J. Physiol. (1976), **254**, *pp.* 743–758
With 5 text-figures
Printed in Great Britain

THE EFFECTS OF OUABAIN AND ETHACRYNIC ACID ON THE INTRACELLULAR SODIUM AND POTASSIUM CONCENTRATIONS IN RENAL MEDULLARY SLICES INCUBATED IN COLD POTASSIUM-FREE RINGER SOLUTION AND RE-INCUBATED AT 37° C IN THE PRESENCE OF EXTERNAL POTASSIUM

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(*Received 3 June 1975*)

SUMMARY

1. The cells in slices cut from the renal outer medulla of normally hydrated adult rats were loaded with Na and depleted of K by incubation for up to 100 min in cold iso-osmolal K-free Ringer containing 180 mM-Na. There was a continuous net cellular water loss during this time; an inverse linear relationship existed between water content and intracellular Na concentration.

2. The original intracellular Na and K concentrations were restored following 60 min re-incubation in warm Ringer (37° C) containing 5·9 mM-K. Restoration of cellular water content was incomplete after re-incubation for up to 120 min.

3. During incubation in cold K-free Ringer the presence of 1 mM ouabain did not affect cellular Na uptake or K and water loss. Ethacrynic acid, 1 mM, completely blocked cellular Na uptake and water loss, without affecting the intracellular K concentration at 100 min. When ouabain and ethacrynic acid were present together water loss was also prevented but intracellular Na concentration rose slightly by 100 min.

4. During re-incubation in warm K-containing Ringer 1 mM ouabain inhibited Na extrusion completely for up to 60 min while only partially preventing K uptake and further depressing the level of cellular hydration. Ouabain in the presence of 1 mM ethacrynic acid had similar effects on intracellular Na and K concentrations, but raised the level of intracellular water above that of cells in control slices.

5. Ethacrynic acid alone, 1 mM, did not interfere with Na extrusion or K uptake, but also raised intracellular water above control values.

6. The results obtained are discussed in relation to (*a*) the nature of the

preparation used, (*b*) the possible membrane transport processes occurring and their known or suggested sensitivity to ouabain and ethacrynic acid, (*c*) the mechanisms which may be responsible for cell volume maintenance in the medulla.

INTRODUCTION

The functional characteristics of renal medullary cells *in vivo* cannot, with the exception of those in the papillary tip, be investigated directly. Wirz, Hargitay & Kuhn (1951) used the technique of serial tissue analysis in order to establish the principal role of the mammalian medulla, and since that time the majority of studies on this region have relied either upon tissue slices and homogenates or upon inferential conclusions based on observations made on whole kidneys *in vivo* or during isolated organ perfusion. The former techniques have admitted physiological drawbacks; exemplifying the possible pitfalls of the latter, and relevant to the present study, is the recent finding of Gutman, Wald & Czazkes (1975) that the binding of ethacrynic acid (EA) in the renal medulla, where its main site of action is believed to be located (see below), differs markedly from that in the cortex, in which (or upon the whole kidney) its action has been chiefly investigated (e.g. Macknight, 1969; Proverbio, Robinson & Whittembury, 1970; Györy, Brendel & Kinne, 1972; Landon & Fitzpatrick, 1972; Podevin & Boumendil-Podevin, 1972; Robinson, 1972; Inagaki, Martinez-Maldonado & Schwartz, 1973; Martinez-Maldonado, Tsaparas, Inagaki & Schwartz, 1974).

The action of diuretic drugs is associated with increased urinary Na excretion, indicating decreased net tubular Na reabsorption (reduced luminar entry and/or peritubular extrusion) in one or more segments of the nephron (see Suki, Eknoyan & Martinez-Maldonado, 1973). The present study was initially designed to examine the effects of ouabain and EA upon the capacity of cells in rat renal medullary slices, loaded with Na and depleted of K by incubation in K-free Ringer at 0° C, to extrude Na and take up K when subsequently incubated in 5·9 mM-K-Ringer at 37° C. The scope of the enquiry was widened in the light of unexpected observations relating to changes in cell volume during the phases of Na-loading and extrusion, and to the effects of EA during the former.

