenched samples obtained from Nuclear Chicago Company were used to prepare quench correction curves by the external standard method and by the method of channels ratio. The results are shown in Appendix B. The quench correction curves were then used to determine the radioactivity in samples quenched with a solution of kidney tissue in NCS (100 mg/ml) and containing ^{14}C-EA. These results are shown in Appendix C. From this investigation it can be seen that the instrument performs adequately when it is used in any one of the three modes, external standard ratio, channels ratio, or the internal standard method. Thus the external standard method, as executed by the LS-100 liquid scintillator was accepted as an adequate measuring device for the degree of quench of the

samples.

The background radioactivity as determined by this machine and with the type of vials used (obtained from Amersham/Searle) was about 30 cpm. As with the tissue counting this value was subtracted from the raw counts.

The counting efficiency for carbon-14 in an aqueous sample was not less than 94% and for the tissue samples it was 85% or better. For tritium the efficiency was not less than 33%.

Solutions

All solutions were made up from distilled water which was passed through a battery of deionizing columns before use to obtain water having a resistance of not less than 9 megohms, measured prior to exposure to air.

Osmolarities of the solutions were determined with a freezing point depression apparatus (Advanced Instruments Inc.), and were maintained at 300 milliosmoles/liter.

Therapy grade oxygen (Liquid Carbonic, Canadian Corporation Ltd.) and grade G nitrogen (Canadian Liquid Air Ltd.) were used for gassing solutions when necessary. Gassing was achieved by vigorous bubbling through the solutions for one hour.

The solutions used for incubation of the tissues are referred to as incubation solutions and those used to bathe the tissues before, and rinse the tissues after the incubation are designated wash solutions.

Wash solutions

Normally the wash solution contained sodium chloride, 143 mM; potassium chloride, 5 mM; calcium chloride, 1 mM; magnesium sulphate, 2 mM; sodium acetate, 10 mM; disodium hydrogen phosphate, 5 mM; and glucose, 10 mM. All the ingredients except calcium chloride and glucose were dissolved in a stock solution which was adjusted to pH 7.4 with 1.0 M hydrochloric acid. This was then diluted to give a concentration of ingredients that was 1.25 times the required concentration. For use, 80 ml of this stock solution was diluted to 100 ml with water containing the required amount of calcium chloride and glucose and other reagents as necessary.

In a few experiments sodium chloride was replaced by potassium chloride, 143 mM. In other experiments it was necessary to maintain the concentration of potassium chloride at 5 mM and vary the sodium concentration upwards from a sodium-free solution. This was achieved by using choline chloride instead of sodium chloride, and substituting acetic acid for sodium acetate and potassium dihydrogen phosphate for disodium hydrogen phosphate. The potassium chloride was omitted and the solutions were buffered to pH 7.4 with 1.0 M Tris base.

Incubation solutions

The incubation solutions were made from the appropriate wash solutions, and contained ^{14}C-EA and where necessary other reagents. These solutions were also gassed prior to use.

Preparation of Tissue Slices

New Zealand, white rabbits, 2.5 to 3.0 Kg in weight were killed by a blow to the head. The kidneys, and on some occasions the liver, were removed immediately and placed in ice-cold wash solution which was maintained at ice temperature during the slicing procedure.

Tissue slices were hand cut with a Stadie-Riggs microtome (Stadie and Riggs, 1944) to a uniform thickness of about 0.5 mm. The first or outer slice was discarded so that all slices used had two cut surfaces. No more than two subsequent slices were cut from each kidney surface. The slices were trimmed to about one square centimetre so that their wet weight was from 45 to 55 mg. The prepared slices were kept moist and cold on a bed of kleenex tissue bathed periodically with ice-cold wash solution. Usually a maximum of sixteen slices was obtained from each animal. Both kidneys were not always necessary, and no attempt was made to randomize the left and right kidney.

For the incubation, the slices were put into 25 ml erlenmeyer flasks containing 5 ml of incubation medium at the temperature required for the experiment. The flasks were agitated at 90 cycles per minute in a Dubnoff metabolic shaking apparatus, that was continuously flushed with oxygen or nitrogen during the period of incubation. At the end of the incubation period the tissue slices were removed from their flasks with forceps, dipped twice in 15 ml of wash solution and drained on Kleenex tissue. The same 15 ml of wash solution was used for all the slices from one experiment. The washed and drained slices were placed into scintillation vials containing NCS and left overnight to dissolve.

Electron Microscopy and Autoradiography*

For electron microscopic examination, tissue slices were rinsed and drained before fixation in 5% glutaraldehyde in phosphate buffer (990 mOsm/litre at room temperature). They were postfixed for 60 min in 1% osmium tetroxide at room temperature, dehydrated by serial passage through graded ethanol and embedded in Epon. Sections were cut on a Porter-Blum MT-2 ultra microtome, stained with uranyl acetate and lead citrate before examination in an EM-7A electron microscope.

For autoradiography slices of kidney tissue were incubated in a medium containing 7.4×10^{-4} M ^{14}C-EA. The high concentration was used in the hope that it would overcome the low specific activity. Incubations were carried out for 30 minutes at two temperatures, 0°C and 37°C, to include the investigation of a possible temperature effect. One slice was prepared for autoradiography without prior incubation. This slice was used as the control to determine background radiation. One of the incubated slices was counted as a control measure to ascertain normal uptake behaviour.

The slices used for autoradiography were rinsed and drained as usual after the incubation, and then plunged into isopentane that had been cooled in liquid nitrogen. To store the frozen slices, they were quickly wrapped in aluminum foil, and placed in vials which were left suspended in liquid nitrogen until used.

Soluble compounds autoradiography according to the method of

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* Electron microscopy and autoradiography were done by Mr. G. Duchon and Dr. R. Varma.

Appleton (1964) was used in this investigation. This author has demonstrated that exposure to isopentane for short periods does not cause a loss of highly soluble compounds.

The frozen tissue is gradually warmed from its storage temperature (-196°C) by first transferring it to dry ice (-76°C) and after equilibration to the cryostat (-25°C). The tissue is frozen in place with a drop of water and sections are cut under full lighting conditions. The cut sections are transferred into cooled coverslips (-5°C). The coverslips are prepared with the appropriate emulsions. During exposure the sections are stored at -25°C. At intervals, the tissues are prepared for microscopic investigation and the films are developed.

Drug Concentration

The recommended diuretic dose of EA for man ranges from 50 to 150 mg. If it were possible to dissolve 100 mg of EA in the total body water of an average man weighing 70 Kg (average 61% water*) the concentration of the drug would be 7.4×10^{-6} M. A few preliminary experiments were carried out at the 10 mg, the 100 mg, and the 200 mg per man dose levels. Even at the lowest dose of EA (10 mg per average man) sufficient radiochemical was recovered to enable experimental examination. This concentration which was equal to 7.4×10^{-7} M was thereafter used as the minimum level in all further investigations.

The radioactivity of every incubation solution that was pre-

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* Documenta Geigy, Scientific Tables, Fifth Edition.

pared, was determined prior to use. The mean count of 80 such determinations from 12 different incubations was 994 dpm/ml with a standard error of 4 dpm/ml. Thus a figure of 1000 dpm/ml was assigned to a 7.4×10^{-7} M EA solution for ease of calculation.

Expression of Results and Sources of Error

Expression of results

Since mannitol is not actively transported into kidney cells (Rosenberg *et al*., 1962) we made use of this fact to measure extracellular space.

When the amount of drug or chemical determined in 1.0 g (wet weight) of tissue, expressed as disintegrations per gram wet weight (i.e., dpm/g w.w.) is reported as a fraction of the amount of drug in 1.0 millilitre of medium expressed as dpm/ml, then a slice:medium ration (S/M) is obtained. Three different phenomena can be defined by the slice:medium ratio:

(a) The drug may passively diffuse only into the extracellular spaces. In this situation the extracellular fluid and the bathing medium may come to equilibrium with respect to drug concentration, and the S/M ratio is less than unity.

(b) The drug may passively diffuse into the extracellular space as well as the intracellular space. Now the S/M ratio is unity or very close to it.

(c) If the S/M is greater than unity it implies that the drug is being concentrated within the tissue without any qualification of the

manner of accumulation. A S/M greater than unity would be expected from non-specific binding of the drug to cell membranes, or from active transport of the drug into the cell, or if a passively diffusing drug is metabolized on entry into the cell. This latter situation only applies to cases where the method used for drug assay does not distinguish between metabolites and native drug, as for example with radioactive counting. So in this work, it was essential to establish that any accumulated drug was not metabolized.

To investigate the possibility that drug metabolites were present, slices of tissue were incubated in a medium containing ^{14}C-EA for six hours. They were removed, rinsed and drained. One slice was dissolved in NCS and counted to establish the presence of an adequate quantity of drug in the tissue. The others were individually extracted as follows:

Slices were left to stand in 2 ml distilled water at 0°C. After two days (a week for two slices) the tissue was disrupted by hand homogenization and the tissue-free extract recovered by centrifugation. This extract was treated with five volumes of cold ethanol and allowed to stand for two hours at 0°C to precipitate dissolved proteins. The alcoholic supernatant was recovered by centrifugation and dried at 50°C. The residue was taken up in a few drops of ethanol. The ethanol extract was spotted on Whatman #1 chromatography paper and then allowed to equilibrate overnight. The chromatogram was run as described earlier, according to the method of Bourke et al., (1966).

Two chromatograms were cut into centimetre squares and counted by liquid scintillation, and two others were scanned on an actigraph (Nuclear Chicago, Actigraph 111). The results (Appendix D) showed no

evidence of more than a single labelled component. Visual comparison showed this spot to have migration characteristics that were similar to those of a spot developed from standard EA.

All numerical data reported in figures and tables are mean values with standard errors.

Sources of error and variation

The largest source of variation was in the response of the animal to the drug. This was accommodated by maintaining a paired control for every treatment.

A source of error that could not be controlled was the morphological depth of the kidney to which slices were cut. Although a total of just three slices were cut counting the first discarded one, there can be no guarantee that morphologically the same depth was achieved every time. The size of the kidneys and the thickness of the different areas within the kidney contribute to this error which is virtually uncontrollable.

Another variation in tissue parameters could have resulted from the thickness of the slice. Although the microtome is intended for the preparation of slices that are 0.5 mm thick, the accuracy of the instrument is subject to the consistency of the kidney tissue and the skill of the operator. The consistency showed some variation between animals.

The volume of the buffer used for the incubation did not contribute any inaccuracies. By way of comparison, a small number of experiments was carried out with 4 ml of incubation medium, but no difference from the control could be detected. These experiments are not re-

ported.

It is unlikely that any error was contributed by the rinsing operation or the draining of the tissue slice. The radioactivity in the rinse solution, after 16 slices had been rinsed, was less than twice the background level for each 0.5 ml aliquot. Thus, if 0.5 ml of rinse solution was transferred into the vial with the slice, the % error would still be very small.

RESULTS AND DISCUSSION

The Extracellular Space

Because the results were expressed as S/M ratios, it was first necessary to establish the pattern of uptake by tissue slices of an extracellular marker. [1-^{3}H]-mannitol at a concentration of 7.5 x 10^{-8} M was used as the marker in these experiments, and the effect of EA on the uptake of this marker was concurrently investigated. Figure 1 shows the pattern of uptake of ^{3}H-mannitol and Table I shows the effect of EA on this uptake.

The mannitol uptake into tissue slices was rapid for the first thirty minutes of incubation and was followed by a marked slowing over the second half hour. This suggests a typical movement of the marker as it equilibrates between the extracellular fluid and the incubation medium. With EA in the incubation medium there was no effect on the extracellular space or membrane permeability to mannitol as determined by S/M ratios. This finding does not directly refute the idea that EA may increase membrane permeability to sodium and anions as mentioned in the introduction (see page 14). It does, however, suggest that if such permeability changes occur, they do so without any change in the extracellular volume which can be measured by these procedures. In the experiment using ^{3}H-mannitol the slices were weighed after the incubation period, but no significant change in the wet weight could be detected (see Table I). The small loss in weight of about 2 mg (about 4%) is within the experimental error and may be attributed to evaporation during the weighing

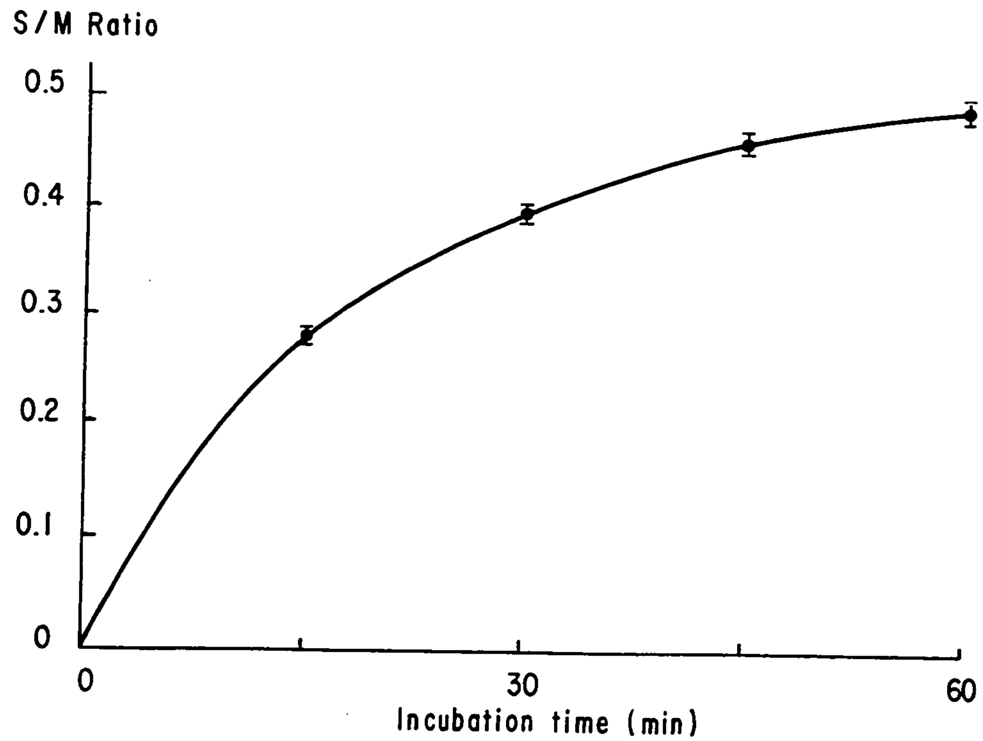

Figure 1. Distribution of ^{3}H-mannitol. Slices of kidney cortex were incubated at 37°C in an incubation medium containing a trace amount of [1-^{3}H]-mannitol. The vertical bars represent standard errors.

TABLE I

EFFECT OF EA ON ^{3}H-MANNITOL SPACE, AND ON THE WET WEIGHT OF TISSUE SLICES

	S/M Ratio at Incubation Intervals t = min				Wet Weight of Slices ($\overline{X}$ mg S.E.)	
					Before Incubation	After Incubation
t	15	30	45	60	0	Variable
Slices in Medium Containing ^{3}H-Mannitol	0.28	0.40	0.46	0.49	51.9±1.0	49.0±2.6
Number of slices	2	4	4	4	6	6
Slices in Medium Containing ^{3}H-Mannitol + EA	0.29	0.39	0,46	0.48	51.3±0.2	49.1±1.1
Number of Slices	2	4	4	4	6	6

process rather than an effect of treatment. Moreover this loss in weight is seen whether or not EA is present in the incubation medium. Quite clearly EA at a concentration of 7.4×10^{-7} M did not influence the so-called "mannitol space", nor did it have any detectable effect on the movement of water into the tissue during the first hour of treatment.

To determine the effect of incubation for longer intervals a similar series of experiments was done. Two extracellular markers, ^{14}C-mannitol and ^{14}C-inulin were used to determine S/M ratios. A concurrent investigation of tissue morphology by electron microscopy was also conducted. Because of this, only two slices were available for determination of the uptake of ^{14}C-mannitol or ^{14}C-inulin. The results we obtained are shown in Table II.

The longer incubation times showed no change in either mannitol or inulin space that could be attributed to EA. After eight hours there is an increase in S/M ratios of about 61% for mannitol and about 73% for inulin over the one hour values. Since neither ratio had reached unity (0.58 for mannitol and 0.33 for inulin) it must be inferred that the cell membranes were still intact, or at the least, their permeability to the markers mannitol and inulin had not changed to any great degree. Perhaps the relatively small increase in S/M ratio of about 8-10% per hour over the last seven hours represents an expansion in extracellular space rather than more complete equilibration. No explanation is available for the difference in the S/M ratios obtained with the two isotopes of mannitol, ^{3}H (Table I) and ^{14}C (Table II). The markers are supplied in an ethanol solution, and the possibility that the difference in S/M ratios was a consequence of the ethanol vehicle was investigated. The

TABLE II

EFFECT OF INCUBATION ON EXTRACELLULAR SPACE

Experimental Conditions*	S/M Ratio at Incubation Intervals t=min							
t	0	$\overline{X}$	30	$\overline{X}$	60	$\overline{X}$	480	$\overline{X}$
^{14}C-EA (7.4 x 10^{-} M)	0.20		5.1		7.9		13.8	
^{14}C-mannitol trace	0.03	0.03	0.36	0.31	0.36	0.36	0.59	0.58
^{14}C-mannitol trace + EA**	0.03		0.26		0.36		0.58	
^{14}C-inulin	0.03	0.02	0.16	0.16	0.19	0.19	0.31	0.33
^{14}C-inulin + EA**	0.02		0.15		0.19		0.35	

*One slice for each condition.

