J. Physiol. (1979), **293**, *pp.* 485–500
With 4 text-figures
Printed in Great Britain

THE INFLUENCE OF CHLORIDE IONS ON RENAL OUTER MEDULLARY CELL VOLUME

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(*Received 10 April 1978*)

SUMMARY

1. Slices of rat renal outer medulla have been incubated in media made hyperosmotic (540 and 1055 m-osmole/kg H_2O) by the addition of urea, and containing variable concentrations of Cl (90, 144, 189 and 215 mM) and constant concentrations of Na (180 mM) and K (5·9 mM). A small number of incubations have been conducted in the presence of 100 mM-Na.

2. Changes in cell volume during incubation have been calculated on the basis of initial and final slice weight and inulin space.

3. The capacity of cells to shrink in response to extracellular osmotic stress was related principally to the external Cl concentration rather than to osmolality, increases in concentration being associated with enhanced shrinkage. Shrinkage was accompanied by net loss of cellular Cl. The ratio between intra- and extracellular Cl concentration (*ca.* 0·41) remained constant in all media.

4. In media containing low Cl concentration (90 mM), reduction of media Na concentration to 100 mM enhanced shrinkage. This effect was not observed when medium Cl concentration was greater than 90 mM.

5. Ethacrynic acid-cysteine (1 mM) significantly impaired the shrinkage response to extracellular osmotic stress, and caused cell swelling in media of relatively low Cl concentration and osmolality. It did not abolish the dependency of cell volume upon Cl concentration. There was marked reduction in the net amount of Cl lost from cells.

6. Ethacrynic acid-cysteine caused an increase in cellular Na content only in media containing 540 m-osmole/kg H_2O and Cl concentrations less than 215 mM.

7. Ouabain (1 mM) inhibited cell shrinkage to a lesser extent than ethacrynic acid-cysteine in all media except that causing the greatest shrinkage under control conditions (215 mM-Cl/1055 m-osmole/kg H_2O). It is suggested that a ouabain-sensitive process may play an increasingly important role in Cl-related cell shrinkage as this becomes more pronounced.

8. The findings are consistent with the view that Cl ions influence cell volume both through their effective external osmotic pressure and by means of Cl-specific process; the latter is associated with net loss of cellular Cl. A dependence of this loss upon Na/K exchange-linked metabolism is inferred, but the present findings do not permit the active or passive nature of the extrusion to be defined.

0022-3791/79/4630-0394 $01.50

INTRODUCTION

Cells in the renal medulla are subject to wide fluctuation in the osmotic character of their environment, in the course of which they are exposed to variable concentrations of solutes with differing degrees of permeancy, notably urea (highly permeant) and the less permeant ions Na and Cl.

When incubated in hyperosmotic media, cells in slices of rabbit outer medulla display a ouabain-sensitive increase in substrate oxidation (Lee, Alexander & Abodeely, 1970), which suggests that they possess the capacity to respond functionally to osmotic stress. Increased activation of Na–K-activated adenosine triphosphatase occurs under these conditions (Alexander & Lee, 1970), though whether this increase is brought about by osmotic stress *per se* or whether it is results from increased concentrations of ions such as Na and Cl may depend not only upon the species but also upon the experimental conditions (see for example the recent studies on activity of this enzyme in rat kidney by Knox, Sax, Wilson & Sen (1977)).

It would be in accord with the general physico-chemical properties of animal cells to expect that cells should lose water (i.e. shrink) in hyperosmotic media. This process might or might not involve solute extrusion. Among renal cells normally exposed to a hyperosmotic environment, those in the diluting segment are characterized by the capacity for extrusion of NaCl. In the rabbit, at least, the driving force for this process is the active transport of Cl ions across the tubular epithelium (Burg & Green, 1973*a*; Rocha & Kokko, 1973). A similar event has been described in the upper part of the human collecting tubule (Jacobson, Gross, Kawamura, Waters & Kokko, 1976).