Considerable evidence supports the view that the medullary thick ascending limb of Henle's loop is a site of the diuretic action of EA. (Goldberg, 1966; Laragh, Cannon, Stason & Heinemann, 1966; Seldin, Eknoyan, Suki & Rector, 1966; Aukland, Johannesen & Kiil, 1969; Davis, 1970). Its mode of action, in terms of interference with metabolic processes and ion transporting systems, is more uncertain (Wolf, Bieg & Fulgraff, 1969; Duggan & Noll, 1972; Landon & Fitzpatrick, 1972; Nechay &

Contreras, 1972; Inagaki *et al.* 1973; Lie, Sejersted, Raeder & Kiil, 1974). Conversely, there is general agreement that at both cortical and medullary levels ouabain acts by the inhibition of Na-K-activated ATPase (Nelson & Nechay, 1970; Allen, Martinez-Maldonado, Eknoyan, Suki & Schwartz, 1971; Torretti, Hendler, Weinstein, Longnecker & Epstein, 1972) but doubt as to whether, in the rat, the ascending limb of Henle's loop is a major site of diuretic action (Streider, Khuri, Weiderholt & Giebisch, 1974).

The results obtained in this study indicate pronounced effects of ouabain and EA, under the defined experimental conditions, in relation to cation and water balance, and suggest that passive as well as active processes may be affected. They are interpreted in the light of possible cation transport processes at the luminar and peritubular membranes.

A brief account of some of this work has been published previously (Law, 1975*c*).

METHODS

The preparation of outer medullary slices (thickness 0·3 mm, weight 3–11 mg) from the freshly excised kidneys of normally hydrated adult rats receiving a diet adequate in Na and K content has been described previously (Law, 1975*a*). Weighed slices were incubated for 5 min in 10 ml. modified Krebs phosphate bicarbonate Ringer constituted as follows (mM): Na^+ 180, K^+ 5·9, Mg^{2+} 1·2, Ca^{2+} 2·6, Cl^- 144, $H_2PO_4^{2-}$ 2·2, HCO_3^- 24·9, SO_4^{2-} 1·2, glucose 10, pyruvate 4·8, glutamate 4·8, fumarate 5·3, urea 144. The availability of metabolites for tissue respiration has been discussed previously (Law, 1975*a*) and the osmolality of this medium (540 m-osmole/kg H_2O) is equivalent to that of outer medullary fluids (Law, 1975*b*). The medium was gassed to pH 7·4 with 95% O_2/5% CO_2 at 37° C. This brief incubation, referred to hereafter as pre-incubation, was designed to stabilize slice composition and provide base-line values to which subsequent compositional changes could be referred. It should be pointed out that neither total slice nor intracellular ion concentrations, or water content, at the end of pre-incubation may necessarily be taken as identical with those obtaining in fresh tissue or *in vivo*.

In a preliminary series of experiments the slice Na, K, and water contents were determined at the end of pre-incubation (see Analytical Methods) and cellular contents determined (see Calculation of Intracellular Composition). In a further series of experiments pre-incubated slices were transferred to 2·5 ml. of a medium otherwise identical but in which KCl and KH_2PO_4 had been replaced by equimolar amounts of choline chloride and NaH_2PO_4 (the slight rise in medium [Na] being considered trivial in the present context) and incubated at 0° C in a shaking incubator (80 c/s) for 10, 20, 30, 50 or 100 min. [^{14}C]carboxyl inulin (The Radiochemical Centre, Amersham) was added to this medium in order to produce a final concentration of approx. 0·5 μc/ml., except in the case of 10 min incubations, as an indicator of extracellular space. The calculation of inulin space and the validity of the assumption that this is an indication of ECS have been described previously (Law, 1975*a*). Since inulin does not equilibrate in medullary slices within 10 min the ECS of these slices, as of pre-incubated slices, was assumed to be 24 μl./100 mg wet weight (Law, 1975*a*). Because the inulin space in renal tissue slowly increases with time, probably due to intracellular penetration (McIver & Macknight, 1974), inulin was added to the incubation media 20 min from the cessation of incubations lasting 30, 50 or 100 min.

This procedure was also followed during the 30, 60 and 120 min incubations in the re-warming period (q.v.). Na loading was either carried out as here described (control) or with the addition to the incubation medium of ouabain (Strophanthin-G, B.D.H.), ethacrynic acid (EA) (Merck, Sharp and Dohme, Ltd) or ouabain + EA. Both drugs were added to concentrations of 1 mM. EA was solubilized as Na ethacrynate in 45 mM-NaOH. Dose dependency was not studied, pronounced responses being obtained with the doses selected for use.

As may be seen from Fig. 1, intracellular concentrations of Na and K ($[Na]_i$ and $[K]_i$), but not the water content (H_2O_c), in control slices achieved constancy within 100 min.

In a third series of experiments control slices which had been loaded with Na in the manner described were transferred to a medium identical with that used for pre-incubation and containing in addition inulin, and, where specified, ouabain, EA or ouabain + EA. During this re-warming period changes in $[Na]_i$, $[K]_i$ and H_2O_c were estimated after 30, 60 or 120 min.