**EA = 7.4 x 10^{-7} M.

addition of 1% ethanol to the incubation medium had no significant effect on the S/M ratios of ^{14}C-mannitol (results not shown here). Further explanation of this anomaly was not pursued but the suggestion is offered that the difference in S/M ratios may have been a concentration phenomenon as it was noted that the concentration of ^{14}C-marker was 13 times that of the ^{3}H-marker.

Morphological Effects

Along with the experiments on extracellular space, we conducted a concurrent examination of the tissue morphology by electron microscopy so as to assess the state of the tissue after varying incubation times. In addition, we attempted a localisation of the drug within the tissue by autoradiography.

For the autoradiographic studies, the slices of tissue were incubated for 30 minutes in a medium containing EA, 7.4×10^{-5} M. It was hoped that the increased uptake resulting from this high concentration of drug would overcome the low specific activity (1.61 μC/μmole) of the drug. After 30 minutes the radioactivity in the slice was 26,000 dpm. An eight week exposure produced no detectable image on the film. Two observations must be made from this experiment. First, it is quite certain that this level of radioactivity failed to make any exposure on a photographic plate, and the use of autoradiography in this project would only be possible with a drug having a higher specific activity. Secondly, since there was no image on the plate, the tentative suggestion is offered that the radioactivity was diffused within the tissue and did not confine itself to the surface of the slice.

The results of the electron microscopic investigation[1] are

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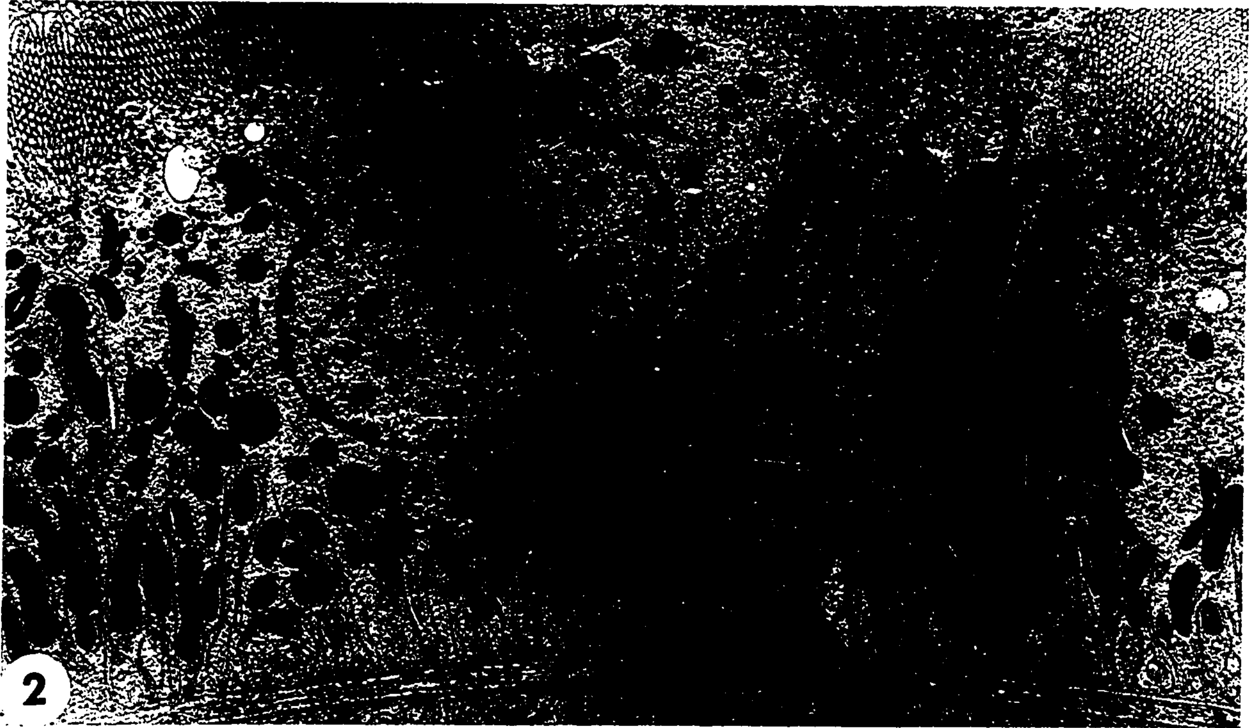
[1] We thank Dr. Rina Varma for her assistance with the autoradiography, and Mr. G. Duchon who performed the electron microscopy. We also acknowledge the contributions of Dr. Ruth M. Henderson who assisted with the interpretation of the electron micrographs.

Figure 2. Electron micrograph of rabbit kidney slice before incubation.

Figure 3. Electron micrograph of rabbit kidney slice after 10 minutes of incubation at 37°C in an oxygenated isotonic buffered medium containing salts and glucose as described in the text.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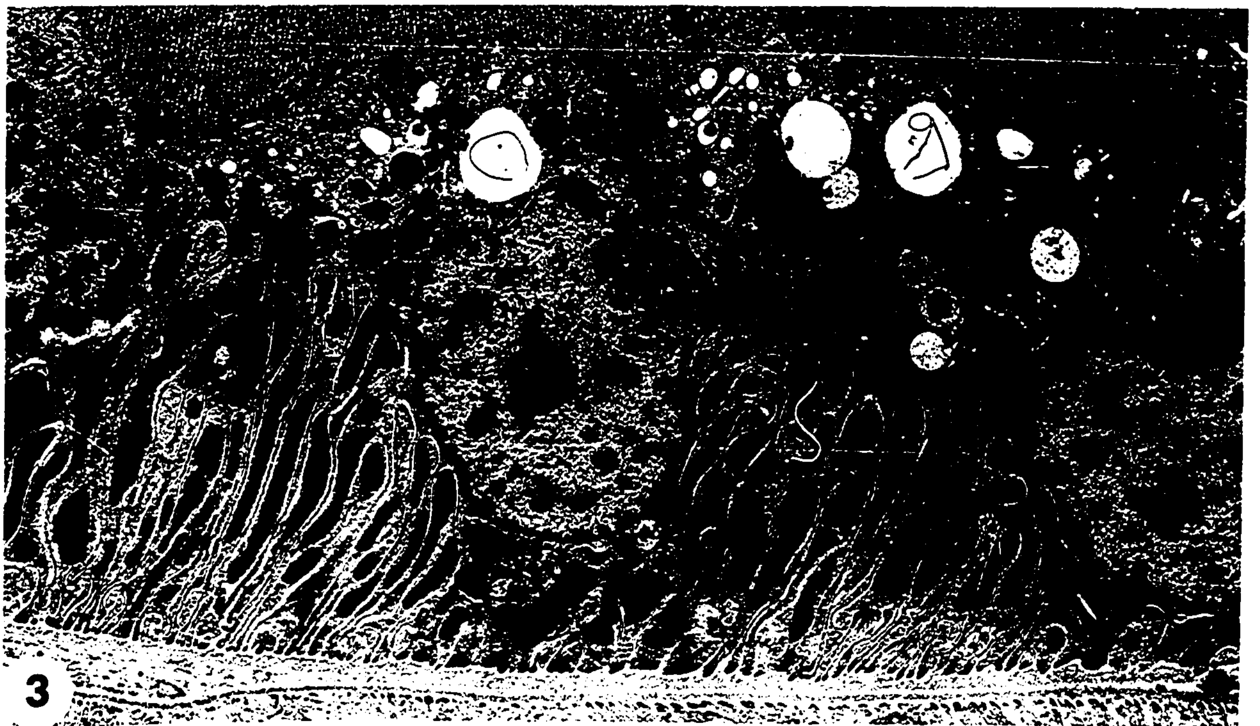
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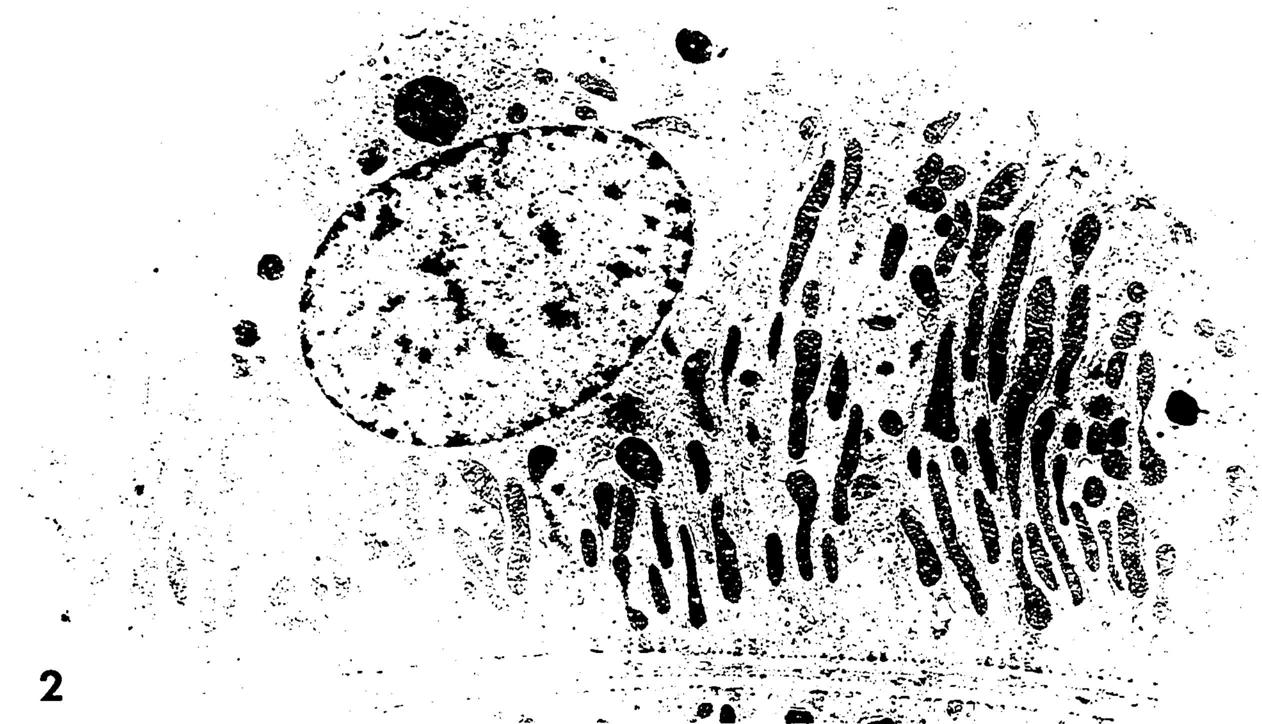

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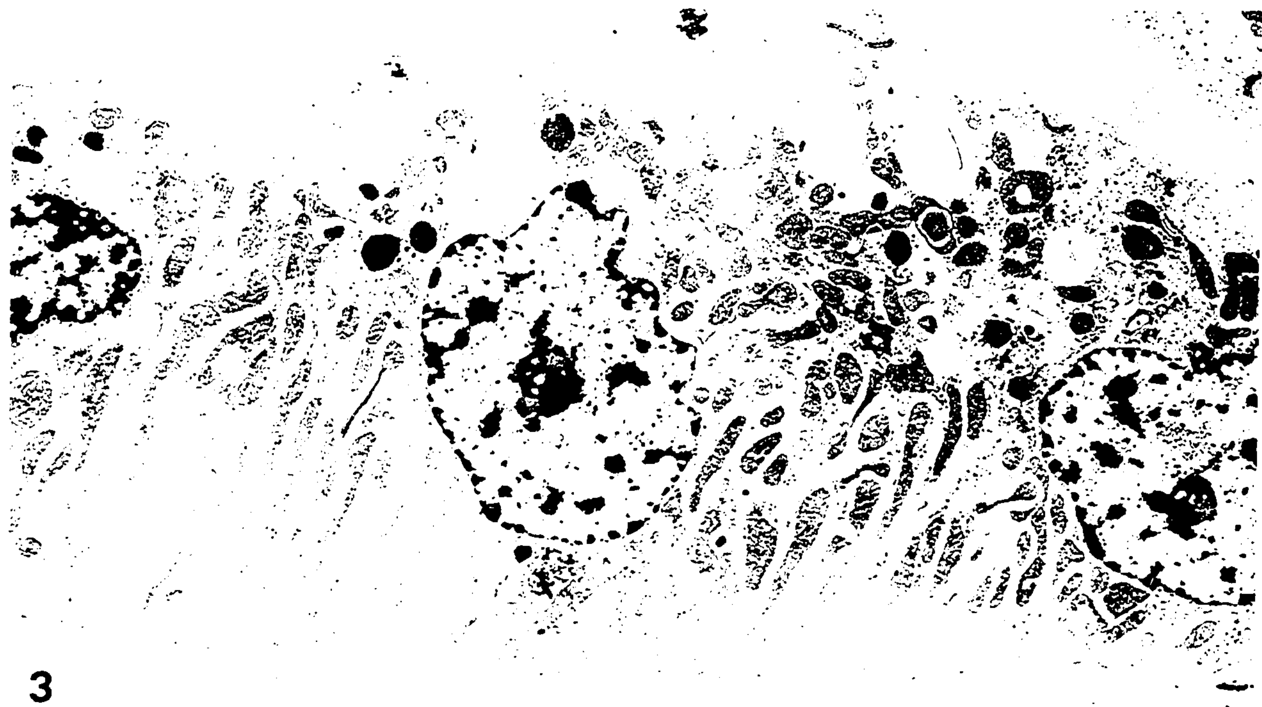

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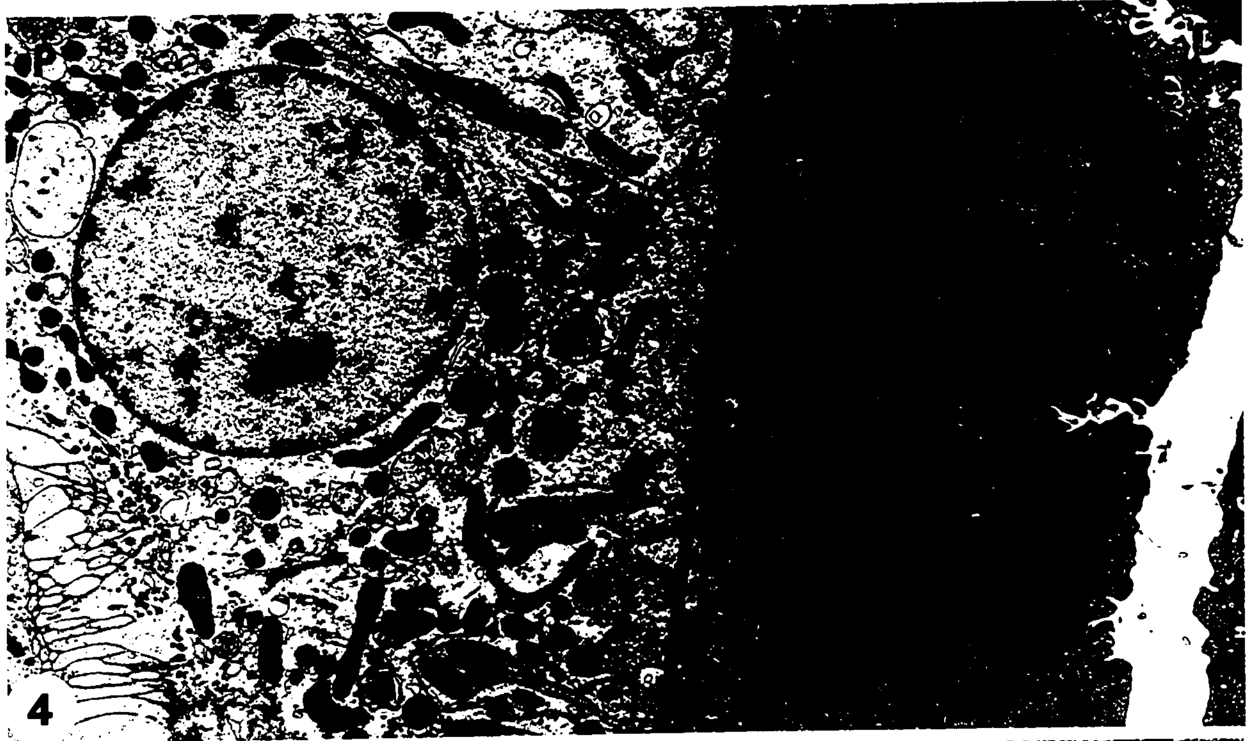
shown in Figures 2, 3, 4, 5 and 6. The effects of two treatments were examined: prolonged incubation alone and in the presence of EA, at a concentration of 7.4×10^{-7} M. This was achieved by incubating slices of tissue in the presence and absence of EA, for varying periods of time, ranging from zero minutes (an unincubated slice that was dropped into the medium and quickly removed) to 480 minutes.

Figure 2 shows a section from a control slice of fresh kidney tissue that had not been incubated. The basal membranes and cell inclusions were all normal with no signs of any swelling or cellular disruption. After a 10 minutes incubation period (Figure 3) some of the mitochondria were distorted whilst others showed no changes. Figure 4 shows a section of a slice of tissue that had been incubated for 30 minutes. Compared with the slice that was incubated for 10 minutes, this slice shows an increase in the number of distorted mitochondria. Tissue swelling is also noticeable in some areas of the section particularly in the packing of the microvilli in the proximal tubules. No changes were seen in the nuclei or nuclear membranes and the basal membranes were still intact. Figure 5 shows a section from a slice that was incubated for 30 minutes in a medium containing EA, 7.4×10^{-7} M. No significant differences could be detected between this section and its paired control. In both sections, control (Figure 4) and treated (Figure 5) the distal tubule appears to be less damaged than the proximal tubule. Generally this was found to be the case in the whole series. Slices that had been incubated for 60 minutes showed marked changes in tissue structure. Vacuolation of the mitochondria and swelling of microvilli in the proximal tubule were more pronounced. Noticeably, how-

Figure 4. Electron micrograph of rabbit kidney slice after 30 minutes of incubation at 37°C in an oxygenated isotonic buffered medium containing salts and glucose as described in the text. D = distal tubule and P = proximal tubule. Magnification = 6×10^3.