The object of the present investigation has been to determine whether Cl ions, in addition to being actively transported by a significant proportion of the outer medullary cell population, are also implicated in cell volume regulation. All cells in the outer medulla face a common problem in regard to volume adjustment in the presence of variable extracellular hyperosmolality, but they may not overcome it by a unique mechanism, and the results obtained in this study cannot therefore be taken as reflecting properties common to all cells in this region.

Urea (in addition to Cl ions) was chosen as a hyperosmotic solute not because its presence exerts high osmotic stress upon the cells (indeed, its high degree of permeancy precludes this (Law, 1975*b*)) but because it is a major medullary solute *in vivo*.

A brief account of some of these findings has been published previously (Law, 1978).

METHODS

Duplicate slices (4–14 mg, thickness not exceeding 0·3 mm) from the outer medulla of normally hydrated adult Wistar rats were prepared as previously described (Law, 1975*a*). Slices were blotted on hard filter paper (Whatman no. 542) and weighed to the nearest 50 μg on a torsion balance.

In an attempt to achieve uniformity of slice composition prior to the start of experimental incubations, all slices were pre-incubated at 37 °C for 5 min in a medium of the following composition (mM): Na^+ 180, K^+ 5·9, Ca^{2+} 2·6, Mg^{2+} 1·2, choline 45, Cl^- 189, HCO_3^- 25, $H_2PO_4^-$ 2·2, SO_4^{2-} 1·2, pyruvate 4·8, glutamate 4·8, fumarate 5·3, glucose 10, urea 54. The calculated osmolality of this medium is 540 m-osmole/kg H_2O, which is approximately equal to that calculated for outer medullary fluids in normally hydrated rats (Law, 1975*b*). The medium was

gassed with 95% O_2/5% CO_2. All reagents were Analar grade where possible. At the end of this period slices were blotted and reweighed.

Slices were then immediately transferred to the medium (2·5 ml.) whose effect on cell volume it was desired to investigate. Incubations were carried out in 5 ml. hard-glass beakers sealed with parafilm and gassed through a pinhole, in a shaking water-bath at 37 °C. The incubation time was 25 min, it being shown in preliminary experiments that this was adequate for the attainment of the maximum cell volume changes caused by the media used. Media were based upon the medium described above, with the following modifications.

(1) *Chloride concentration and osmolality.* By appropriate equimolar substitution of Na gluconate for NaCl and of choline chloride for urea, media were prepared containing 90, 144, 189 and 215 mM-Cl. By addition of extra urea or urea plus choline chloride each of the above media was prepared to a final osmolality of 540 or 1055 m-osmole/kg H_2O. In the present text, in order to avoid lengthy description when media are referred to in terms of both Cl concentration and osmolality, they will be described as 189/1055, etc., as appropriate.

(2) *Inhibitors of ion transport.* Strophanthin G (ouabain) (British Drug Houses) (1 mM) and ethacrynic acid (Merck, Sharp & Doehm, Ltd) (1 mM) were added separately or together to certain media as specified in the text. Ethacrynic acid was used in the form of its adduct with L-cysteine (Sigma Chemical Co.), freshly prepared according to the method of Burg & Green (1973*b*). This adduct will be referred to as EA-Cys.

(3) *Low Na.* By equimolar substitution of choline chloride for NaCl, media were prepared containing chloride and osmolal concentrations as described in (1) but with 100 mM-Na in place of 180 mM-Na.

Determination of cell volume and intracellular composition

Because of the small size of the slices used it was found impracticable to estimate intracellular Na, K and Cl in the same slice. Initial attempts to equilibrate intracellular Cl with ^{36}Cl, thus enabling very much lower concentrations of intracellular Cl to be estimated than is possible using conventional conductimetric technique, were abandoned due to the unacceptably wide variations in concentrations so determined. It was therefore necessary to perform experiments under two general headings.