Intracellular Cl^- was not estimated in the present study.

Analytical methods. Slices removed from incubation media at the appropriate time were rinsed briefly in inulin-free medium, blotted gently but firmly on hard filter paper (Whatman no. 542) and reweighed to the nearest 50 μg. They were then leached for 18 hr in 5 ml. distilled water. This process is adequate for the extraction of Na, K and inulin from such small pieces of tissue (Law, 1975*a*). Leaching fluid was analysed for Na and K by flame photometry against 0·1 or 0·2 mM standards. In certain experiments (see below) urea was also determined by a modification of the Berthelot reaction (Boehringer, Mannheim). [^{14}C]Carboxyl inulin concentrations in leaching fluid and 200 × dilutions of incubation media were determined in a Packhard TriCarb Liquid Scintillation Spectrometer.

Dry weights and total solute contents. As pointed out previously (Law, 1975*a*) it is difficult to obtain consistent values for slice water content, and thus for content of solute-free dry solids (SFDS), by the conventional technique of drying to constant weight, but values obtained on larger segments of medulla appear to be applicable. In a separate series of experiments the % SFDS and medullary fluid osmolality (as 2([Na] + [K]) + [urea] (Schmidt-Neilson & O'Dell, 1961)) were checked. The values obtained were SFDS 11·2 ± 0·3 % (w/w in fresh tissue dried to constant weight at 105° C, means ± S.E., n = 19), osmolality 549 ± 10 m-osmole/kg H_2O (19). These checks were made randomly throughout the course of the investigation, and it was assumed that the values obtained were applicable to all *fresh* slices. It was further assumed that the total slice content of SFDS remained unchanged throughout the incubations, percentage content thus varying with the degree of hydration.

Calculation of intracellular composition. For inulin space (ECS) see Law (1975*a*).

It is convenient to relate H_2O_c to a slice of standard weight, e.g. 10 mg at the time of weighing. In this case

$$H_2O_c\ (\mu l./mg\ SFDS) = \frac{\left[10 - \left(\frac{1{\cdot}12 \times 10}{\text{f.w.wt.}}\right)\right] - [ECS\ (\mu l./100\ mg\ \text{f.w.wt.})]}{\left(1{\cdot}12 \times \frac{10}{\text{f.w.wt.}}\right)},$$

where f.w.wt. = final wet weight (mg). Intracellular cations were calculated as

$$\text{concn. (mM)} = \frac{(\text{whole slide concn.} \times [\text{cell vol.} + ECS]) - (\text{medium concn.} \times ECS)^*}{\text{cell volume}},$$

* = 0 for K in K-free medium.

in which (for a slice of f.w.wt. 10 mg), ECS = μl./10 mg,

$$\text{cell volume} = 10 - \left[\left(\frac{1{\cdot}12 \times 10}{\text{f.w.wt.}}\right) + \text{ECS}\right].$$

Assumptions. In addition to those already referred to in the text the following assumptions were made:

(i) Sp.gr. of renal tissue is 1·0.

(ii) Weight changes of slices during incubation represent gain or loss of water without significant contribution from solutes.

(iii) The volumes of incubation media were sufficiently great by comparison with slice sizes for their composition to be unaffected by the uptake or loss of solutes by the slices.

(iv) Intracellular water content is indicative of cell volume.

RESULTS

Changes in $[Na]_i$, $[K]_i$ *and* H_2O_c *during Na-loading and re-warming*

Fig. 1 shows the changes in $[Na]_i$, $[K]_i$ and H_2O_c which occurred in control slices during Na loading for up to 100 min at 0° C and subsequently upon transference to medium at 37° C containing 5·9 mM-K. Values at 0 min are those obtained after 5 min pre-incubation (see Methods). Net Na-K exchange during Na-loading was complete within 50 min, a mean increase in $[Na]_i$ of 42 mM being accompanied by a mean decrease in $[K]_i$ of 33 mM. Original (zero time) intracellular concentrations were almost exactly restored following 1 hr re-warming. H_2O_c during Na-loading did not reach a stable value, falling from $5{\cdot}54 \pm 0{\cdot}16$ (11) to $4{\cdot}25 \pm 0{\cdot}18$ (14) μl./mg SFDS and continuing to fall during the first hour of re-warming ($3{\cdot}36 \pm 0{\cdot}20$ (15) μl./kg SFDS at 1 hr). Thereafter some net water uptake was apparent.