Figure 5. Electron micrograph of rabbit kidney slice after 30 minutes of incubation at 37°C in an oxygenated isotonic buffered medium containing salts and glucose and EA, 7.4×10^{-7} M as described in the text. D = distal tubule, and P = proximal tubule. Magnification = 6×10^3.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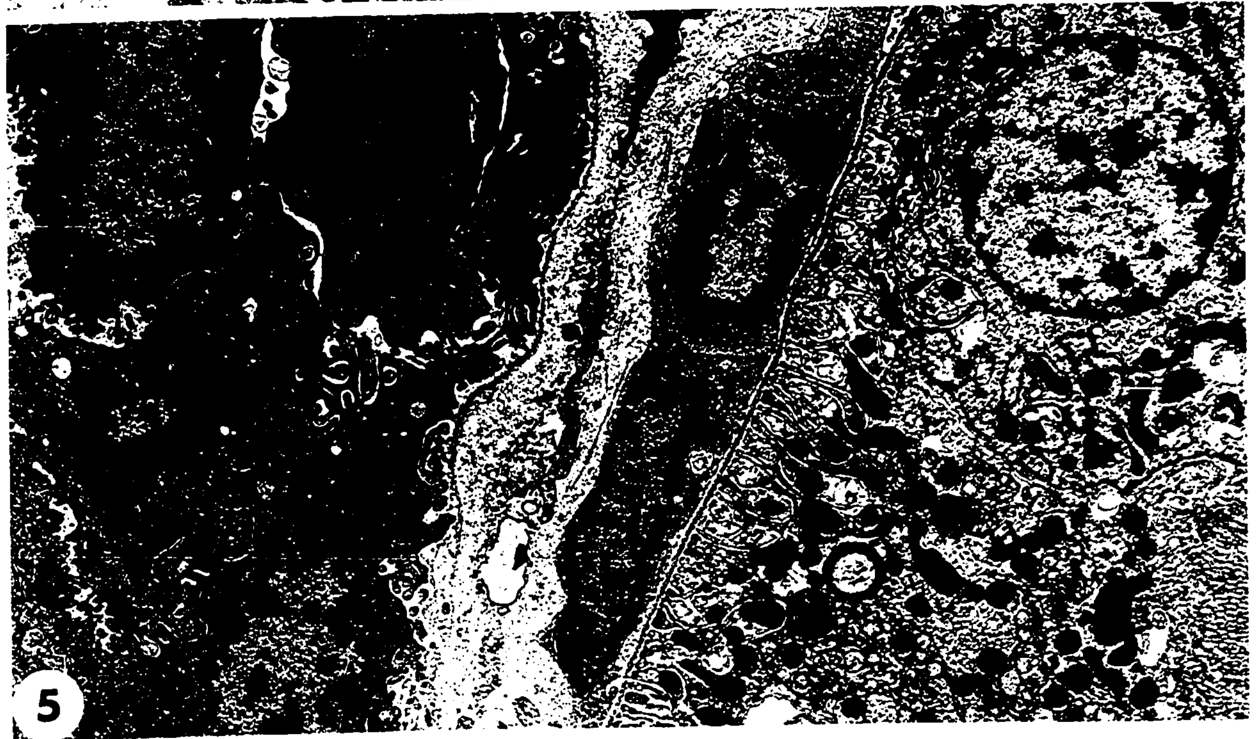
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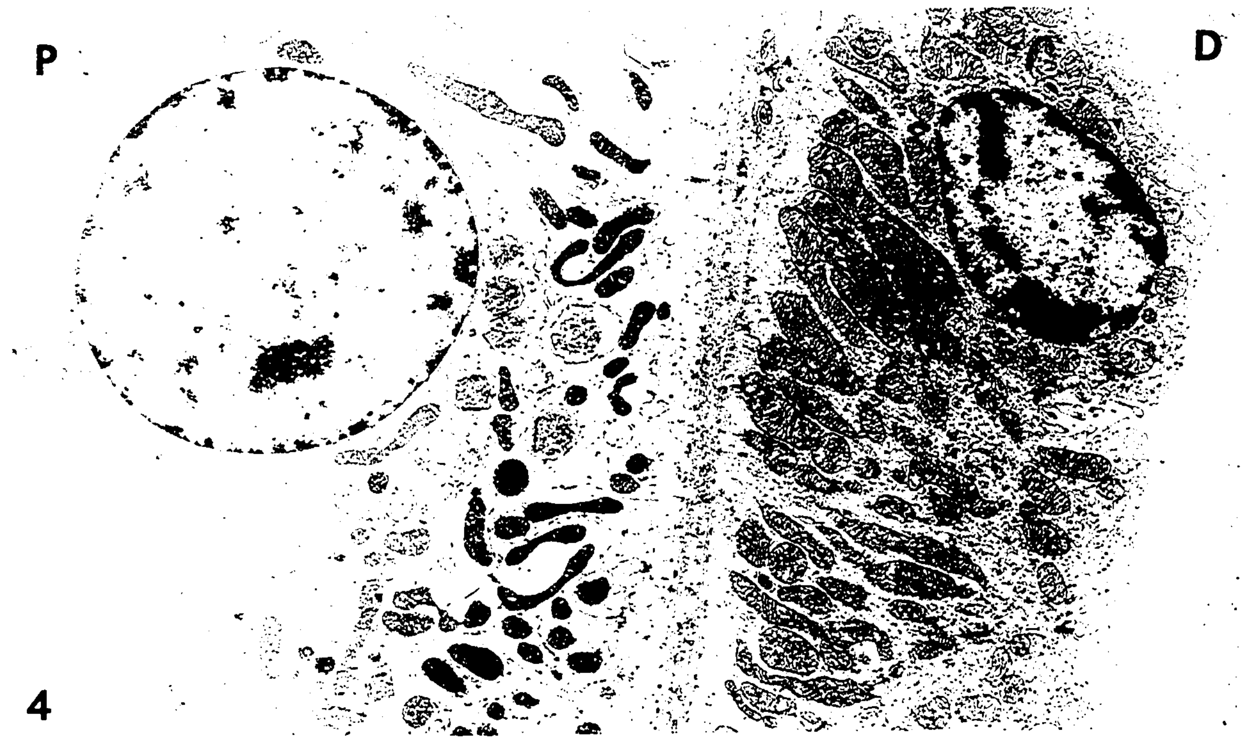
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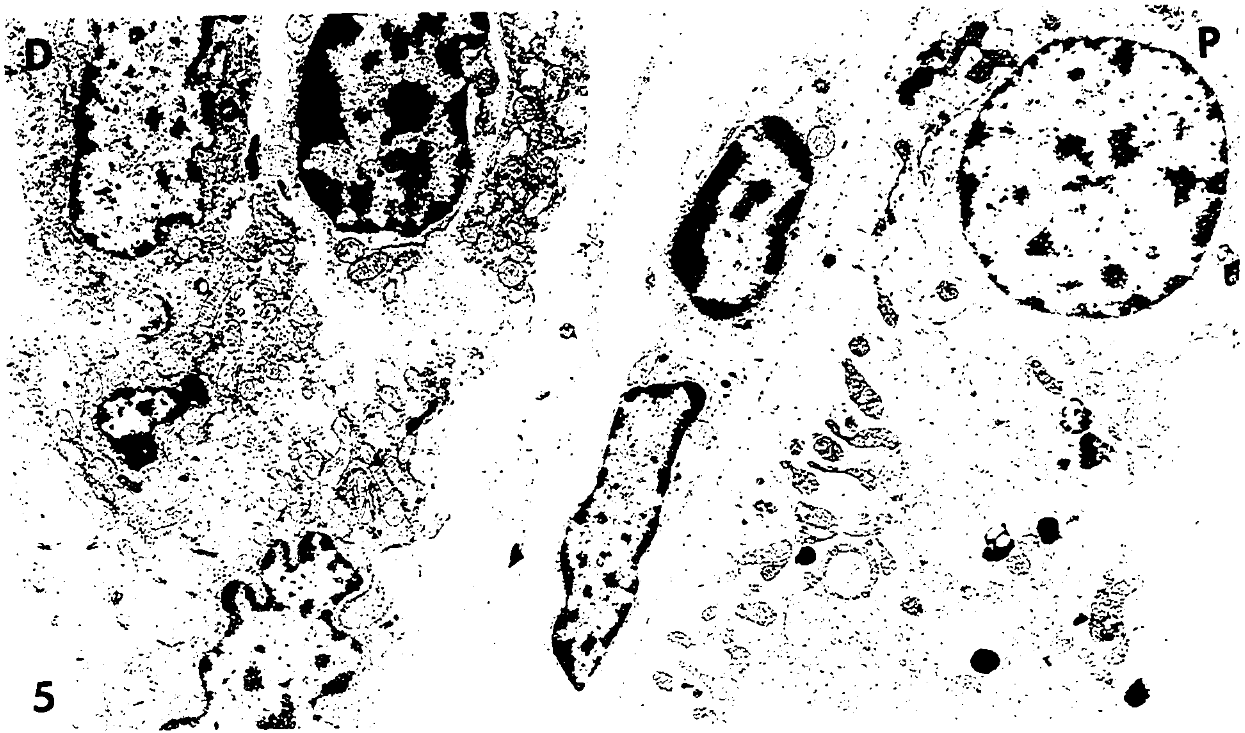
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ever, these changes were not evident throughout the section. Some areas showed minimal changes in the appearance of the microvilli. In these areas mitochondria could be detected with no vacuolations and with well defined cristae.

After eight hours, gross changes in tissue morphology are clearly seen, both in the control and in the treated slice. Figure 6 shows a section from a slice that was incubated without EA. The majority of the mitochondria were swollen and did not show well defined cristae; many contained dense granules thought by some investigators to be deposits of calcium phosphate (Greenwalt _et al_., 1964). The nucleus was not enlarged but frequently contained clumps of chromatin granules. The microvilli were almost entirely absent, but numerous myelin figures were now evident. Both the proximal and the distal tubules showed considerable swelling and disruption, but again the basal membranes appeared intact. Thus electron microscopic examination of tissue slices at the level of magnification used did not reveal any differences which could be attributed to the presence of EA, at a concentration of 7.4×10^{-7} M. However, this study strongly suggested that incubation experiments with this tissue should not be carried out for more than 60 minutes. The optimal incubation period would appear to be about 30 minutes.

We have observed by electron microscopy that gross tissue damage is evident after incubation for 8 hours, but the basal membranes appear intact. We have also demonstrated that after 8-hour incubations the S/M ratios for ^{14}C-mannitol and ^{14}C-inulin are well below unity (0.58 and 0.33 respectively) which supports the electron microscopic evidence for intact basal membranes. There is, however, a significant

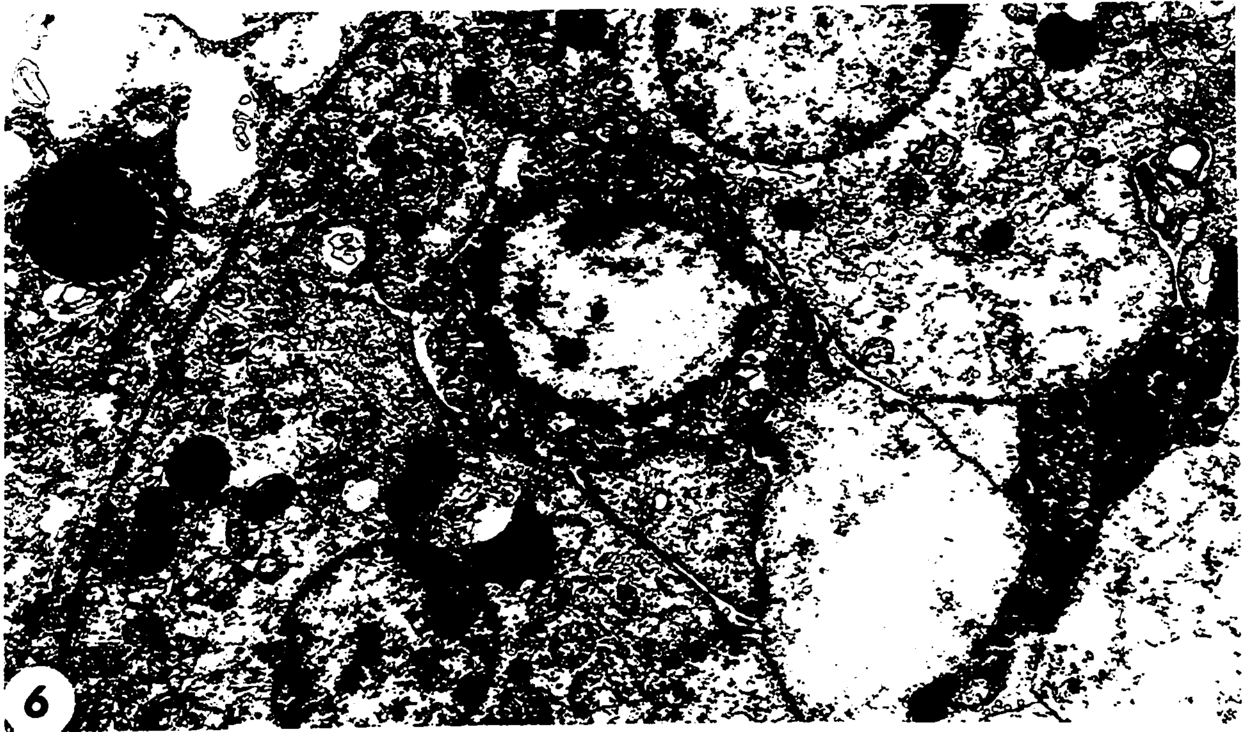

Figure 6. Electron micrograph of rabbit kidney slice after 480 minutes of incubation at 37°C in an oxygenated isotonic buffered medium containing salts and glucose as described in the text.

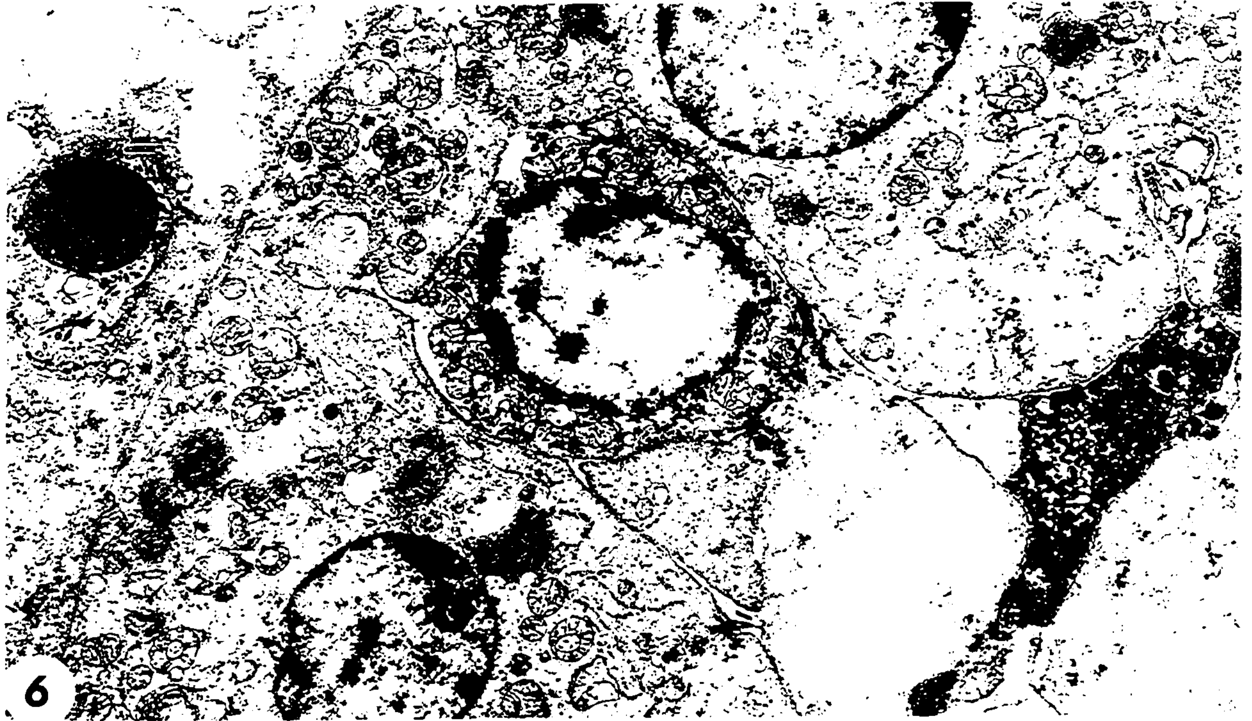

Figure 6. Electron micrograph of rabbit kidney slice after 480 minutes of incubation at 37°C in an oxygenated isotonic buffered medium containing salts and glucose as described in the text.

change in S/M ratios during the last seven hours (0.36 to 0.58 for mannitol and 0.19 to 0.33 for inulin). It was suggested earlier that this increase could be due to expansion of the extracellular space but it was not possible to offer further comment on the nature of this expansion. Since the electron microscopy demonstrated swelling of the tissue (see Figure 6) it is reasonable to assume that tissue swelling is the cause of expansion of the extracellular space. Thus, when considered in apposition the two studies on the extracellular space and the tissue morphology complement each other.