(*a*) *Cell volume, Na and K.* [^{14}C]Carboxyl inulin (Radiochemical Centre, Amersham) was added to incubation media (final conc. *ca.* 0·5 μc/ml.). Twenty-five minutes is sufficient for the penetration of this marker into the extracellular space (Law, 1975*a*) while minimizing any error in this determination due to cellular penetration (McIver & Macknight, 1974). After removal from incubation medium, slices were briefly rinsed in identical 'cold' medium, blotted and reweighed. They were then leached overnight in 3 ml. distilled water. The activity of the leachate, and of a 1250× dilution of the original incubation medium, was determined using a Packard TriCarb Liquid Scintillation Spectrometer. The inulin space was expressed absolutely (μl.) by direct comparison of the activities of these fluids, and as μl./100 mg final wet weight (%), calculated as described previously (Law, 1975*a*). The co-identity of the inulin space and the extracellular space has been discussed elsewhere (McIver & Macknight, 1974; Law, 1975*a*) and will be assumed in this paper.

Cell volume change during incubation was assessed on the basis of alteration in cell water content. Macknight & Leaf (1977) have recently discussed the validity for this method and have concluded that save in the case of grossly swollen tissues it is acceptable. In calculating the initial cell volume certain assumptions, based on previous experience of work with outer medullary slices, have been necessary. (i) The solute-free dry matter in fresh slices is approximately 11% (w/w) (Law, 1975*a*, 1976). (ii) In fresh tissue the extracellular space is approximately 24 μl./100 mg wet wt. (Law, 1975*a*); this is equivalent to 27 μl./100 μl. tissue fluid. With only minor variations these values have been confirmed in subsequent work, as has the observation that fresh slices lose up to 10% of their initial weight during the first few minutes of incubation (Law, 1975*a*, *b*). For present purposes it will be assumed that this loss occurs from the cellular compartment (damaged or detached cells) and extracellular region (blood, luminal fluid, etc.) in proportion to their relative dimensions (i.e. that the extracellular space, as a proportion of total slice fluid, is not affected by this loss); also that the specific gravity of renal tissue is 1·0. It must be accepted that uniform application of the values quoted above must inevitably lead to quantitative errors in individual slices. Percentage change in cell volume during 25 min incubation was calculated as follows (initial volume = 100):

$$\% \text{ change} = \frac{[\text{final wt.} - (0{\cdot}11 \times \text{fresh wt.})] - \text{inulin space } (\mu\text{l.})}{[\text{initial wt.} - (0{\cdot}11 \times \text{fresh wt.})] \times (1 - 0{\cdot}27)} \times 100.$$

The numerator and denominator represent the final and initial cells volume (μl.) respectively. Weights are in mg wet tissue. Values greater than 100 represent cellular swelling, values less than 100 shrinkage.

The concentrations of Na and K in the leachate were estimated by flame photometry against 0·2 or 0·3 mM standards, and the intracellular concentrations calculated according to Law (1976).