The initial values for $[Na]_i$, $125 \pm 5{\cdot}8$ mM (9), and $[K]_i$, $63{\cdot}1 \pm 1{\cdot}8$ mM (9), are somewhat greater than might have been expected as being physiologically normal. For example, medullary microsomal (Na+K)-activated ATPase has been implicated in the renal concentrating process (Martinez-Maldonado, Allen, Eknoyan, Suki & Schwartz, 1969; Beyth & Gutman, 1970). But Wald, Gutman & Czaczkes (1974) have shown that in the rat medulla this enzyme is maximally activated by Na concentrations considerably below those reported here in normally hydrated rats, which would appear to leave no scope for increased activation during hydropaenia. It must be remembered that the values quoted are for the end of 5 min pre-incubation, and do not necessarily represent *in vivo* levels, but it is improbable that there would have been a marked cellular influx of Na during this brief period in view of the freshness of the slices, the presumed favourable metabolic conditions, and the presence of external K.

Khuri, Agulian & Kalloghlian (1972) found a mean $[K]_i$ of 46·5 mM in distal tubular cells of rats. Since cortical whole slice K concentrations are considerably in excess of those in the medulla (Atherton, Hai & Thomas, 1968) it might be expected

that $[K]_i$ would also be lower, and not, as found in the present experiments, about 50% higher. But the heterogenicity of the cell population in the present study (see Discussion) renders the comparison a doubtful one: moreover, Khuri *et al.* were studying activities rather than chemical concentrations.

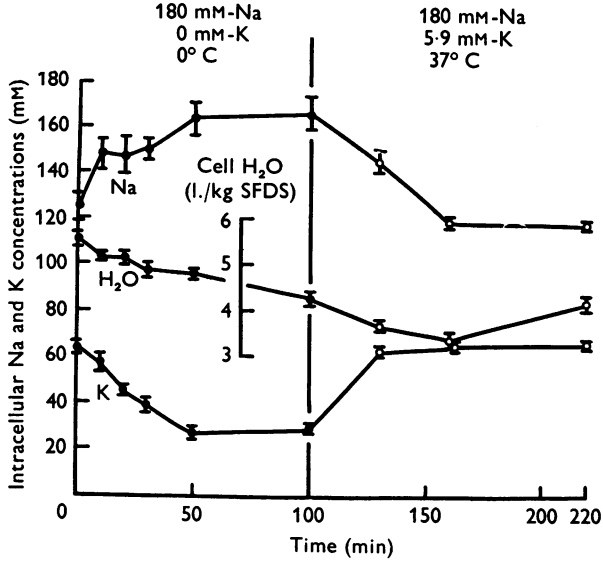

Fig. 1. The changes in renal medullary intracellular Na and K concentrations (mM) and water content (l./kg SFDS) during 100 min incubation in K-free Ringer solution at 0° (filled circles) and subsequent re-incubation for 120 min in the presence of 5·9 mM external K at 37 °C (open circles). Values are mean ± 2 × S.E.

Effects of ouabain and EA during Na-loading

In Table 1 are shown the values for $[Na]_i$, $[K]_i$ and H_2O_c at 100 min when 1 mM ouabain, EA or ouabain + EA were incorporated in the K-free medium. Also shown are the initial (i.e. post pre-incubation) values and those obtained when ouabain or EA were omitted (controls).

It may be seen that ouabain did not significantly affect $[Na]_i$ or H_2O_c attained by 100 min, whereas EA completely prevented net Na gain and water loss. When ouabain and EA were present together water loss was likewise prevented, but net Na gain only partially so, $[Na]_i$ rising to a value intermediate between those found in control slices and slices incubated in EA alone. Neither drug, alone or in combination, altered $[K]_i$ decrease.

TABLE 1. Intracellular Na and K concentrations (mM) and water content (l./kg SFDS) in medullary slices before incubation (initial) and after incubation in K-free medium for 100 min without (control) or with the addition of 1 mM ouabain, EA or ouabain + EA

	Na	K	H_2O
Initial	125 ± 5·8***	63·1 ± 1·8***	5·54 ± 0·16***
Control	***167 ± 8·1	***29·8 ± 1·4	***4·25 ± 0·18
Ouabain	***171 ± 8·1	***25·3 ± 2·1	**4·72 ± 0·21
EA	122 ± 4·1***	***27·6 ± 1·3	5·79 ± 0·15***
Ouabain + EA	138 ± 3·3*	***26·7 ± 1·8	5·51 ± 0·12***

Values are mean ± S.E.; $16 \geqslant n \geqslant 8$. Pre-fixed asterisks show significance of differences *vs.* initial values, suffixed asterisks *vs.* control values, as follows: * = $P < 0{\cdot}025$, ** = $P < 0{\cdot}01$, *** = $P < 0{\cdot}001$. Other differences are not significant.