In general, we confined our incubations to a maximum of 60 minutes where uptake was studied against time; in experiments where the incubation time was constant, a 30-minute incubation was used.

Ethacrynic Acid Uptake

Kidney tissue slices were incubated at 37°C for varying time intervals in a medium containing ^{14}C-EA, at a concentration of 7.4×10^{-7} M. This concentration of drug was used as the control for all treatments. The response of the animals to the drug varied considerably. In one series the S/M values for the 60-minute incubation (29 replicates) extended from 6.6 to 16.2 with a mean value of 10.7 ± 0.49 S.E.

Figure 7 is a plot of drug uptake against time. There is little doubt that EA is being accumulated within the tissue slice. Compared with mannitol, in 60 minutes EA attains a 21-fold concentration within the tissue (S/M ratios of 0.5 and 10.7 respectively). This observation does not indicate the type of uptake or the nature of the concentrating mech-

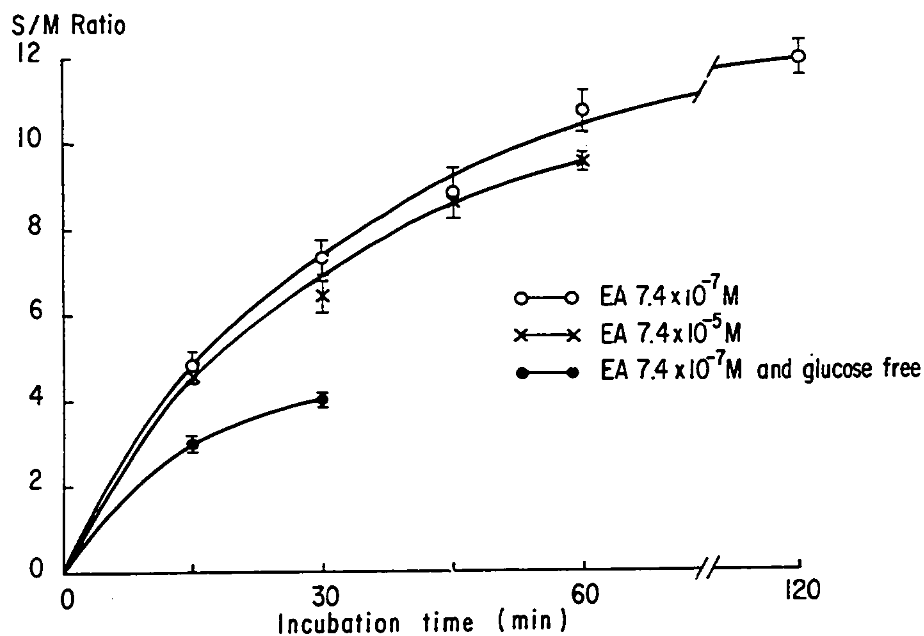

Figure 7. Uptake of ethacrynic acid. Slices of kidney cortex were incubated at 37°C. The figure shows the uptake of EA at two concentrations of drug. The lower concentration, 7.4×10^{-7} M is the cumulative result of 29 experiments. (N = 29 for each of the first four time periods and N = 4 for the last point). The higher concentration 7.4×10^{-7} M is the result of a single experiment with 4 replicates. The uptake of EA in the absence of glucose is also shown. The vertical bars represent S.E.

anism. From these data it is not possible to infer a saturable process. The rate of uptake is very rapid for the first 15 minutes and is followed by a marked decrease which is maintained for the duration of the experiment (120 minutes). To further investigate the possibility of saturation of the concentrating process, the pattern of uptake was determined with a 100-fold increase in drug concentration. The results of this experiment showing the uptake by tissue slices from a medium containing 7.4 x 10^{-5} M EA are also given in Figure 7. Despite the 100-fold difference in the concentration of EA, there is a marked similarity in the uptake patterns. After incubation for 60 minutes at the higher drug concentration, the S/M ratio is reduced by just 10%. It appears that a rapid uptake of EA is first achieved followed by a further accumulation at a slower rate. If the fast uptake is saturated within the first 30 minutes, as seems likely from the data, then two processes could account for the slow accumulation that is seen with both concentrations of EA. First, it is possible that with time there is a slow penetration of EA into the intracellular organelles. This interpretation is supported by our electron microscopic studies which have demonstrated the presence of vacuolations with time. Secondly, part of this slow accumulation may be a consequence of non-specific binding of EA to low affinity binding sites on the membrane. It is also possible that as the integrity of the membrane deteriorates with time, these sites could increase giving rise to an apparently non-saturable system.

Active Transport

The previous experiments had shown that slices of kidney tissue accumulate EA against a concentration gradient. In 1 hour the concentration within the tissue is approximately 11 times that of the medium. If it can be shown that this uptake process is related to the expenditure of metabolic energy, then it could be classified as an active transport process. Furthermore, if this concentrating mechanism were shown to be exclusive to the kidney, then an involvement with kidney function could be entertained.

Experiments were carried out in a medium devoid of glucose. The results are shown in Figure 7. After 30 minutes incubation the uptake of EA was reduced to 55% of the control value. This provided the first clue that the process was possibly an active one. To further explore the possibility of active transport, some uptake experiments were carried out at 0°C to investigate the effect of temperature, and others were done under anoxic conditions to determine the need for oxygen. In the latter experiments the incubation medium was gassed with nitrogen and the incubations carried out in an atmosphere of nitrogen. The results are shown in Figure 8. The absence of oxygen reduces the uptake of EA by about 50%. But it may not be interpreted that this represents a complete inhibition of active uptake, as no tests were carried out to ensure complete absence of oxygen in the system. The possibility definitely exists that a portion of the uptake was due to residual energy or oxygen or both. Another possibility is that substitution with nitrogen results in a partial block of cellular metabolism. This becomes more

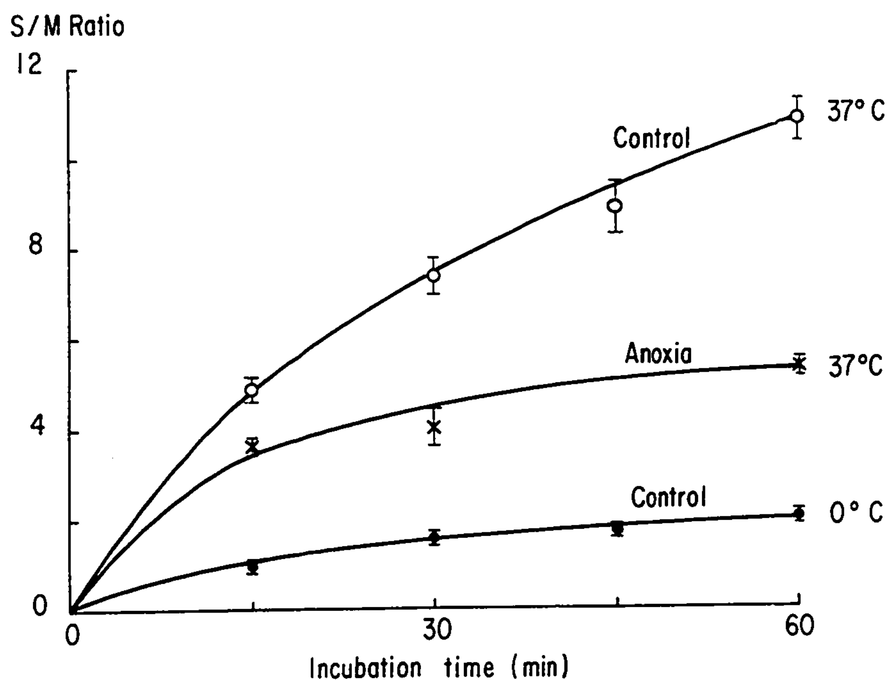

Figure 8. Effects of anoxia and temperature on uptake. Slices of kidney cortex were incubated in a medium containing EA, 7.4 x 10^{-7} M, for varying time periods at 37°C and at 0°C to show the effects of temperature. For the anoxic conditions nitrogen was used instead of oxygen. The vertical bars represent standard errors.

convincing if this result is compared with the findings from the experiment carried out at 0°C, also shown in Figure 8. The decrease in uptake seen with reduced temperature is far more dramatic than that seen under anoxic conditions reaching only 19% of the control after 60 minutes. However, the inhibition of active transport may not be the only contribution to this decrease. We can offer no justification for the assumption frequently made by other workers that low temperature does not inhibit non-specific binding of the drug to kidney tissue, as no studies were made to determine this.

Beyer et al. (1965) have shown that as much as 48% of an intravenously administered dose of EA can find its way into the intestine via the bile. Because of this, it was necessary to explore the potential of liver slices for uptake. The results are shown in Figure 9. Slices of liver appear to accumulate EA but the uptake is considerably less than that seen with kidney slices. Furthermore, the initial rapid uptake phase that is seen in kidney is relatively shortlived in the liver, possibly less than 15 minutes, while in 30 minutes the uptake is practically saturated. It is noticeable that the uptake by liver slices is far in excess of what would be expected from diffusion alone. Perhaps liver tissue also binds this drug non-specifically. Nevertheless we interpret this result as evidence for a concentrating mechanism in the kidney, which is not equally apparent in the liver.

Since the accumulation of EA by kidney slices requires glucose and is inhibited by anoxia and low temperature, the case for active uptake of this drug becomes much stronger. To further establish the metabolic requirement for the uptake of this drug, the effects of 2,4-di-

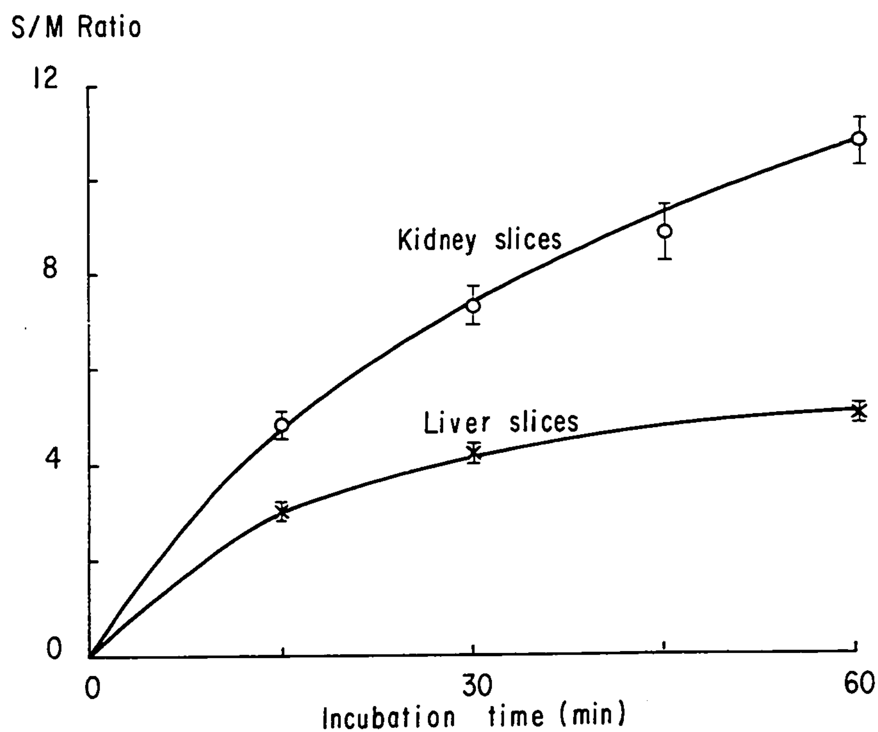

Figure 9. Uptake of ethacrynic acid by liver slices. Slices of kidney cortex (control) and liver were incubated at 37°C in a medium containing EA, 7.4×10^{-7} M. The vertical bars represent standard errors.

nitrophenol (2,4-DNP) and sodium azide were studied. These agents are both effective inhibitors of oxidative phosphorylation and if they inhibit the uptake of EA, the suggestion that the process is dependent on metabolic energy should be strengthened further.

The concentration of sodium azide which would inhibit drug uptake was first established. Slices of tissue were incubated for 30 minutes in media containing various concentrations of sodium azide. From the results shown in Table III, 5 mM sodium azide was selected as the concentration to be used for the investigation. An entirely different criterion determined the concentration of 2,4-DNP that was used. In a preliminary experiment, slices of tissue were incubated in a medium containing 1 mM 2,4-DNP. The degree of quench caused by the yellow colour from the 2,4-DNP in these slices was far in excess of that which could be accommodated by the counting system being used. Rather than embark on a project to overcome the problem of excessive colour quenching, it was decided to determine a concentration of 2,4-DNP that would permit counting under the conditions in use for liquid scintillation. The concentration arrived at was 0.1 mM 2,4-DNP.

Hence uptake studies were carried out with sodium azide, 5 mM and 2,4-DNP, 0.1 mM. The results are shown in Table IV. Both agents produced a marked inhibition of uptake of EA. After incubation for 1 hour the S/M ratios in both cases were half that of the control. The significant finding from these experiments is that 2,4-DNP and sodium azide will inhibit the accumulation of EA by slices of kidney tissue thereby characterising the uptake as an active process that requires metabolic energy.

TABLE III

THE EFFECT OF SODIUM AZIDE ON THE UPTAKE OF ^{14}C-EA

Concentration of Sodium Azide In Incubation Medium (mM)	S/M Ratio ± S.E. after 30 min Incubation (N=3 for all Treatments)
0.0	13.33 ± 1.23
0.5	8.52 ± 0.40
1.0	9.69 ± 1.05
10.0	5.69 ± 0.38
100.0	4.56 ± 0.56

TABLE IV

THE EFFECTS OF METABOLIC INHIBITORS ON THE UPTAKE OF ^{14}C-EA

Treatments	No. of Experiments	S/M Ratio ± S.E. at Time Interval t = min			
t		15	30	45	60
Control	29	4.85±0.03	7.34±0.04	8.81±0.07	10.69±0.49
2,4-DNP (0.1 mM)	4	3.64±0.19	3.85±0.28	5.07±0.64	5.03±0.29
Sodium Azide (5 mM)	4	2.21±0.17	3.87±0.34	4.36±0.23	4.77±0.19

Ouabain and probenecid are two pharmacological agents that are known for their effects on transport. Although neither agent is totally specific, they have the advantage of being extensively used which has resulted in much information being made available. Ouabain is known to inhibit Na-K-ATPase. This enzyme affects the active transport of sodium which is commonly called the Sodium Pump. If it also inhibits EA uptake in tissue slices, then the possibility arises that this uptake occurs by a co-transport mechanism. Similarly, probenecid is regarded as the prototype inhibitor of classical organic acid transport systems. If this drug inhibits EA uptake, then further investigation into the organic acid transport mechanism for EA becomes worthwhile. Thus the effects of ouabain (1 mM) and probenecid (0.1 mM) were studied and the results are shown in Table V.

Ouabain, 1 mM caused a 50% inhibition of the uptake of EA by a kidney slice that had been incubated for 1 hour. It is possible that the inhibition was not maximal and a higher concentration of ouabain might have shown a more dramatic effect.

Inhibition of EA uptake by probenecid was more severe than that produced by ouabain despite its lower concentration. If the results are retabulated as a percentage of the corresponding control, this comparison is seen to better advantage. Thus in Table VI we see that with probenecid there is a 12% increase in inhibition over ouabain. It is also of note that the difference in efficacy is remarkably consistant throughout the incubation period. To explain this constant difference in inhibition by the two drugs, two hypotheses were considered:

(a) There is one site of inhibition which is sensitive to both

TABLE V

EFFECTS OF OUABAIN AND PROBENECID ON ETHACRYNIC ACID UPTAKE

Treatments	No. of Experiments	S/M Ratio ± S.E. at Time Interval t = min			
t		15	30	45	60
Control	29	4.85±0.03	7.34±0.04	8.81±0.07	10.69±0.49
Ouabain (1 mM)	7	2.95±0.21	3.45±0.13	4.45±0.31	5.11±0.33
Probenecid (0.1 mM)	4	1.80±0.11	2.54±0.13	3.25±0.19	3.74±0.26

TABLE VI

COMPARISON OF THE INHIBITORY EFFECTS OF OUABAIN AND PROBENECID

Treatments	Uptake at Time Interval t = min Originally Determined as dpm/g Wet Weight of Tissue, Expressed as % of Paired Control for 60 min			
t	15	30	45	60
Control	45	69	82	100
Ouabain	35	42	54	61
Probenecid	23	33	42	49
Difference between Ouabain & Probenecid	12	9	12	12

agents.

(b) There are two sites of EA accumulation such that probenecid inhibits one and ouabain inhibits both sites. This latter situation is not unlikely as the inhibition of organic acid transport by ouabain has been demonstrated (Burg et al., 1962 and Schultz et al., 1970). It is noted that this ouabain inhibition of the organic acid transport excludes the third hypothesis, namely, two sites of EA accumulation one of which is inhibited exclusively by ouabain and the other by probenecid.

If the first hypothesis were correct, then the constant difference (12%) could be explained by a more complete inhibition of the process by probenecid, and incomplete or partial inhibition by ouabain. Alternatively, if the second hypothesis is correct, then the constant difference (12%) could result from total inhibition of one site, say the Na-K-ATPase transport system, and partial inhibition of the other, the organic acid transport system.