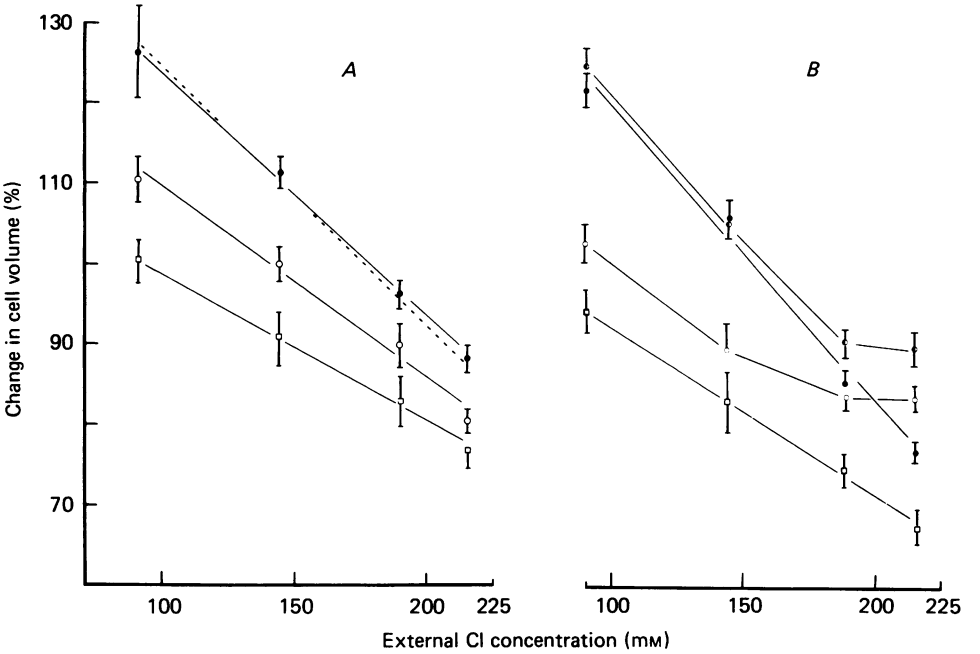

Fig. 1*A*, *B*. Percentage change in outer medullary cell volume (initial volume = 100) following incubation for 25 min in media of 540 m-osmole/kg H_2O (Fig. 1*A*) and 1055 m-osmole/kg H_2O (Fig. 1*B*) containing 90, 144, 189 and 215 mM-Cl and 180 mM-Na, under control conditions (□) and in the presence of EA-Cys (●), ouabain (○) and EA-Cys plus ouabain (◐) (all 1 mM). In media of 540 m-osmole/kg H_2O the results obtained using EA-Cys plus ouabain were nearly identical with those obtained with EA-Cys alone. Individual points for the former have therefore been omitted in the interests of clarity, and represented by a dashed regression line. Points show mean ± S.E. of mean (+ or − S.E. of mean where error bars would otherwise overlap) ($16 \geqslant n \geqslant 6$).

(*b*) *Chloride*. After incubation and reweighing slices were leached overnight in 250 μl. distilled water. The Cl concentration in the leachate was estimated using a Corning-EEL 920 Chloride Meter. By setting the meter to read the Cl concentration of 20 μl. samples, but administering aliquots of 200 μl., the functional sensitivity of the meter can be increased by a factor of 10×. Readings obtained by this method are reliable and reproducible and allow determinations to be made within the low Cl concentration range found in the slice leachates. Following incubation in media containing 90 mM-Cl it was necessary, except for relatively large slices, to incubate slices in pairs in order to bring the leachate Cl concentration within the range of sensitivity of the instrument. Intracellular Cl concentration was calculated as in Law (1977), the values used for the inulin space being those obtained in parallel studies of cell volume and cation content as described under (*a*). Note that in calculating intracellular Cl concentration, unlike Na and K concentrations, the fluid volume of the slice is not negligible by comparison with that of the leachate, and must be allowed for.

RESULTS

Cell volume and Cl content

In Fig. 1*A* and *B* are shown the percentage changes in medullary cell volume resulting from incubation in media of 540 and 1055 m-osmole/kg H_2O and containing 180 mM-Na and 90, 144, 189 and 215 mM-Cl. Results are shown under control conditions and in the presence of EA-Cys, ouabain and EA-Cys plus ouabain. Under control conditions there exists a rectilinear relationship between cell volume, as percentage of the initial volume (= 100), and the external Cl concentration, $[Cl]_o$. For each value of $[Cl]_o$ the decrease in cell volume was less in media containing 540 m-osmole/kg H_2O (Fig. 1*A*) than in those of 1055 m-osmole/kg H_2O (Fig. 1*B*). The effects of EA-Cys, ouabain and EA-Cys plus ouabain may be summarized as follows. In media containing 540 m-osmole/kg H_2O (*a*) both drugs inhibited cell shrinkage, but at the concentrations used (1 mM) the effect of EA-Cys was the greater, (*b*) the degree of swelling or impairment of shrinkage due to EA-Cys plus ouabain was indistinguishable from that caused by EA-Cys alone, suggesting that the inhibitory effects of these drugs are not additive under these conditions, and (*c*) cell volumes in the presence of each drug remained related to $[Cl]_o$. In media of 1055 m-osmole/kg H_2O a similar pattern of response was found when $[Cl]_o$ was 90 or 144 mM. But in medium 189/1055 a change in this pattern may be discerned which becomes accentuated and statistically significant in medium 215/1055 (the medium causing the greatest cell shrinkage under control conditions). In this medium the shrinkage after adding ouabain ($83{\cdot}3 \pm 1{\cdot}4\,\%$ (7)) was less than that when EA-Cys alone was present ($77{\cdot}4 \pm 1{\cdot}3\,\%$ (15); $P < 0{\cdot}025$) (values are mean ± S.E. of mean (*n*)). When both drugs were present shrinkage was further impaired ($89{\cdot}8 \pm 1{\cdot}6\,\%$ (7); $P < 0{\cdot}01$ *vs.* ouabain alone and $< 0{\cdot}001$ *vs.* EA-Cys alone).