Relationship between $[Na]_i$ and cell volume during Na-loading

During Na-loading it was noted that cell volume, as indicated by H_2O_c, appeared to be linearly related to $[Na]_i$. This is shown in Fig. 2, which includes values obtained during control incubations for various times and also 100 min values when ouabain and/or EA were present. As mentioned in the Discussion, this relationship may or may not be causal. In particular it may be noted that whereas $[Na]_i$ stabilized during the period

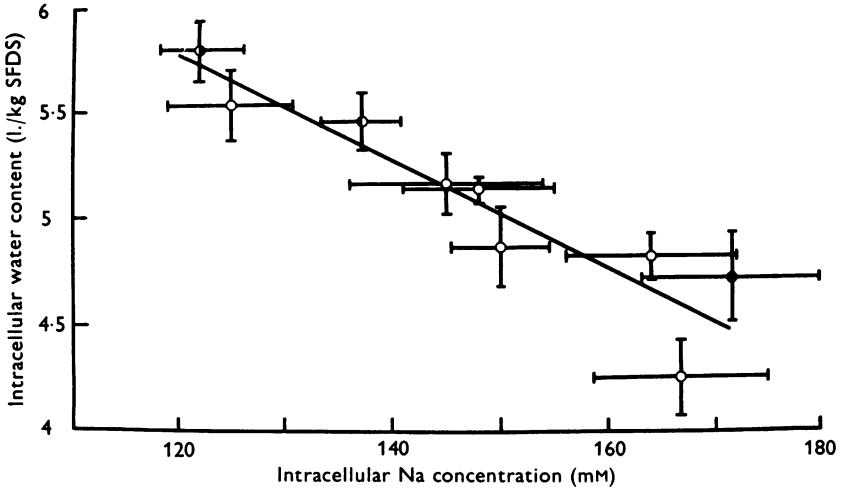

Fig. 2. The intracellular water content (l./kg SFDS) of renal medullary cells as a function of intracellular Na concentration during incubation in K-free Ringer solution at 0° C. Open circles represent incubation for varying times up to 100 min under control conditions. ●, ◑, and ◐ represent incubation for 100 min in the presence of 1 mM ouabain, EA acid or ouabain + EA respectively. Values are mean ± 2 × S.E.

50–100 min (in control slices) H_2O_c continued to fall; nor did any consistent relationship exist during re-warming under various conditions.

$[Na]_i$ during re-warming

Fig. 3 shows the time course for net Na extrusion during Re-warming in the presence of 5·9 mM external K and of ouabain, EA, or ouabain + EA. The interrupted line represents controls, adapted from Fig. 1. EA did not affect the time course of Na extrusion by comparison with control slices, but ouabain, whether or not accompanied by EA, completely prevented net Na extrusion for 1 hr. The fall in $[Na]_i$ during 60–120 min need not imply net Na extrusion since it was accompanied by an approximately equivalent increase in H_2O_c (see Fig. 5 and Discussion).

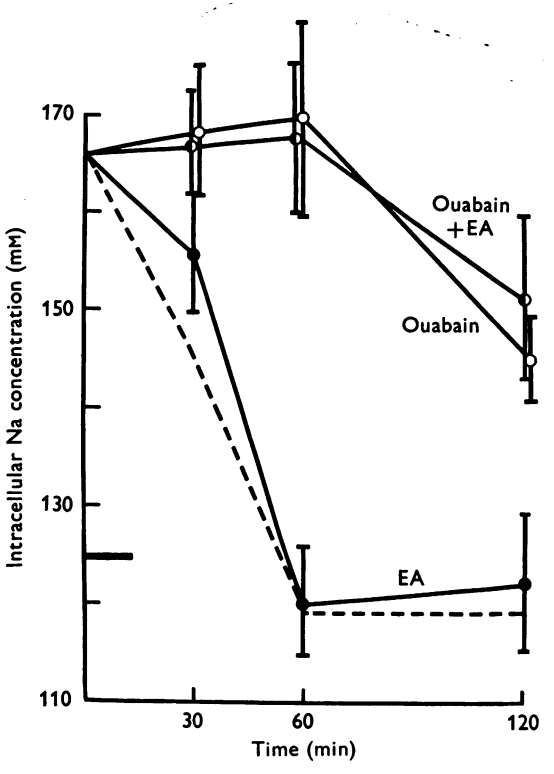

Fig. 3. The changes in renal medullary intracellular Na concentration in K-depleted Na-loaded slices incubated at 37° C in Ringer solution containing 180 mM-Na and 5·9 mM-K in the presence of 1 mM ouabain (○), EA (●) or ouabain + EA (◐). The dashed line indicates the control levels (from Fig. 1). The thick horizontal bar indicates the mean intracellular Na concentration in these cells before the commencement of K-depletion and Na-loading. Values are mean ± 2 × S.E.