From the data in Table VI it is not possible to say which of these two explanations is the more likely. The critical experiment at this point would be to establish the concentration of ouabain necessary for maximal inhibition of the uptake of EA. If the ouabain concentration used in the above experiment (1 mM) produced maximal inhibition, then the possibility of two different systems for the uptake of EA must be entertained. These systems could be a sodium-dependent-ouabain-inhibited process, and an organic-acid-transport-probenecid-inhibited process which may be ouabain inhibited.

The results of an investigation into the efficacy of ouabain as an inhibitor of EA uptake are shown in Table VII. To overcome animal

TABLE VII

THE EFFECT OF VARYING CONCENTRATIONS OF OUABAIN ON ETHACRYNIC ACID UPTAKE

Ouabain Concentration (mM)	No. of Experiments	S/M Ratio ± S.E. at Time Interval t = min		
t		15	30	60
0	29	4.84±0.32	7.34±0.38	10.69±0.49
1	7	2.95±0.21	3.45±0.13	5.11±0.33
3	4	2.10±0.21	3.49±0.39	4.66±0.65
5	4	1.91±0.04	3.48±0.20	4.67±0.16

variation, the S/M ratios have again been represented as a percentage of the control value during a 60-minute incubation period. Ethacrynic acid accumulation after a 30-minute incubation period is reduced by 37% in the presence of 1 mM ouabain and this inhibition does not increase with 3 mM or with 5 mM ouabain. These findings support the suggestion that the uptake of EA into slices of kidney tissue involves more than one process.

If there are two sites of uptake for EA [as discussed above in (b)], then there are two approaches by which this concept may be examined further: A demonstration that ouabain causes potentiation of the inhibition of EA uptake by probenecid, would show that the two sites for accumulation of this drug are capable of operating concurrently. Alternatively, these two transport processes could be further characterised by subjecting them to procedures used to classify ouabain-sensitive transport systems on the one hand, and organic acid transport systems on the other. It was deemed that the latter investigation was more productive and therefore should be attempted first.

Sodium Dependence

Because of its sensitivity to ouabain, it is reasonable to postulate the involvement of a co-transport system for the accumulation of EA. To test this postulate, the influence of sodium on EA uptake was examined. A preliminary experiment was conducted to determine the uptake of EA in a sodium-free incubation medium. The results are shown in Table VIII. It appears from these data that sodium is not an absolute

TABLE VIII

UPTAKE OF ^{14}C-EA FROM A SODIUM-FREE INCUBATION MEDIUM

Treatments	No. of Experiments	S/M Ratio ± S.E. at Time Interval t=min		
t		15	30	60
Sodium-free	4	3.60±0.47	5.88±0.85	8.30±0.84
163 mM Na	4	4.82±0.63	7.58±1.00	9.05±1.02
*Significant at 5%		+	+	N.S.

* Paired "t" test.

requirement for EA accumulation but a degree of dependence on this cation does exist. The difference is significant at the 5% level for the 15- and 30-minute incubation periods. After incubation for 60 minutes there is only a 7% inhibition of uptake, which is considerably smaller than the corresponding value of about 50% for ouabain (see Table V). If EA is accumulated by a co-transport process which can be inhibited by ouabain, then there must be an explanation for this discrepancy. To some extent this could be a consequence of animal variation, but it may also be due to the presence of sodium introduced into the medium by diffusion from the tissue down a concentration gradient. To examine the latter possibility, the experiment was repeated using a leach period prior to the incubation. The slices were leached individually in 10 ml beakers placed in 5 ml of ice-cold choline-substituted sodium-free wash solution. After 30 minutes the solution was aspirated and replaced by a fresh 5 ml aliquot of sodium-free wash solution and the leaching process continued for a further 30 minutes. The slices were then incubated and treated as before. From the results shown in Table IX, slices incubated in a sodium-free medium will accumulate EA despite a pre-incubation leach period in a sodium-free choline chloride solution. It is apparent that leaching did not interfere with the uptake of EA under normal conditions. Even the need for a recovery period by the control slices was not evident from the results. Judging from the results of the control experiment, a further deduction can be made: Exposure to 163 mM choline chloride for 60 minutes does not seriously impair the ability of tissue slices to take up EA.

With respect to the influence of sodium, two observations can

TABLE IX

THE EFFECT OF LEACHING ON THE UPTAKE OF ^{14}C-EA FROM A SODIUM-FREE MEDIUM

Treatment	No. of Experiments	S/M Ratio at Time Interval t = min				
t		15	30	45	60	90
Sodium-free	2	2.7	4.5	6.0	7.5	8.4
163 mM Na	2	4.6	6.5	8.4	10.1	11.6

be made from these preliminary experiments: The presence of sodium in the incubation medium enhanced the uptake of EA but was not an absolute requirement, and secondly a large fraction of EA is accumulated in the absence of sodium. This could again mean the existence of two mechanisms for the accumulation of EA.

To expand the information of sodium-dependent transport, experiments were designed to study the uptake of EA using 4 different concentrations of the drug ranging from 7.4×10^{-7} M to 7.4×10^{-4} M and 6 different concentrations of sodium ranging from 0 mM to 163 mM. The incubations were carried out for 30 minutes. The leach period was not included. The detailed results of this experiment are shown in Table X. Figure 10 is a histogram which for clarity shows only the effects of sodium on the uptake of EA at four drug concentrations.

From the results shown in Table X, there is little doubt that sodium when present in the incubation medium will enhance the accumulation of EA. Furthermore this sensitivity to sodium is not apparent at sodium concentrations above 50 mM.

The histogram in Figure 10 shows the uptake of EA in the absence and in the presence of sodium. In the absence of sodium the S/M ratios show no response to changes in the medium concentration of EA. Alternatively in the presence of sodium, the S/M ratios were reduced when the medium concentration was raised 100-fold and 1000-fold. For a 100-fold increase in medium concentration of EA (7.4×10^{-7} M to 7.4×10^{-5} M) the S/M ratio was reduced by 20% and for a 1000-fold increase of medium concentration of EA the decrease in S/M ratio was 34%. Thus

TABLE X

SODIUM ENHANCEMENT OF UPTAKE OF ^{14}C-EA

Ethacrynic Acid Concentration (M)	Uptake of Ethacrynic Acid (S/M Ratio) at Various Sodium Concentrations in the Incubation Medium.					
No. of Experiments	8	6	6	4	4	4
Sodium Conc. (mM)	Zero	25	50	100	130	163
7.4×10^{-7}	4.81±0.42	6.41±0.52	7.89±0.55	9.69±0.32	8.12±0.80	7.72±0.32
7.4×10^{-6}	5.23±0.20	7.35±0.26	8.06±0.51	8.32±0.21	8.18±0.50	8.36±0.51
7.4×10^{-5}	4.86±0.26	6.04±0.24	5.60±0.20	6.52±0.36	6.21±0.96	6.19±0.34
7.4×10^{-4}	4.19±0.21	4.63±0.24	4.74±0.19	5.21±0.12	5.21±0.26	5.09±0.22

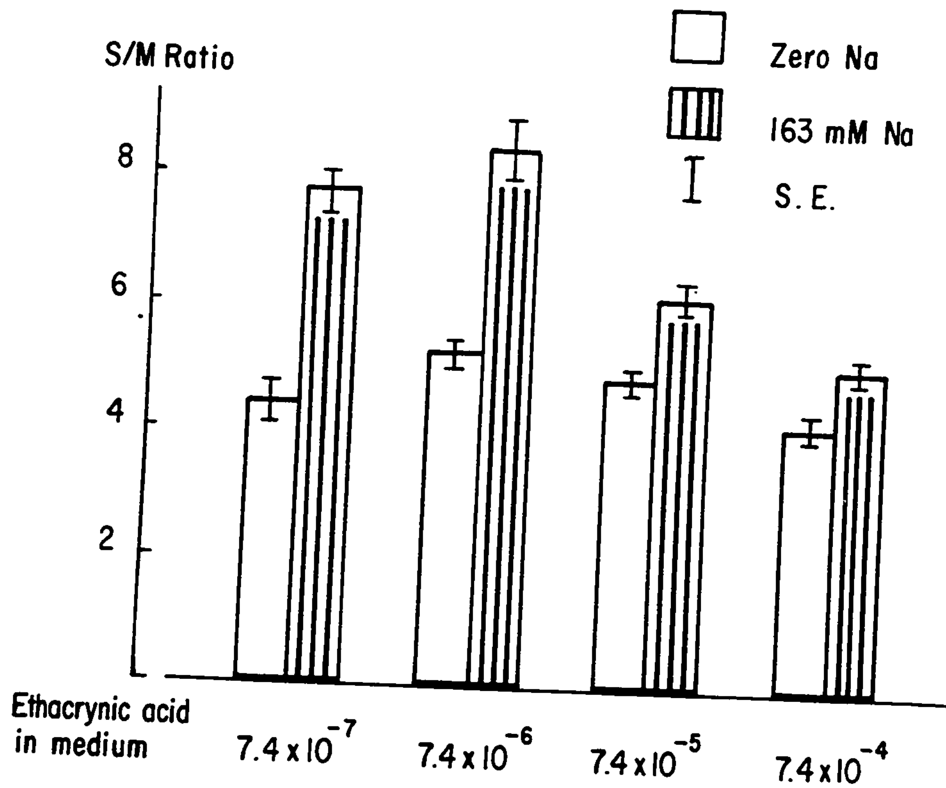

Figure 10. Enhancement of ethacrynic acid uptake by sodium. Slices of kidney cortex were incubated at 37°C in the presence and absence of sodium, with varying concentrations of ethacrynic acid.

the change in EA concentration of the medium is not in proportion to changes in S/M ratios.

The experiments carried out so far have not determined whether the uptake process is saturable or not. This ambiguity could be due to the presence of more than one mechanism for the accumulation of EA. The inhibition of one system would leave another system operating well enough to mask the evidence for or against saturation.

A brief recapitulation of the information acquired at this point shows the following:

(a) Kidney tissue slices are able to concentrate EA.

(b) The process requires metabolic energy.

(c) The transport of EA can be inhibited to a degree by ouabain or probenecid. Inhibition of uptake is also seen if sodium is omitted from the incubation medium.

(d) About 50% of the total uptake of EA is not inhibited either by metabolic inhibitors or by ouabain or probenecid. Furthermore, seen from the study on sodium dependence, a similar fraction of the uptake of EA is independent of the effects of sodium.

In considering the last point, the information acquired suggests two conflicting possibilities. Either it is totally an outcome of non-specific binding; for example, to the cut cell surfaces, or in addition to this adsorption another uptake mechanism is also involved. If the theory of non-specific binding is postulated then two pieces of evidence that were discussed earlier must be considered anew: If slices are incubated at low temperature (0°C) then a maximal inhibition of uptake is observed (see Figure 8). The uptake is reduced to 19% of the control

value after 60 minutes incubation. This low level might more truly reflect the non-specific binding which must occur to some extent. All other inhibitions imposed on this uptake process are far less effective; for example, the corresponding value for probenecid was 48%. Secondly we consider anew the effect of the medium concentration of EA on uptake. The data in Figure 10 show that the S/M ratios for EA uptake in Na-free solutions were similar at the highest and lowest concentrations of EA. This implies that at the high concentration, 7.4×10^{-4} M the absolute uptake of EA was 1000 times the uptake at the low concentration examined, 7.4×10^{-7} M. Expressed as µmoles EA/gram wet weight of tissue, these uptake values are 3.1 µmoles and 3.6×10^{-3} µmoles respectively. This large capacity of a non-specific uptake process might be expected to completely mask any active component. From our experiments this does not seem to be the case. As has been shown earlier, a 50% inhibition of uptake was common to a number of metabolic inhibitors and this represents a change from 1×10^{-2} µmoles per gram wet weight tissue to 5×10^{-3} µmoles EA per gram wet weight of tissue. Thus it is unlikely that 50% of the uptake of EA under control conditions is due to non-specific binding. At this point two questions may be asked: first, can the organic acid transport system be involved in the uptake and second, could it be a cationic transport system that is sensitive to sodium but not exclusive to it?

Organic Acid Transport

In a paper defining "substrate"[1] specificity in renal organic anion transport, Despopoulos (1965) suggested some criteria which he felt were "ideal physiological characteristics". These are as follows:

(a) Transport against an electrochemical gradient, both *in vivo* and *in vitro*.

(b) Metabolically dependent transport.

(c) Depression of transport by probenecid.

(d) Depression of transport by metabolic inhibitors.

(e) Independence of renal excretion and urinary pH within physiological limits.

(f) Saturation of transport.

(g) Enhancement of transport by acetate, pyruvate or lactate.

We have shown that the transport of EA occurs against a concentration gradient *in vitro*, it requires metabolic energy, it is inhibited by probenecid and it is depressed by metabolic inhibitors. If the 'ideal' physiological characteristics' of Despopoulos (1965) are accepted as suitable criteria for substrate specificity of organic anion transport then the possibility definitely exists that EA is a substrate for this transport process. From the list shown above, three requirement (e, f and g) remain to be fulfilled. Independence of renal excretion and urinary pH within physiological limits is not strictly applicable to an

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[1] Despopoulos (1965) has referred to the agent that is transported as the "substrate" and this practice has been adopted in this dissertation.

in vitro preparation. It has, however, been reported that the excretion of EA is subject to changes in pH (Beyer *et al.*, 1965 and Mudge, 1970), but it is not clear whether these pH changes comply with the 'physiological limits' implied by Despopoulos (1965). Secondly, our work has demonstrated that the question of saturation cannot be easily resolved with the slice technique. Finally, there remains the enhancement of transport by acetate, pyruvate or lactate. We felt that enhancement of EA transport by acetate would further strengthen the postulate that the organic acid carrier was responsible for the accumulation of this drug. We also reasoned that if EA was transported in this manner, then it would be possible to demonstrate this phenomenon by competitive inhibition. Thus the uptake of EA was determined in the absence of acetate and in the presence of p-aminohippurate (PAH) which is generally considered to be a typical substrate for the organic anion system. The results of these two experiments are shown in Table XI.

Initially the response to the omission of acetate is not as marked as most of the other inhibitors examined previously, but at the longer incubation periods the effect became more apparent. After 60 minutes the uptake is reduced to about 65% of the control value which is comparable to inhibition by ouabain (61%, see Table VI). This evidence supports the premise that the presence of acetate enhances the uptake of EA. This in turn supports the view that EA could enter the cell by an organic acid carrier mechanism.

To study the effects of an organic acid on the uptake of EA, experiments were conducted with two concentrations of PAH, 100 and 10,000 times the concentration of EA. The lower concentration of PAH did not

TABLE XI

EFFECTS OF ACETATE AND PAH ON ^{14}C-EA UPTAKE

Treatments	No. of Experiments	S/M Ratio ± S.E. at Time Interval t = min		
t		15	30	60
Control	29	4.84±0.32	7.34±0.38	10.69±0.49
Acetate-free	4	3.16±0.36	4.20±0.34	7.04±0.52
PAH (7.4 x 10^{-5} M)	4	3.96±0.47	7.42±0.55	10.16±0.86
PAH (7.4 x 10^{-3} M)	4	2.56±0.17	4.62±0.15	5.47±0.28

affect the uptake of EA. This demonstration that PAH does not compete with EA for the transport sites, despite its 100-fold excess concentration argues against the idea of a common carrier for both drugs.[1] Yet, in an earlier experiment (see Figure 7) a 100-fold increase in EA caused 10% reduction in S/M ratio after incubation for 60 minutes, which contradicts this finding. However, if the PAH concentration is raised to 10,000 times the concentration of EA (7.4×10^{-3} M PAH and 7.4×10^{-7} M EA) then accumulation of EA is significantly reduced (about 51%). It is possible that this inhibition may be due to competition for a common carrier, but it is also possible that the high concentration of PAH interfered with the biochemical and physiological function of the cells. No experiments were done to determine the effects of high concentrations of PAH on cell function.

On reviewing our results from the experiments designed to examine the involvement of EA uptake with the organic acid carrier the following observations can be made:

(a) Probenecid is thought to inhibit PAH competitively (Burg et al., 1969).

(b) PAH is a typical substrate for the organic acid carrier.

(c) PAH 7.4×10^{-5} M, will not inhibit the uptake of EA.

(d) PAH 7.4×10^{-3} M, will inhibit the uptake of EA.

(e) Probenecid 10^{-4} M, will inhibit the uptake of EA.

If EA and PAH share the same carrier we must conclude from our data that

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[1] Because PAH was not being measured, this statement only holds true if the affinities of the two drugs EA and PAH for the carrier are similar.

unless the combined concentration of the drugs in the medium exceeds 1 mM, the demonstration of competitive inhibition is not possible. This implies that the capacity of the transport mechanism for organic acids is very high. On the one hand the high capacity concept is supported by the study on the sodium dependence of EA uptake (see Figure 10). In that experiment a 1000-fold increase in substrate concentration which is a total concentration of organic acid of 0.74 mM, did not show a marked decrease in S/M ratio. On the other hand, the inhibition by probenecid refutes this idea in that EA uptake was inhibited by a concentration of 0.1 mM. If, however, the latter phenomenon is a peculiarity of probenecid, then EA would still qualify as a substrate for the organic acid carrier.

Alternatively, if EA is not a substrate for the organic acid carrier and is transported by an exclusive mechanism, then the EA carrier is very similar to the carrier for the organic acids. Perhaps the suggestion that EA is accumulated by more than one mechanism is nearest the truth.