It may be seen from Fig. 1*A* that slices incubated under control conditions in medium 189/540 shrank to about 86% of their original volume. Since both the osmolality and Cl concentration of this medium have been considered as typical of outer medullary interstitial fluid (Law, 1975*b*, 1977), this shrinkage requires explanation. It is very unlikely that the quantitative assumptions referred to in the Methods section were grossly erroneous when applied to the slices used in the present study. By the substitution of alternative values for percentage solute-free dry matter and initial inulin space in the expression used to calculate final cell volume, it may be shown that both would require to be consistently and considerably in error if even moderately inaccurate values for final cell volume and intracellular concentrations were to be derived. For example, even if the true percentage solute-free dry matter and inulin space were respectively 20% less than and greater than the values actually employed, the apparent 86% shrinkage would rise only to 91%.

A more probable explanation derives from the difference between the cellular environment *in vivo* and *in vitro*, and in particular the likelihood that cells swell during the 5 min pre-incubatory period. Cell volume at the start of the experimental incubation (initial volume) would thus be over-estimated. In confirmation of frequent observations during similar previous studies, slices lost up to 10% of their fresh weight during the 5 min pre-incubatory period; but if this loss is assumed to consist chiefly of cell debris, adherent blood and luminal fluid which were not removed by the initial blotting, there is no reason why cell swelling should not occur simultaneously. The degreee of this swelling would serve merely to modify the net weight loss. The conditions under which the preincubations were carried out were characterized by two factors which might be expected to cause cell swelling: (i) osmotic entry of water into the cells due to absence from the incubation medium of protein, which is normally present in rat medullary interstitial fluid (Williams, Moffat & Creasey, 1971) and (ii) reduction or absence of the hydrostatic constraint

TABLE 1. The effect of extracellular Cl concentration ($[Cl]_o$) on medullary intracellular Cl concentration ($[Cl]_i$) and content in media of 540 and 1055 m-osmole/kg H_2O under control conditions and in the presence of EA-Cys (1 mM) and ouabain (1 mM). $R = [Cl]_i/[Cl]_o$. Values are mean ± S.E. of mean (n). In all cases n for concentration and content are identical. The significance of differences between values in the presence of EA-Cys or ouabain and those under control conditions are represented thus: * $P < 0.05$; ** $P < 0.025$; *** $P < 0.001$