$[K]_i$ *during re-warming*

Increase in $[K]_i$ was not affected by EA, but whereas ouabain or ouabain + EA had completely inhibited net Na extrusion they only partially prevented net K uptake (see Fig. 4). That cells were able to maintain intracellular/extracellular (i.e. medium) [K] ratios of between

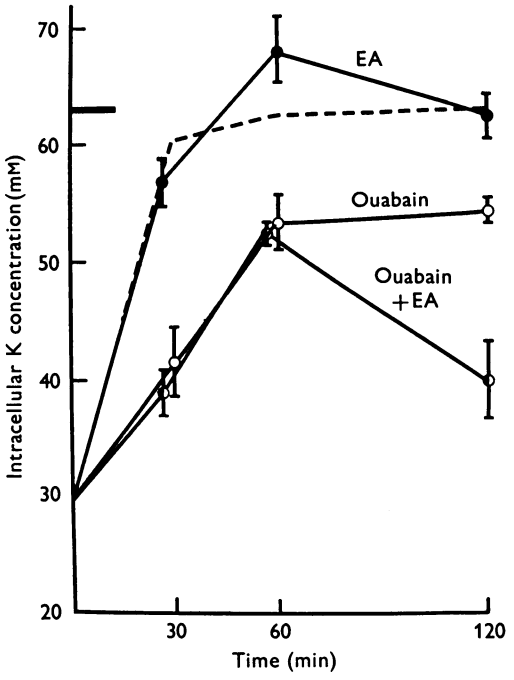

Fig. 4. The changes in renal medullary intracellular K concentration in K-depleted Na-loaded slices incubated at 37° C in Ringer solution containing 180 mM-Na and 5·9 mM-K in the presence of 1 mM ouabain (○), ethacrynic acid (●) or ouabain + ethacrynic acid (◐). The dashed line indicates the control levels (from Fig. 1). The thick horizontal bar indicates the mean intracellular K concentration in these cells before the commencement of K-depletion and Na-loading. Values are mean ± 2 × S.E.

6·5 and 10·5 at 120 min (i.e. nearly 4 hr after the start of the sequence of incubations) may be taken as indicative of continuing metabolic integrity. Nevertheless the values of $[K]_i$ at 1 hr for cells incubated in ouabain (53·3 ± 2·4 mM (15)) or ouabain + EA (52·3 ± 1·1 mM (15)) were significantly lower than corresponding values in control slices (63·9 ± 1·4 (15)) ($P < 0·001$). Control values, having regained their original (post pre-incubation) levels, remained unchanged during 60–120 min, as also did

levels in cells incubated in ouabain or EA separately. However, when both ouabain and EA were present in the medium $[K]_i$ fell by 120 min to $39{\cdot}9 \pm 3{\cdot}3$ mM (8). From Fig. 5 it may be seen that during this period the H_2O_c in these slices rose markedly, the over-all effect being to maintain the intracellular K *content* constant at approximately 188 m-equiv/kg SFDS. It has been the practice in this paper to refer to cellular composition in terms of concentration rather than content in so far as the former is more likely to be the determinant of transmembrane ion (and water) movement. Changes in ion content, however, may usefully reflect the occurrence of such movements, and have been referred to in the text when it seemed appropriate to do so.

H_2O_c during re-warming

Fig. 5 shows the net changes in H_2O_c during re-warming in the presence of 5·9 mM external K. Under all conditions studied the pattern of H_2O_c change was basically similar, consisting of a continued decrease for 1 hr followed by a gradual increase. However, when EA or ouabain + EA were present (*a*) the decrease was very small (30 and 60 min levels were not

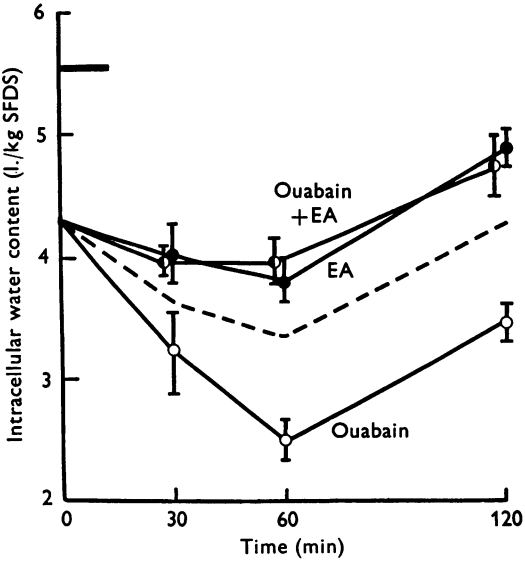

Fig. 5. The changes in renal medullary intracellular water content in K-depleted Na-loaded slices incubated at 37° C in Ringer solution containing 180 mM-Na and 5·9 mM-K in the presence of 1 mM ouabain (○), (EA) (●) or ouabain + EA (◐). The dashed line indicates the control levels (from Fig. 1). The thick horizontal bar indicates the mean intracellular water content in these cells before the commencement of K-depletion and Na-loading. Values are mean ± 2 × S.E.