Our working hypothesis is that the uptake of EA can be divided into two fractions: one portion that is resistant to the action of metabolic and transport inhibitors and another portion that is sensitive to these agents. It is emphasized that the terms 'resistant' and 'sensitive' are purely operational and meant for ease of discussion. It is not clear whether the resistant fraction is accumulated by non-specific binding alone or in addition to some other mechanism. The work described so far has provided circumstantial evidence for both situations. In like manner, the other fraction of EA uptake, the sensitive portion, is

irregular in its behaviour when tissue slices are subjected to metabolic inhibitors. On the one hand it is inhibited by ouabain and demonstrates sodium dependence while on the other hand it qualifies in many respects as a substrate for the organic acid carrier. The experiments that have been discussed up to this point have not been able to demonstrate one of these mechanisms to the exclusion of the other. In an attempt to do this, we considered the possibility of phloretin-2'-glucoside (also called phloridzin or phlorrhizin or phlorizin) as a selective inhibitor of the sodium dependent transport.

Using mongrel dogs, Cho and Cafruny (1970) used retrograde intraluminal injection to study the reabsorption characteristics of the renal tubule. They showed that PAH when introduced by retrograde intraluminal injection into the kidney, finds its way into the plasma. They also showed that in the presence of probenecid the plasma level was reduced. Phlorizin, however, did not influence PAH transport but was specific for glucose reabsorption. The authors suggest that these two agents, phlorizin and probenecid are capable of demonstrating specific inhibition if used in this way. Some diversity of opinion exists about probenecid, but this is not so for phlorizin. This glycoside and its aglycone form, are recognized as specific inhibitors of sugar transport processes (Lotspeich, 1961). Although the inter-relationships between sugar transport and sodium ions are complex, it is known that for active transport of sugar in the kidney (Kleinzeller and Kotyk, 1961), the simultaneous presence of sugar and sodium is essential. We have demonstrated that the presence of sodium enhances the uptake of EA, and we have also shown inhibition of uptake by probenecid. Furthermore, we have

argued that both these mechanisms may be in operation simultaneously. Thus far we have not demonstrated that these two processes are capable of concurrent operation. On these grounds it seemed reasonable to investigate the effects of phlorizin on the uptake of EA, both by itself and in the presence of probenecid. At a concentration above 10^{-4} M, phlorizin has been shown to inhibit aerobic oxidative metabolism while at 10^{-4} to 10^{-6} M it is relatively specific for active sugar transport (Britten and Blank, 1969). Consequently, we used a concentration of 10^{-4} M phlorizin and the results can be seen in Table XII. The inhibition by phlorizin is quantitatively similar to the effect produced by the omission of sodium from the incubation medium, and this augurs well for the concept of a sodium-dependent uptake component. However, when phlorizin and probenecid are present together the results are no different from probenecid alone. These results do not necessarily contradict the work of Cho and Cafruny (1970), because a number of differences exist in the two experimental approaches. The retrograde intraluminal injection method is more specific in that it estimates transport from the lumen to the peritubular fluid. This is far removed from the uptake of EA by slices of kidney cortex, where both secretion as well as reabsorption could operate. Furthermore, to directly compare our slice preparation with the retrograde intraluminal injection of Cho and Cafruny (1970) it would be necessary to expand our findings by first establishing the lowest effective concentration of phlorizin and probenecid, and then to repeat this experiment with these concentrations. Finally, we must emphasize, that the phlorizin-probenecid experiment was undertaken in an attempt to demonstrate the simultaneous functioning of two mech-

TABLE XII

THE EFFECTS OF PHLORIZIN & PROBENECID ON THE UPTAKE OF ^{14}C-EA

Treatments	No. of Experiments	S/M Ratios ± S.E. After Incubation for 30 Minutes
Control	29	7.34±0.38
Phlorizin (10^{-4})	4	5.05±0.32
Phlorizin (10^{-4}) & Probenecid 10	4	2.80±0.34
Probenecid	4	2.69±0.22

slices by two mechanisms, organic acid transport and Na-dependent co-transport both of which function simultaneously. These experiments have not demonstrated these processes in isolation.

Cation Gradient

Since the uptake of EA in sodium-free solutions is not totally inhibited the question must be entertained as to whether or not a potassium gradient is also involved. The potassium gradient as a source of energy for transport has been associated with transport in carcinoma cells (Eddy, 1968). An experiment was undertaken to investigate the effect of replacing sodium with potassium in the test solution. The slices were prepared for incubation in a wash solution with the sodium replaced by potassium (K-rich wash); these slices were then incubated in a normal solution for the control experiment, and a K-rich medium for the test experiment. The tissue slices were found to be swollen prior to the incubation, and had increased their weight by about 65%. The results of the uptake of ^{14}C-EA are shown in Table XIII. The control values compare favourably with the accumulated controls from other experiments, again suggesting that the swelling of the slices did not interfere with the accumulation of EA. There can be no doubt that potassium does not substitute for sodium in this system. Another point of interest was the failure of the slices to regain their normal volume during the incubation in a normal medium. This finding supports the work of Daniel _et al_. (1971) and others who showed that the recovery of cell volume is inhibited by EA. We note further that the swelling

TABLE XIII

UPTAKE OF ^{14}C-EA FROM A K-RICH MEDIUM

Treatments	No. of Experiments	S/M Ratios at Time Interval t = min			
t		15	30	45	60
Accumulated Controls	29	4.8	7.3	8.8	10.7
Prepared in K-rich & Incubated Normal	2	2.7	3.0	7.5	9.2
Prepared in K-rich & Incubated in K-rich	2	3.2	3.6	4.7	4.2

of the tissues occurred prior to incubation in EA and not as a consequence of it. Thus we support our earlier finding with mannitol (see Table I), in which we showed that EA did not cause any change in wet weight.

In a separate experiment, slices of kidney cortex were prepared in a wash solution with the sodium chloride replaced by choline chloride, and with the potassium chloride unchanged (5 mM). The slices were incubated for 30 minutes in two groups: a control group was incubated in a medium similar to the wash solution and a second group was incubated in a K-rich medium. The results of this experiment have not been reported in tabular form, but the following comment is made: the slices did not swell prior to incubation. Swelling only occurred in the slices that were exposed to the K-rich medium. In this group, after a 30-minute incubation period the wet weight had increased by 67% with a standard error of 3%. There was no difference in the uptake of EA, both groups attaining a S/M ratio of about 4, which is similar to the results reported in Table XIII. Thus we are able to confirm our earlier findings: tissue swelling is due to potassium and not due to EA, this swelling does not influence the uptake of EA, and finally, potassium does not substitute for sodium in this system. These findings are an important contribution to the implication of the organic acid carrier in the transport of EA. Foulkes and Miller (1961) have shown with potassium-depleted slices, that for the accumulation of PAH, an active accumulation of cation (K) was more important than its actual intracellular concentration. Moreover, Burg and Orloff (1962) had reported that the maximal S/M ratios of PAH are obtained with 4 - 5 mM potassium

and concentrations above this cause a decline in the uptake. Our experiments have demonstrated that slices which have been swollen by exposure to high potassium concentrations retain their ability to accumulate EA; so the two latter characteristics of PAH transport, namely, the need for an active accumulation of cation (K) and an optimal potassium concentration of 4 - 5 mM, are not applicable to the transport of EA. Thus, these findings together with the competition study done with PAH (see Table XI) provide us with three pieces of evidence that dissociate the uptake mechanisms of PAH and EA into slices of kidney cortex. If PAH is the typical substrate for the organic acid carrier then the hypothesis of a similar mechanism for EA must be held in doubt.

CONCLUSIONS

The findings from this work are varied and at the same time interrelated, and therefore prevent any rigid classification. They include some intracellular effects, some membrane functions, and some findings that must be discussed in association with the extracellular space.

Beginning in reverse order we must conclude that EA at a concentration of 7.4×10^{-7} M does not influence the extracellular space as defined by trace amounts of mannitol or inulin. After a prolonged incubation of up to eight hours, the S/M ratio did not change appreciably, suggesting that the permeability of the cell membrane to EA was not changed. The integrity of the membrane was substantiated by electron microscopic examination of paired tissues. The cell membrane showed no signs of disintegration, although the intracellular organelles showed damage that increased with the duration of incubation. A thirty minute incubation was found to be optimal, while sixty minutes was deemed the maximal duration of incubation. After a sixty minute incubation, the microvilli in the proximal tubules are markedly swollen and the mitochondria become vacuolated. The distal tubules are more resistant to this damage. We have deliberately avoided the use of the 'stages of ultra-structural change' as defined by Ginn and his colleagues (1968) and also by Trump and Ginn (1968). In their experiments the change in morphology was produced by exposure to high concentrations of potassium chloride which caused swelling; these conditions do not apply here. The presence of EA in the medium at the concentrations we employed, does not

potentiate nor accelerate the morphological changes. Thus the changes cannot be attributed to the presence of EA. In this respect we have also shown that addition of EA did not cause any change in wet weight of the tissue slices. We must conclude from this evidence that EA at a concentration of 7.4×10^{-7} M produces no swelling or dehydration, and no cellular changes that can be observed by electron microscopy.

The events at the cell membrane are perhaps the most interesting and they continue to be a challenge. There is little doubt that a transport mechanism for EA exists as a membrane phenomenon. This process can be inhibited by ouabain and it has a requirement for sodium in the bathing medium. Thus it is probably associated with the Na-K-ATPase in the membrane. Whether or not this enzyme is itself a carrier for EA remains to be proved, but from our evidence we anticipate that a co-transport mechanism may be more likely. Potassium will not replace sodium in the medium, so a simple cation gradient as the mechanism of transport must be excluded. Phlorizin, a plant glycoside that is known to inhibit Na-dependent sugar transport (Britten and Blank, 1969) also inhibits EA uptake and this is again interpreted as evidence in support of a co-transport mechanism.

The Na-dependent uptake component was examined in greater detail. Uptake was studied under conditions of varying sodium concentrations from 0 to 163 mM, and varying drug levels from 7.4×10^{-7} to 7.4×10^{-4} M. From this work we established that sodium concentrations above 50 mM do not influence the transport of EA. However, in the absence of sodium the uptake is not abolished, as about 40% of the total uptake is still achieved. This study also provided evidence to support

the concept of two transport processes (see Figure 9), and one of these mechanisms showed no signs of saturation despite a 1000-fold increase in drug concentration in the medium.

In addition to this display of co-transport characteristics, the uptake of EA identifies strongly with the transport of organic acids in the kidney. Thus accumulation against a concentration gradient, dependence on metabolic energy, sensitivity to acetate, inhibition by metabolic inhibitors and depression by probenecid are all characteristics of this uptake process. However, PAH, the classical organic acid, does not demonstrate competition with EA for a common "carrier" even in a 100-fold excess of the competitor. Unlike PAH the uptake of EA is not dependent on a cation gradient nor is it inhibited by a high concentration of potassium. Thus the concept of the organic acid "carrier" as a vehicle for EA must continue to remain speculative. We have not succeeded in demonstrating the presence of either mechanism to the exclusion of the other. Phlorizin used in combination with probenecid did not decrease the uptake of EA any more than did probenecid alone.

Finally we report some metabolic relationships that complement the transport phenomenon. The uptake of EA is inhibited if the slices are deprived of oxygen or glucose, which suggests a need for energy that comes from oxidative metabolism. This information was expanded by demonstrating inhibition of the uptake of EA with 2,4-DNP and sodium azide. From these experiments we must infer that oxidative phosphorylation represents a portion of the energy supply for this uptake system.

The marked inhibition of uptake that was seen with low temperature incubations was a unique finding. None of the inhibitors used was able to duplicate this result. We offer two observations by way of interpretation. First we note that a reduction in temperature should inhibit all processes to some degree whether they are mechanisms for transport or reactions for the generation of energy, whereas the effect of inhibitors would be less comprehensive. Secondly we believe that the low temperature incubations might also inhibit any non-specific binding of drug. Thus the marked reduction in uptake at low temperature is not without explanation although none can be regarded as unequivocal.

In large measure this project has succeeded in finding answers to the questions that were posed at the outset. However, rather than serve to end a project, these answers have generated considerable speculation that has led to a new series of questions. This speculation although not strictly a part of this dissertation is considered to be of some importance and has been included for the sake of completeness in a final chapter under the heading "future projections".

FUTURE PROJECTIONS

This study was not intended as a physiological investigation and in no way can it be interpreted as such. Nevertheless, an attempt to apply the findings from this work to the living animal poses a number of interesting questions.

Beyer et al. (1965) have reported that with low doses of EA, diuresis is preceded by a delay period. They interpreted this finding as accumulation of the drug at an active site. Our work has demonstrated the presence of a concentrating mechanism in a slice of kidney cortex, which supports the interpretation made by Beyer et al. (1965). Furthermore, we have shown that this concentrating mechanism is not found to the same degree in the liver. Thus the evidence to support the concept of a concentrating mechanism in the kidney is fairly strong.

If EA is to be concentrated within the kidney as a prerequisite to natriuresis, then the most likely location for this mechanism would be in the region of the tubule, where sodium pumping is most active. Thus the proximal tubule and the ascending limb of the loop of Henle would be the two areas where a concentrating mechanism might be located. Probenecid is known to inhibit the transport of organic acids in the region of the proximal tubule, and ouabain inhibits Na-K-ATPase in both the proximal and the distal tubules. Since both these inhibitors effect EA uptake, both the proximal tubule and the distal tubule must be considered as prospective sites for a drug concentrating mechanism.

The movement of sodium out of the tubule involves a passive diffusion from the lumen to the tubule cell, followed by active pumping

by Na-K-ATPase, which moves the sodium into the interstitium. Thus Na-K-ATPase is thought to be located on the peritubular membrane. It is conceivable that EA can move from the lumen into the tubule cell, down its own concentration gradient and also be co-transported. In a similar manner, once EA has entered the tubule cell, it could enter the interstitium. From here it would enter the circulation again. If, however, EA is a substrate for the organic acid secreting mechanism[1], then some of the EA could be returned to the tubule cell. Thus this hypothesis sees the tubule cell as the site of accumulation of EA. Once a critical concentration of EA has accumulated within the tubule cell it could cause metabolic changes such as depletion of ATP or inhibition of its formation. This would shut down the Na-K-ATPase and with it the sodium pump. The reabsorption of sodium in the proximal tubule would then cease, and with it the transport of EA into the tubule cell. The concentrating phenomenon could then be repeated at the ascending limb of the loop. Although the organic acid secretory process is absent in this region, a co-transport of EA out of the lumen of the loop would be expected, and a similar incapacitation of the pump would result. This would lead to natriuresis and diuresis.

This extensive series of speculations would explain a number of the properties of EA that have been observed both with *in vivo* and *in vitro* experiments. Examples of some of these characteristics are as

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[1] 'Organic acid secretion' and 'Organic acid transport' are used interchangeably. In this context it refers to the movement of substrate from the interstitium towards the tubular lumen.

follows:

(a) The short duration of action of the drug is well explained. If the accumulation of EA within the tubule cell causes inhibition of sodium transport, then this inhibition would in turn block the transport of EA. Any EA still in the blood would now be filtered and consequently excreted in the urine.

(b) We have shown that the transport of EA into slices of kidney cortex bears resemblance to a co-transport system as well as an organic acid transport system. This dual nature of the uptake of EA is in accord with the postulated model.

(c) This hypothesis does not exclude the concept of increased membrane permeability induced by EA. Once the tubule cells acquire a critical concentration of EA it is possible that the membrane undergoes some changes. The property of selective permeability which is characteristic of the tubule might be lost at high levels of EA.

(d) The one aspect that defies explanation within this model is the hitherto apparent inability of a number of workers to demonstrate concentration of EA within the kidney. By our hypothesis, the elevated drug concentration in the kidny is short lived. Thus the time taken for the drug to act would be a critical factor in any attempt to estimate drug levels in the kidney. Nechay et al. (1967) attempted a determination of the drug concentration within the kidney, during diuresis. Radiolabelled EA was administered intravenously to anaesthetized dogs and the kidneys removed 15 and 30 minutes later. Microsomal preparations from these kidneys were counted to assess the EA that was bound. After 15 minutes the bound drug was estimated at 0.24 μmoles/g protein and

after 30 minutes the concentration had fallen to 0.15 μmoles/g protein. The authors have compared these figures to the amount of drug that was bound to a microsomal preparation during an in vitro determination of microsomal ATPase that was 50% inhibited by EA. In this experiment 29 μmoles of EA adhered to each gram of microsomal protein. From these figures the authors claim that 1000 to 2000 times less drug is bound to the kidney than is bound to the in vitro preparation. These figures are somewhat misleading and might be questioned thus: In the first instance the figures might be questioned on grounds of precision and accuracy. Secondly the time lag of 15 and 30 minutes can be questioned. Beyer et al. (1965) had shown that with a minimal effective dose the peak natriuretic response occurred 15 minutes after the drug was administered. At 30 minutes, sodium excretion had almost decreased to its normal level. Thus it is possible that in the experiments of Nechay et al. (1967) the maximum sodium excretion had already been effected in 30 minutes. If our hypothesis is correct then possibly the bulk of the EA in the kidney will have also been excreted prior to removal of the kidney. The authors also report that the concentration of EA in the urine, measured 15 minutes after administration of the drug was 0.137 μmoles/minute. Compared to the figure of the excreted drug (0.015 μmoles/g protein), this represents a 10-fold loss of drug for each minute. Had the urine been assessed earlier for labelled EA, the figure could possibly have been even higher.