		Control			Ethacrynic acid-cysteine			Ouabain		
$[Cl]_o$ (mM)	Osmolality (m-osmole/kg H_2O)	$[Cl]_i$ (mM)	R	Cl content (μequiv/g SFDW)	$[Cl]_i$ (mM)	R	Cl content (μequiv/g SFDW)	$[Cl]_i$ (mM)	R	Cl content (μequiv/g SFDW)
90	540	36·2 ± 4·1 (7)	0·40	194 ± 22	39·2 ± 1·6 (7)	0·44	282 ± 6***	31·3 ± 3·2 (7)	0·35	181 ± 12
	1055	40·3 ± 3·5 (6)	0·45	212 ± 16	38·4 ± 3·4 (6)	0·43	272 ± 24	33·8 ± 3·1 (8)	0·38	194 ± 14
144	540	55·6 ± 7·2 (8)	0·39	270 ± 27	55·9 ± 2·7 (8)	0·39	340 ± 13*	44·5 ± 1·7 (7)	0·31	240 ± 7
	1055	61·2 ± 3·1 (6)	0·43	273 ± 13	55·7 ± 2·4 (14)	0·39	324 ± 14*	52·7 ± 3·1 (7)	0·37	264 ± 15
189	540	77·3 ± 7·7 (8)	0·41	357 ± 29	79·3 ± 4·0 (8)	0·42	417 ± 13	62·6 ± 3·0 (8)	0·33	299 ± 12
	1055	82·3 ± 10·1 (6)	0·44	333 ± 41	87·6 ± 4·9 (7)	0·46	393 ± 19	65·8 ± 5·5 (8)	0·35	299 ± 21
215	540	87·6 ± 2·8 (7)	0·41	390 ± 11	93·0 ± 5·6 (8)	0·43	473 ± 21**	73·4 ± 4·8 (8)*	0·34	324 ± 13**
	1055	96·1 ± 4·9 (16)	0·45	358 ± 18	96·3 ± 5·2 (8)	0·45	418 ± 15*	81·8 ± 2·3 (8)	0·38	366 ± 8

imposed by an intact basement membrane (and perhaps also the renal capsule). It has recently been suggested that hydrostatic and colloidal osmotic forces play a major role in determination of cortical tubular cell volume (Linshaw, Stapleton, Cuppage & Grantham, 1977), and that the high effective osmotic pressure within outer medullary cells causes transitory swelling in protein-free media (Bullivant, 1978).

In a parallel series of studies $[Cl]_i$ and Cl content were estimated at the end of incubation in each medium. It was found that under control conditions the ratio $[Cl]_i/[Cl]_o$ remained approximately constant within the range of $[Cl]_o$ studied. Values fell within the range 0·39–0·45 (mean 0·41) (see Table 1). This ratio is slightly

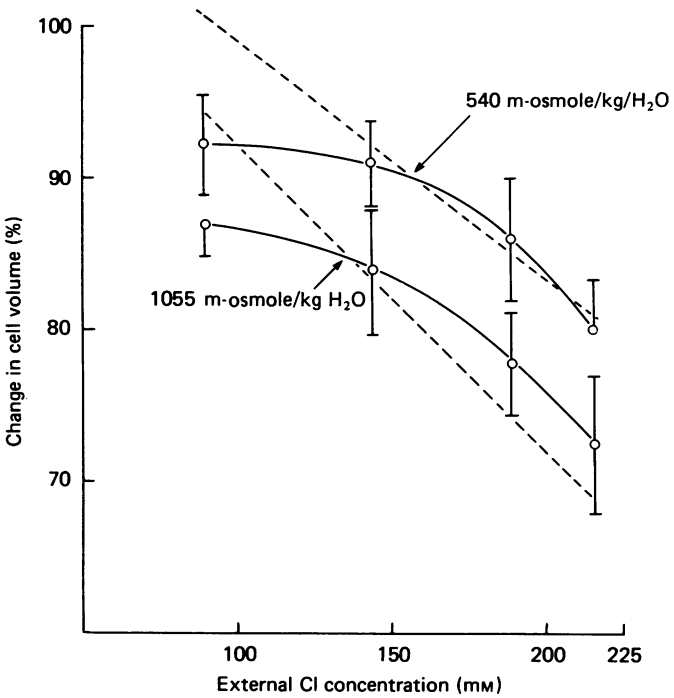

Fig. 2. Percentage change in outer medullary cell volume (initial volume = 100) following incubation for 25 min in media of 540 and 1055 m-osmole/kg H_2O containing 90, 144, 189 and 215 mM-Cl and 100 mM-Na. The dashed lines represent volumes in the presence of 180 mM-Na (from Fig. 1*A* and *B*). Points show mean ± S.E. of mean (+ or − S.E. of mean where error bars would otherwise overlap) ($10 \geqslant n \geqslant 6$).

lower than that reported previously (Law, 1977), but the discrepancy is not important in the present context: what is relevant is that cell shrinkage is thus seen to be directly related (although not necessarily causally) to $[Cl]_i$ as well as to $[Cl]_o$.