significantly below that after 100 min K-depletion) and (*b*) levels were consistently higher than those in control slices. Conversely, in the presence of ouabain, levels were at all times below those in control slices.

DISCUSSION

Some general considerations

The experiments described here reflect the collective properties of at least three types of structurally and functionally diverse cells, namely those in the thin descending and thick ascending limbs of Henle's loop, and in the collecting ducts. The thick ascending limb of Henle's loop, in which the hyperosmotic transport of NaCl occurs, may well be the area accounting for the major share of the medullary metabolic expenditure, but it cannot be assumed that these cells necessarily make the greatest contribution to the occurrences observed in the slices used in these experiments, and none of the suggestions put forward below makes such assumptions in respect of any one part of the cell population.

The changes in intracellular cation concentrations observed in these slices, and the effects of ouabain and EA upon them, suggest the activity of several ion transporting mechanisms. Since marked alterations in cellular volume accompany these changes, it is relevant to enquire whether the mechanisms responsible for transepithelial ion transport are identical with, or include, those responsible for cell volume maintenance. Maude (1969), working on rat proximal tubule, concluded that so far as Na and water transport are concerned the processes are largely independent. Such an idea is intuitively attractive; it is inherent in the 'dual pump' hypothesis reviewed by Giebisch, Boulpaep & Whittembury (1971), and an analogous duality has recently been suggested for the epithelium of the toad urinary bladder (Macknight, Civan & Leaf, 1975). The present findings cannot provide any direct answer to this question, since it is unclear whether or not there is residual luminal membrane function in renal slices, which would be necessary for the full manifestation of transepithelial transporting processes. The findings are, however, more readily explainable if it is assumed that there is residual luminar membrane function and it will be tentatively suggested that certain processes involved in transepithelial movement of ions also play a role in the regulation of cell volume.

Changes in intracellular composition during Na-loading

The changes in cell volume (H_2O_c) present a puzzling feature of this study, especially the decrease observed during Na-loading, which cannot be ascribed to adverse metabolic (Law, 1975*a*) or osmotic conditions (Law, 1975*b*). Cortical cells, incubated under comparable conditions

(Whittembury, 1968), display the 'textbook' response of (reversible) swelling. From Fig. 2 it can be seen that during cell shrinkage an inverse linear relationship existed with $[Na]_i$; the effect of this was to maintain near constancy of cellular Na content (at approx. 700 m-equiv/kg SFDS). But to suggest that this relationship simply reflects water loss unaccompanied by net Na loss would imply either (*a*) that water loss in a *primum mobile*, which is inadmissible, or (*b*) that it results from K loss. The latter is improbable in view of the fact that $[K]_i$ fell to an equal extent under all conditions studied whereas when EA or ouabain + EA were present cell volume remained high (and $[Na]_i$ low) (Table 1). Presumably during net Na-loading Na (probably as NaCl) and water were extruded by some mechanism analogous to the electrogenic pump of Whittembury & Proverbio (1970). Passive luminar entry of Na, down its concentration gradient, would account for the rise in $[Na]_i$ to a level only slightly below that in the incubation medium. This leaves unexplained the effects of ouabain and of EA. It may be assumed that under these conditions all active transport processes were blocked by hypothermia. The observed effects of ouabain and EA on the behaviour of the cells must, *a priori*, occur by means of interference with passive processes, e.g. charge effects or the competitive inhibition of binding sites. Thus the effect of EA could be explained by assuming that it inhibited peritubular extrusion of Na (or NaCl) and water, causing cell volume to be maintained, while at the same time blocking the passive entry of Na across the luminar membrane. Such a multiple role may be not inconsistent with the known actions of EA. Several groups of workers have cast doubt upon the specificity of EA in regard to its diuretic action and its effects at enzymic level (Macknight, 1969; Poat, Poat & Munday, 1970; Epstein, 1972; Martinez-Maldonado *et al.* 1974). This interpretation would imply that in the absence of EA extrusion of Na (or NaCl) plus water continued in the cold, which would be inconsistent with a wholly electrogenic character for such extrusion.