To experimentally establish this hypothesis, the foremost requirement would be a demonstration that EA is concentrated in the kidney. Nechay and Chinoy (1968) have described a dog under anaesthesia that

was prepared for constant infusion and for independent urine collection from each kidney. By injecting the test drugs unilaterally into a renal artery they were able to use one kidney as the test organ and the other as the control. For our purpose, a rabbit could be similarly prepared for constant saline infusion. With this preparation a dose response curve could be established to determine the lowest diuretic dose of EA. Using ^{14}C-EA the excretion patterns for sodium, water and ^{14}C-EA in urine, could be established. The kidneys could be removed at different time intervals with respect to the peak diuretic effect. If the different cell fragments of these kidneys are examined for ^{14}C-EA content it should be possible to determine whether diuresis and concentration of EA in the kidney occurred together. This experiment is similar to that of Nechay _et al_. (1967), but it is no longer subject to the criticisms that have been made.

To investigate the possibility that the tubule cell is the locus of drug accumulation the method of Burg _et al_. (1966) could be used. These workers developed a method for the isolation and study of renal tubular segments from rabbits. Their method permits study of the movement of substances from a bathing solution into the tubule cells and the lumen. The reverse movement of substances, that is, from within the tubule outwards could also be studied by perfusing the tubule with a solution containing the test substance. This would be a suitable test system for the two methods of transport for EA that have been postulated; namely, the ouabain sensitive sodium dependent process for movement of EA from lumen to interstitium, and the organic acid transport process for the movement of EA from the intersitium

to the tubule cells. If our speculations were realised to this point a number of the experiments described in this thesis could be applied to the isolated tubule. These would include the effects of metabolic and transport inhibitors and the use of autoradiography. The involvement of ATP, using the ^{32}P-labelled nucleotide could also be included in these experiments.

ADDENDUM

At the completion of this work, two papers (Epstein, R.W., Biochim. Biophys. Acta., 274: 119-127 and 128-139, 1972) were published which pertain to results reported herein. This addendum is a comment on these publications in accordance with a request by the examining committee.

In the first of these articles, the author has claimed that the accumulation of EA by slices of rabbit kidney cortex is a chemical association rather than a conventional transport process. The following observations drawn from his work, form the major support for his hypothesis:

(a) The uptake process was not saturable.

(b) It was not inhibited by 0.1 mM 2,4-DNP.

(c) It was not inhibited by low temperature.

(d) The uptake process was independent of the presence or absence of sodium in the incubation medium.

(e) Replacing oxygen with nitrogen in the experimental procedure did not influence the uptake.

These findings are directly opposed to our own. An explanation of this work (Epstein, 1972) is not intended, but attention is drawn to two methodological differences of considerable importance.

In the first instance it is noted that in this work (Epstein, 1972) the slices of rabbit kidney cortex were first subjected to a preincubation period of 40 minutes at 25°C, followed by incubation for 60 minutes at the same temperature. Investigations carried out in this

laboratory have shown that the duration of incubation is an important factor in assessing the viability of the tissue slice. Incubation causes definite damage to the intracellular organelles with time and although the basal membranes appear intact the assumption cannot be made that the surface properties of the membrane remain unchanged. Thus, the possibility exists, that as the integrity of the membrane deteriorates with time, the number of nonspecific binding sites could increase giving rise to an apparently non-saturable system.

A second major difference is in the concentration of the drug used in this work (Epstein, 1972). The experiments were carried out in a medium containing 2×10^{-3} M EA, which is about 3000 times the concentration of drug used in the experiments described in this thesis. In contrast to the findings of Epstein, the "sensitive" portion of the accumulated EA observed in this study, and which is defined on page 80 of this thesis, approaches saturation after 30 minutes incubation in a medium containing EA, 7.4×10^{-4} M (see fig. 10, p. 73). Complete saturation of this portion might be expected with 2×10^{-3} M EA.

Thus the conclusions reported in the first of these papers are drawn from experimental procedures that are very different from those used in this thesis and the results from the two works do not bear ready comparison.

In the second paper, the effects of EA on a number of cellular functions are described. The author reports the following:

(a) EA reduced the accumulation of sugars by slices of rabbit kidney cortex, regardless of whether these transport processes were

Na-dependent or not. Ouabain on the other hand only inhibited the Na-dependent processes, suggesting that ouabain inhibition and EA inhibition of sugar transport are not similar.

(b) Slices incubated in the presence of EA cause the Na content to increase and the potassium content to decrease. Water content of the slices also increases.

(c) EA depresses oxygen uptake of the tissue regardless of the presence or absence of sodium in the incubation medium.

(d) The total ATP content of slices of kidney cortex is decreased when the slices are incubated in a medium containing EA.

In referring to these characteristics the author (Epstein, 1972) states: "It is now difficult to understand how such a diversity of effects can be attributed to a single master effect of EA on biological systems." Be that as it may, it is more difficult to understand how such a diversity of effects can be achieved by EA if it is present only in the extracellular space. That this has occurred to the author is apparent from the following statement made by him: "However, the sum total of the results presented above do not necessarily argue against the possibility that EA, in some of its effects, at the beginning of its action acts at the cell membrane, and then as it penetrates more and more to the inside, continues blocking cell metabolism."

This addendum has presented some of the contentious aspects of the two papers (Epstein, 1972). Notwithstanding the view held by the author, the results reported in these papers support rather than dispute the findings documented in this thesis.

REFERENCES

Allen, J.C.; Martinez-Maldonado, M.; Eknorgan, G.; Suki, W.; Huggins, R.A. and Schwartz, A. Correlation of renal Na^+, K^+-ATPase with digoxin induced Na^+ diuresis. Fed. Proc. 28: 649, 1969.

Appleton, T.C. Autoradiography of soluble labelled compounds. J. Roy. Micr. Soc. 83: 277-281, 1964.

Baer, J.E.; Russo, H.F.; Michaelson, J.K. and Beyer, K.H. New class of diurectic saluretic agents, the α,β-unsaturated ketone derivatives of aryloxyacetic acids. Pharmacologist. 4: 158, 1962.

Baer, J.E.; Michaelson, J.K.; Russo, H.F. and Beyer K.H. 2,3-Dichloro-4-(2-methylene butyryl)-phenoxyacetic acid (I). Fed. Proc. 22: 598, 1963.

Barger, A.C. Renal hemodynamic factors in congestive heart failure. Ann. N. Y. Acad. Sci. 139: 276-284, 1966.

Beyer, K.H. Factors basic to the development of useful inhibitors of renal transport mechanisms. Arch. Int. Pharmacodyn. 98: 97-117, 1954.

Beyer, K.H.; Baer, J.E.; Michaelson, J.K. and Russo, H.F. Renotropic characteristics of ethacrynic acid: a phenoxyacetic saluretic-diuretic agent. J. Pharmacol. Exp. Ther. 147: 1-22, 1965.

Binder, H.J.; Katz, L.A.; Specer, R.P. and Spiro, H.M. The effect of inhibitors of renal transport on the small intestine. J. Clin. Invest. 45: 1845-1858, 1966.

Bourke, E.; Ledingham, J.G.G. and Stokes, G.S. Effects of intravenous ethacrynic acid on the renal handling of citrate and urate in man. Clin. Sci. 31: 231-690, 1966.

Britten, J.S. and Blank, M. The action of phlorizin and sugars on (Na^+-K^+)-activated ATPase. J. Membrane Biol. 1: 238-247, 1969.

Burg, M.B. and Orloff, J. Effect of strophanthidin on electrolyte content and PAH accumulation of rabbit kidney slices. Am. J. Physiol. 202: 565-571, 1962.

Burg, M.B.; Grantham, J.; Abramov, M. and Orloff, J. Preparation and study of fragments of single rabbit nephrons. Am. J. Physiol. 210: 1293-1298, 1966.

Burg, M.B. and Orloff, J. p-Aminohippurate uptake and exchange by separated renal tubules. Am.J. Physiol. 217: 1064-1068, 1969.

Burns, R. To a mouse. In: The complete works of Robert Burns. Ed; G. Gebbie. 1: 153, 1886.

Cade, J.R.; Shalhoub, R.J.; Canessa-Fischer, M. and Pitts, R.F. Effect of strophanthidin on the renal tubules of dogs. Amer. J. Physiol. 200: 373-379, 1961.

Cannon, P.J.; Ames, R.P. and Laragh, J.H. Methylene butyryl Phenoxy-acetic Acid. J. Am. Med. Assoc. 185: 854-863, 1963.

Cannon, P.J.; Heineman, H.O.; Statson, W.B. and Laragh, J.H. Ethacrynic acid. Effectiveness and mode of diuretic action in man. Circulation. 31: 5-18, 1965.

Charnock, J.S. Ethacrynic Acid. Inhibition of renal (Na + K)-ATPase. Clin. Res. 18: 149, 1970.

Charnock, J.S.; Potter, H.A. and McKee, D. EA inhibition of (Na^+ + K^+)-Activated Adenosinetriphosphatase. Biochem. Pharmacol. 19: 1637-1641, 1970.

Charnock, J.S.; Cook, D.A. and Casey, R.E. The Role of Cations and other Factors on the Apparent Energy of Activation of (Na^+ + K^+)-ATPase. Arch. Biochem. Biophys. 147: 323-329, 1971.

Cho, K.C. and Cafruny, E.J. Renal tubular reabsorption of p-amino-hippuric acid (PAH) in the dog. J. Pharmac. Exp. Ther. 173: 1-12, 1970.

Cole, C.H.; Blythe, W.B. and Welt, L.G. Lack of correlation between diuretic action and inhibition of ouabain sensitive ATPase. J. Clin. Invest. 48: 17a, 1969.

Cooke, C.R. and Lindeman, R.D. The site and action of ethacrynic acid and its effect on the renal cortico-medullary sodium gradient. Bull. Johns Hopkins Hosp. 117: 271-285, 1965.

Daley, D. and Evans, B. Diuretic Action of Ethacrynic Acid in Congestive Heart Failure. Brit. Med. J. 2: 1169-1171, 1963.

Daniel, E.E.; Kidwai, A.M.; Robinson, K.; Freeman, D. and Sylvia Fair. The mechanisms by which EA affects ion content, ion fluxes, volume and energy supply in the rat uterus. J. Pharmacol. Exp. Ther. 176: 563-579, 1971.

Davis, P.W. Inhibition of renal Na^+-K^+-activated Adenosine Triphosphatase acitivity of ethacrynic acid. Biochem. Pharmacol. 19: 1983-1989,1970.

Deetjen, P. und Kramer, K. Die Abhangigkeit des O_2 -Verbrauch der Niere bei osmotischer Belastung. Pflugers Arch. ges. Physiol. 273: 636-650, 1961.

Deetjen, P.; Buntig, W.E.; Karen Hardt and Rohde, R. Diuretic effect of EA in the rat: a micropuncture study concerning relationship of site and mode of diuretic action. Progress in Nephrology - Ed: Peters and Roch-Ramel. p. 255, 1969

Despopoulos, A. and Callahan, P.X. Molecular features of sulfonamide transport in renal excretory processes. Amer. J. Physiol. 203: 19-26, 1962.

Despopoulos, A. A definition of substrate specificity in renal transport of organic anions. J. Theoret. Biol. 8: 163-192, 1965.

Duggan, D.E. and Noll, R.M. Effects of ethacrynic acid and cardiac glycosides upon a membrane adenosinetriphosphatase of renal cortex.

Dunham, E.T. and Glynn, I.M. Adenosinetriphosphatase activity and the active movements of alkali metal ions. J. Physiol. Lond. 156: 274-293, 1961.

Earley, L.E. and Friedler, R.M. Renal tubular effects of ethacrynic acid. J. Clin. Invest. 43: 1495-1506, 1964.

Ebel, H.; Ehrich, J. und De Santo, N.G. Die Wirkung von Diuretica auf die ATPase-aktivatat einer plasma-zellmembranreichen fraction (PMF) der Ratteniere. Pfulugers Arch. ges. Physiol. p. 307, R77, 1969.

Eddy, A.A. The effects of varying the cellular and extracellular concentrations of sodium and potassium ions on the uptake of glycine by mouse Ascites-Fumour cells in the presence and absence of sodium cyanide. Biochem. J. 108: 489-498, 1968.

Eigler, J.; Carl, H. und Edel, H.H. Zum wirkungmechanismus der Etacrynsaure. I. Der einfluss von etacrynsaure und Furosemid auf membranpotential und Kuzschlussstrom an der Krotenhaut. Klin. Wschr. 44: 417-421, 1966.

Fanestil, D.D. Renal Na-K ATPase relationship to total functional renal mass. Nature Lond. 218: 176-177, 1968.

Farber, S.J.; Alexander, J.D.; Pellegrino, E.D. and Earle, D.P. The effect of intravenously administered digoxin on water and electrolyte excretion and on renal function. Circulation. 4: 378-386, 1951.

Flannigan, W.J. and Ackerman, G.L. Site of action of ethacrynic acid. Arch. Intern. Med. 118: 117-122, 1966.

Foltz, E.L. Preliminary clinical observation with an aryloxyacetic acid diuretic. Fed. Proc. 22: 598, 1963.

Foulkes, E.C. and Miller, B.F. The role of potassium in the renal transport of p-aminohippurate. In: Membrane Transport and metabolism. Ed: A. Kleinzeller and A. Kotyk. N.Y. Academy, p.559-565, 1961.

Frazer, J.W. Properties of non-activated ATPase from renal particulate fractions of rats. Fed. Proc. 22: 213, 1963.

Fülgraf, G. Effects of diuretics on the relation between oxygen consumption and sodium transport. Proc. 4th. Int. Congr. Nephrol. 2: 119-126, 1969.

Fülgraf, G.; Greven, J.; Holzhüter, H.; Nünemann, H.; Oswald, H. und Sudhoff, D. Die Wirking von Furosemid und Ethacrynsaure auf die kohlenhydrat und fettsaureaufnahme und auf ern Glykogengehalt der Hundeniere in vivo.- Naunyn-Schmiedebergs Arch. Pharmak. 270: 117-135, 1971.

Fujimoto, M.; Nash, F.D. and Kessler, R.H. Effects of cyanide, Qo, and dinitrophenol on renal sodium reabsorption and oxygen consumption. Amer. J. Physiol. 206: 1327-1332, 1964.

Gertz, K.H.; Braun-Schubert, G.; Brandis, M. and Boylan, J.W. The effect of saline infusion and haemorrhage on glomerular filtration pressure and single nephron filtration rate. Pflugers Arch. ges. Physiol. 310: 193-205, 1969.

Ginn, F.L.; Shelburn, J.D. and Trump, B.F. Disorders of cell volume regulation. I. Effects of inhibition of plasma membrane adenosine triphosphatase with ouabain. Amer. J. Path. 53: 1041-1071, 1968.

Glynn, I.M. The action of cardiac glycosides on Na and K movements in human red cells. J. Physiol. 136: 148-173, 1957.

Glynn, I.M. Transport adenosinetriphosphatase in electric organ. The relation between ion transport and oxidative phosphorylation. J. Physiol. Lond. 169: 452-465, 1963.

Goldberg, M.; McCurdy, D.K.; Foltze, E.L. and Bluemle, L.W., Jr. Effects of ethacrynic acid (a new saluretic agent) on renal diluting and concentrating mechanisms: Evidence for site of action in the loop of Henle. J. Clin. Invest. 43: 201-216, 1964.

Goldberg, M. Ethacrynic acid: site and mode of action. Ann. N. Y. Acad. Sci. 139: 443-452, 1966.

Gordon, E.E. and de Hartog, Maria. The relationships between cell membrane potassium ion transport and glycolysis. J. Gen. Physiol. 54: 650-663, 1969.

Greenwalt, J.W.; Rossi, C.S. and Lehninger, A. Effect of active accumulation of Ca and PO_4 ions on rat liver mitochondria. J. Cell. Biol. 23: 21-38, 1964.

Hagedorn, C.W.; Kaplan, A.A. and Hulet, W.H. Prolonged administration of ethacrynic acid in patients with renal disease. New England J. Med. 272: 1152-1155, 1965.

Heidenreich, O.; Laaff, H. und Fülgraf, G. Der einfluss von Calcium-und Kaliumionen auf die diuretische Wirkung von Herzglykosiden biem Hund. Arch. esp. Path. Pharmakol. 255: 317-327, 1966.

Herms, H. and Hofmann, K.E. Untersuchengen an der Froschhaut zur Kenntnis des Wierkungmechanismus von Diuretica an transportaktiven. Arch. esp. Path. Pharmakol. 251: 355-374, 1965.

Hoffman, J.F. and Kregenow, F.F. The characterisation of new energy dependent cation transport processes in RBCs. Ann. N. Y. Acad. Sci. 137: 566-576, 1966.

Holzgreve, H.; Frick, A.; Rumrich, G.; Wiederholt, M. and Ullrich, K.J. Wirkungsweise von diuretica auf den transtubularen transport von natriumchlorid. Normale und pathologisch Functionen des Nierentubulus. Ullrich und Hierholzer (Huber, Bern/Stuttgart, 1965).

Hook, J.B. A positive correlation between natriuresis and inhibition of renal Na-K-ATPase by ouabain. Proc. Soc. exp. Biol. N.Y. 131: 731-734, 1965.

Hook, J.B. and Williamson, H.E. Lack of correlation between natriuretic activity and inhibition of renal Na-K-activated ATPase. Proc. Soc. exp. Biol. & Med. 120: 358-360, 1965.

Horster, M. and Thurau, K. Micropuncture studies on the filtration rate of single superficial and juxtamedullary glomeruli in the rat kidney. Pflugers Arch, ges. Physiol. 301: 162-181, 1968.

Hyman, A.L.; Jaques, W.E. and Hyman, E.S. Observation on the direct effect of digoxin on renal excretion of sodium and water. Amer. Heart J. 52: 592-608, 1956.

Jones, V.D.; Lockett, G. and Landon, E.J. A cellular action of mercurial diuretics. J. Pharmacol. Exp. Ther. 147: 23-31, 1965.

Katz, A.I. and Epstein, F.H. The role of sodium-potassium-activated adenosine triphosphatase in the reabsorption by kidney. J. Clin. Invest. 46: 1999-2011, 1967.

Kessler, R.H.; Weinstein, S.W.; Nash, F.D. and Fujimoto, M. Effects of chlormerodrin, p-chlormercuribenzoate and dichlorphenamide on renal sodium reabsorption and oxygen consumption. Nephron. 1: 221-229, 1965.

Kleinzeller, A. and Kotyk, A. Cations and transport of galactose in kidney-cortex slices. Biochim. Biophys. Acta. 54: 367-369, 1961.

Kleinzeller, A. and Knotkova, A. The effect of ouabain on the electrolyte and water transport in kidney cortex and liver slices. J. Physiol. 175: 172-192, 1964.

Landon, E.J. and Norris, J.L. Sodium- and potassium-dependent adenosine triphosphatase activity in a rat kidney endoplasmic reticulum fraction. Biochem. Biophys. Acta. 71: 266-276, 1963.

Lotspeich, W.D. Phlorizin and the cellular transport of glucose. Harvey Lect. 56: 63-91, 1961.

MacGaffey, K.; Le Zotte, Jr., L.A.; Snyder, J.H.; Moore, E.W. and Jick, H. Proc. Soc. Exptl. Biol. 116: 11-16, 1964.

MacKnight, A.D.C. The effects of ethacrynic acid on the electrolyte and water contents of rat renal cortical slices. Biochim. Biophys. Acta. 173: 223-233, 1969.

Mathog, R.H. and Klein, W.J. Ototoxicity of ethacrynic acid and aminoglycoside antibiotics in uremia. New England J. Med. 280: 1223-1224, 1969.

Melvin, K.E.W.; Farrelly, R.O. and North, J.D.K. Ethacrynic Acid: A New Oral Diuretic. Brit. Med. J. 1: 1521-1524, 1963.

Meriwether, W.D.; Mangi, R.J. and Serpick, A.A. Deafness following standard intravenous dose of ethacrynic acid. J.Amer.Med.Ass. 216: 795-798,1971.

Nechay, B.R. and Pardee, L.M., Jr. Inhibition of N-methylnicotinamide secretion by ouabain in the chicken kidney. J. Pharmacol. exp. Ther. 147: 270-276, 1965.

Nechay, B.R.; Palmer, R.F.; Chinoy, D.A. and Posey, V.A. The problem of Na^{+}-K^{+} adenosine triphosphatase as the receptor for diuretic action of mercurial and ethacrynic acid. J. Pharmacol. exp. Ther. 157: 500-617, 1967.

Nechay, B.R. and Chinoy, D.A. Effect of ouabain on renal transport of p-aminohippuric acid (PAH) and blood flow in the dog. Eur. J. Pharmacol. 3: 322-329, 1968.

Nechay, B.R. and Contreras, R.R. In vivo inhibition of dog renal ATPase by EA. Lack of inhibition in the rat. Pharmacologist. 13(648): 308, 1971.

Nelson, J.A. and Nechay, B.R. Renal Na + K ATPase activity: Reduction in vivo by diuretic doses of ouabain in the dog. Fed. Proc. 28: 547, 1969.

Orloff, J. and Burg, M. Effect of Strophanthidin on electrolyte excertion in the chicken. Amer. J. Physiol. 199: 49-54, 1960.

Palmer, R.F. and Nechay, B.R. Biphasic renal effects of ouabain in the chicken: correlation with a microsomal Na^+ - K^+ stimilated ATPase. J. Pharmacol. exp. Ther. 146: 92-98, 1964.

Pillay, V.K.G.; Schwartz, F.D.; Aimi, K. and Kark, R.M. Transient and permanent deafness following treatment with ethacrynic acid in renal failure. Lancet. 1: 77-79, 1969.

Potter, D.; Sakai, T.; Harrah, J. and Holliday, M.A. Renal function and structure within 18 hours following uninephrectomy. Clin. Res. 17: 444, 1969.

Proverbio, F.; Robinson, J.W.L. and Whittembury, G. Sensitivities of (Na^+-K^+)-ATPase and Na^+ extrusion mechanisms to ouabain and EA in the cortex of the guinea pid kidney. Biochim. Biophys. Acta. 211: 327-336, 1970.

Rádo, J.P.; Szende, L.; Tako, J.; Bános, C.S. and Borbély, L. Further studies on the site of action of clopamide and furosemide. J. Clin. Pharmacol. 9: 99-103, 1969.

Rádo, J.P.; Szende, L.; Tako, J. and Borbély, L. Studies on the sites of action of ethacrynic acid and furosemide during angiotensin infusion. J. Clin. Pharmacol. 10: 375-381, 1970.

Raison, J.K.; Lyons, J.M.; Mehlhorn, R.J. and Keith, A.D. Temperature induced phase changes in mitochondrial membranes detected by spin labelling J. Biol. Chem. 246: 4036-4040, 1971.

Reiter, M. Wirdung von Strophanthin auf Kontractionskraft und Saurstoffverbrauch des Herzstreifens der Ratte. Arch. Exptl. Pathol. Pharmakol. 219: 315-332, 1953.

Rendi, R. and Uhr, M.L. Sodium, Potassium-requiring adenosinetriphosphatase activity. I. Purification and properties. Biochim. Biophys. Acta. 89: 520-531, 1964.

Rennick, B.R. and Pryor, M.Z. Effects of autonomic drugs on renal tubular transport of catecholamines in the chicken. J. Pharmac. exp. Ther. 148: 262-269, 1965.

Rosenberg, L.E.; Downing, S. and Segal, S. Extracellular space estimation in rat kidney slices using ^{14}C-saccharides and phlorizin. Am. J. Physiol. 202: 800-804, 1962.

Schatzmann, H.J. Herzglykoside als Hemmstoffe für den aktiven Kalium und Natriu transport durch die Erythrocytenmembran. Helv. Physiol. Pharmac. Acta. 11: 346-354, 1953.

Schultz, E.M.; Craboe, E.J.; Bicking, J.B.; Bolhofer, W.A. and Sprague, J.M. α,β-Unsaturated ketone derivatives of aryloxyacetic acids, a new class of diuretics. J. Med. Pharm. Chem. 5: 660-662, 1962.

Schultz, S.G. and Curran, P.F. Coupled transport of sodium and organic solutes. Physiol. Revs. 50: 637-718, 1970.

Senft, G.; Losert, W.; Sitt, R.; McEvoy, J. und Koess, H. Vergleichende Untersuchungen Uber den einfluss von 6-aminonicotinamide und 2,4-dinitrophenol auf den Natrium-und Kaliumtransport verschiedener Gewebe. Arch. Pharm. esp. Path. 255: 388-397, 1966.

Skou, J.C. The influence of some cations on an adenosine triphosphatase from peripheral nerves. Biochim. Biophys. Acta. 23: 394-401, 1957.

Skou, J.C. Studies on the Na^{+}-K^{+} activated ATP hydrolysing enzyme system. The role of SH groups. Biochem. Biophys. Res. Commun. 10: 79-84, 1963.

Skou, J.C. Enzymatic basis for active transport of Na and K across cell membranes. Physiol. Revs. 45: 596-617, 1965.

Stadie, W.C. and Riggs, B.C. Microtome for the preparation of tissue slices for metabolic studies of surviving tissues _in vitro_. J. Biol. Chem. 154: 687-690, 1944.

Swanson, R.E. and Hakim, A.A. Stop-flow analysis of creatinine excretion in the dog. Amer. J. Physiol. 203: 980-984, 1962.

Taylor, C.B. The effect of mercurial diuretics on adenosinetriphosphatase of rabbit kidney _in vitro_. Biochem. Pharmacol. 12: 539-550, 1963.

Thurau, K. Renal Na-reabsorption and O_2-uptake in dogs during hypoxia and hydrochlorothiazide infusion. Proc. Soc. exp. Biol. N.Y. 106: 714-717, 1961.

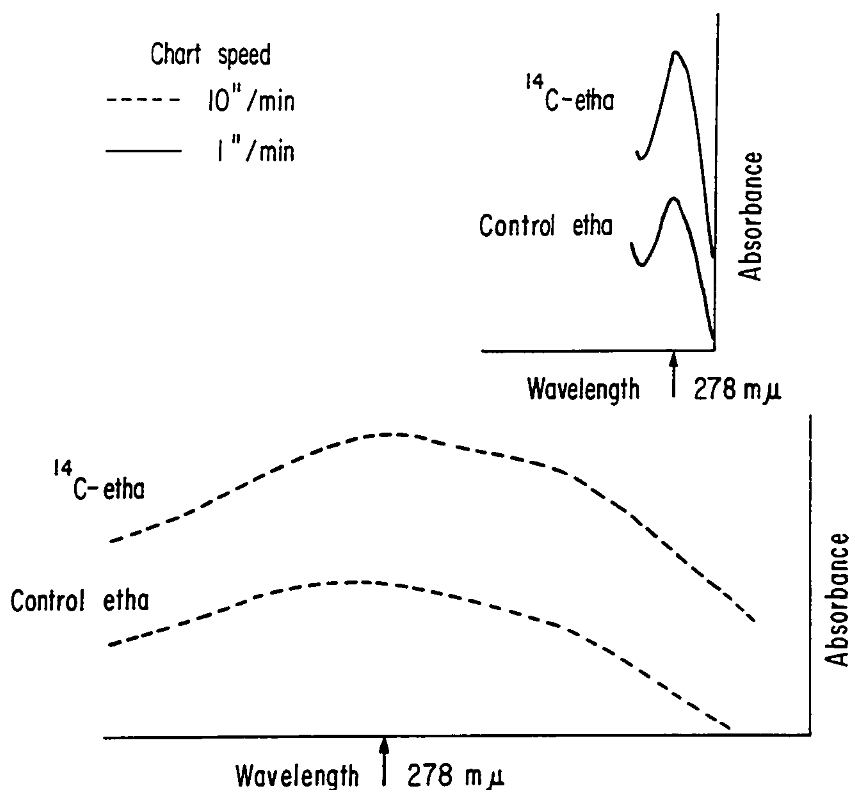
Trump, B.F. and Ginn, F.L. Studies of cellular injury in isolated flounder tubules. II. Cellular swelling in high potassium media. Lab. Invest. 18: 341-351, 1968.

Whittam, R. and Wheeler, K.P. Transport across cell membranes. Ann. Rev. Physiol. 32: 21-60, 1970.

Whittembury, G. and Proverbio, F. Two modes of Na extrusion in cells from guinea pig kidney cortex slices. Pflugers Arch. 316: 1-25, 1970.

Zins, G.R.; Walk, R.A.; Gussin, R.Z. and Ross, C.R. The diuretic activity of ethacrynic acid in rats. J. Pharmac. exp. Therap. 163: 210-215, 1968.

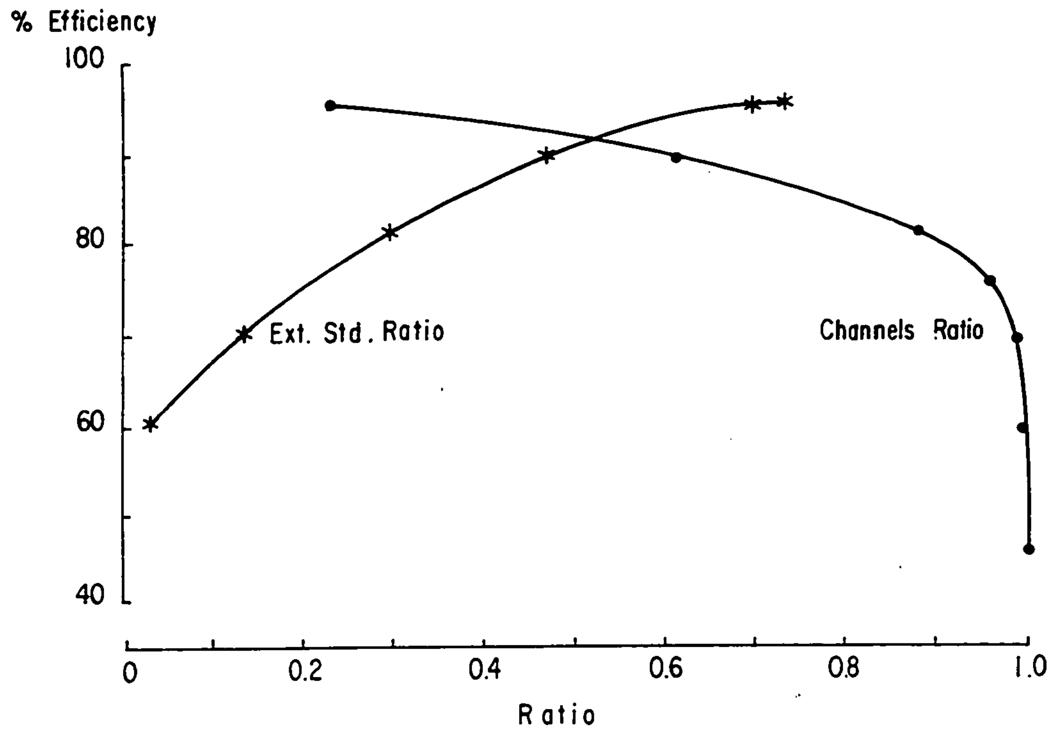
APPENDIX A



<u>Spectral purity of ^{14}C-ethacrynic acid</u>

The radiochemical is compared with a standard made from the unlabelled drug of certified purity. Both solutions were scanned at two speeds. Ethacrynic acid has a characteristic absorbance peak at 278 mμ. These peaks are shown by the arrows.

APPENDIX B



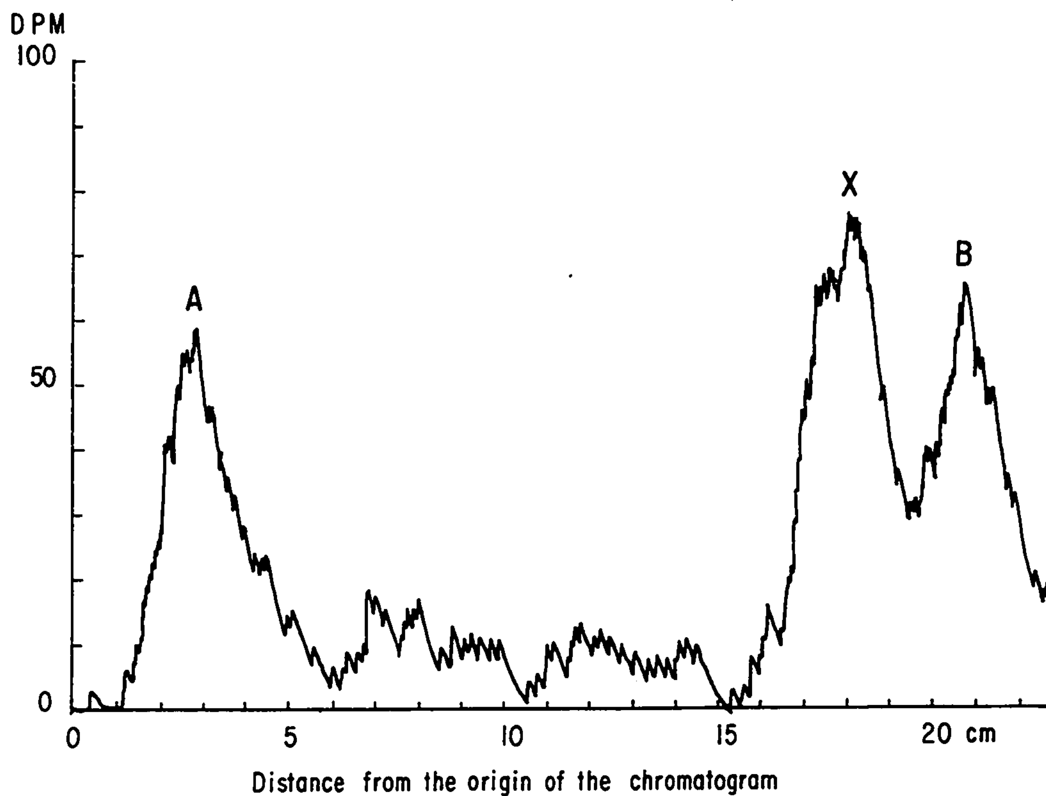
^{14}C-quench correction curves prepared for the LS-100 liquid scintillation system, using factory prepared standards. Two methods of quench correction are compared.

APPENDIX C

Addition	1	2	3	4	5
Quench Solution - ml	1.0	0.5	0.5	0.5	-
BBS-2-Fluor - ml	10	10	10	10	10
^{14}C-Ethacrynic Acid - dpm	10,000	10,000	6,000	-	10,000
Efficiency by Ext. Std. Ratio - %	81	88	88	86	95
Efficiency by Channels Ratio - %	81	87	87	84	95
Radioactivity - Ext. Std. Ratio - dpm	9546	9738	5738	*	9263
Radioactivity - Channels Ratio - dpm	9546	9849	5804	*	9263

* This vial was used as the background radioactivity and was 34 cpm.

APPENDIX D



Actigraph tracing of a chromatogram showing a single peak x for the radioactive components. A and B are markers in the chromatogram to denote points before the origin and beyond the solvent front. The absorbance was recorded at a fixed wavelength of 278 mμ, with maximum gain.

END OF REEL

ARC-30