Ouabain did not affect changes of $[Na]_i$, $[K]_i$ or H_2O_c by comparison with controls, but ouabain + EA brought about a small rise in $[Na]_i$, and hence also an increase in cell Na content, since H_2O_c did not decrease under these conditions (Table 1). While this would be consistent with ouabain not interfering with the suggested effects of EA, it would imply that in the absence of ouabain more Na was being extruded from the cells than when ouabain was present. Again, passive mechanisms must be involved.

$[K]_i$ appears unrelated to cell volume during Na-loading. It is surprising that in all incubatory conditions $[K]_i$ remained as high as 25–30 mM after 100 min in K-free medium. Presumably the intracellular concentration would have fallen further during more prolonged incubation.

Changes in intracellular composition during re-warming

Although net K uptake and Na extrusion occurred rapidly upon re-warming (Figs. 3, 4) there was a delay of about 1 hr before H_2O_c began to increase. The continued decrease in H_2O_c during the initial stage of re-warming was accentuated by ouabain but largely abolished by EA or ouabain + EA (Fig. 5), which would be consistent with an inhibitory effect of EA on a Na + water extrusion mechanism. From Figs. 3 and 4 EA does not appear to interfere with net Na/K exchange. However, it may be seen that when ouabain + EA were present H_2O_c was still above control levels (Fig. 5) while $[Na]_i$ remained high (Fig. 3). No net gain of intracellular Na need be envisaged during the period 0-60 min, since both H_2O_c and $[Na]_i$ remained almost constant. But during the period 60–120 min as H_2O_c rose $[Na]_i$ fell (Fig. 3) to such an extent that cellular Na content remained at about its previous level. Thus cellular water increased without concomitant Na uptake. Since the presence of ouabain + EA should have blocked peritubular Na extrusion mechanisms it is possible that luminar Na entry, for which there would otherwise have been an increasingly favourable concentration gradient during this period, was blocked. Such a role for EA was suggested in the preceding subsection of this Discussion. But in the absence of osmotic imbalance water must have entered the cells as a result of ionic movement, the relevant ion under these conditions probably being K. When ouabain was present, peritubular K entry via a Na/K exchange pump should not have occurred; entry, which must be active, might therefore have taken place at a luminal site. Active K uptake is believed to occur in the distal tubule of the rat (Malnic, Klose & Giebisch, 1966*a*, *b*; De Mello-Aires, Giebisch, Malnic & Curran, 1973), and although ouabain may inhibit this process (Duarte, Chométy & Giebisch, 1971; Streider *et al.* 1974) it seems improbable that it did so in the present experiments, unless some third ouabain-insensitive process is to be invoked, since even when ouabain was present $[K]_i$ rose markedly. This rise was less than that observed when ouabain was absent (Fig. 4) and the levels of H_2O_c remained below control values (Fig. 5). Maude (1969) has also observed a depression of H_2O_c in the presence of ouabain in cortical cells.

Concluding remarks

The foregoing hypotheses have been put forward in an attempt to account for these findings in terms of the known or conjectured transport properties of the medullary cell membranes and of the effects which ouabain and EA are believed to have upon these processes there and at other sites in the kidney. It is not intended to represent that these explanations uniquely fit the observed occurrences. There are indications

that some uncharacterized ion pump mechanisms may be present and that, since they can be blocked by drugs at 0° C, at least some of them must be passive by conventional criteria. The effects of EA may be widespread; in particular the findings would be consistent with an inhibition of passive luminar Na entry and with the existence of a passive component of Na (or NaCl) linked water extrusion.

It appears to be a collective property of outer medullary cells to lose water under adverse conditions (zero external K and hypothermia). $[Na]_i$ and intracellular K content may be among the factors determining cell volume; since net transport of both these ions occurs in certain cells of the medulla it would be of interest to know whether these factors bear any relation to cell volume in other epithelia involved in net ion transference, e.g. salivary gland cells. It would be in accordance with the observed facts to suggest that cell volume is normally maintained by the opposing forces of two regulator mechanisms, namely an EA-sensitive water-extruding mechanism tending to reduce H_2O_c and a resistance to water loss which is dependent upon maintenance of relatively low $[Na]_i$ and high intracellular K content.

In so far as the transmembrane processes regulating the latter may be those associated with transepithelial ion movement, the regulation of cell volume in the medulla would be seen as an integral part of such movements.

I am grateful to Miss Diane Kirkland for skilled technical assistance. Some of these experiments were carried out in collaboration with Miss Sian M. Jones and were incorporated in her Third Year Project Report.

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