Intracellular Cl concentration was also estimated at the end of 5 min pre-incubatory periods and found to be 80·3 ± 2·1 mM (10) ($[Cl]_i/[Cl]_o$ = 0·42). Although the true extracellular space cannot be measured by inulin penetration over so short a period, if a value of 25 ml./100 g wet weight is assumed, this coupled with 11% SFDW yields an initial Cl content of approximately 440 μequiv/g SFDW.

EA-Cys did not significantly affect $[Cl]_i$ in any medium, but it caused net retention of Cl as indicated by the increased content, as compared with control levels (although

this did not reach significance at the level $P < 0{\cdot}05$ in all media; see Table 1). Ouabain slightly but consistently reduced $[Cl]_i$, but its effect in this respect was less marked than in earlier studies (Law, 1977) and only in medium 215/540 did this reduction achieve significance at $P < 0{\cdot}05$ (Table 1). This medium was the only one in which Cl content was significantly reduced by ouabain.

When the external Na concentration was reduced from 180 to 100 mM, the relationship between cell volume and $[Cl]_o$ under control conditions changed from rectilinear (Fig. 1) to curvilinear (Fig. 2). However, only in medium 90/1055 were the individual

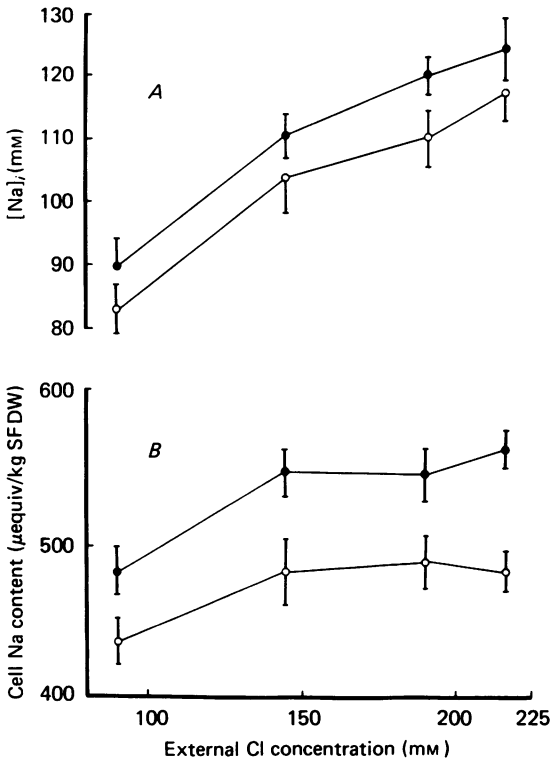

Fig. 3*A*, *B*. Intracellular Na concentration (mM) (Fig. 3*A*) and content (μequiv/g SFDW) (Fig. 3*B*) of outer medullary cells following incubation for 25 min in media of 540 m-osmole/kg H_2O (●) and 1055 m-osmole/kg H_2O (○) containing 90, 144, 189 and 215 mM-Cl and 180 mM-Na. Points show mean ± S.E. of mean (+ or − S.E. of mean where error bars would otherwise overlap) ($16 \geqslant n \geqslant 6$).

values for cell volume significantly different under the two conditions (volume in the presence of 180 mM-Na = 94·6 ± 2·7 (8); in the presence of 100 mM-Na = 86·9 ± 2·0 (8); $P < 0{\cdot}05$) (for medium 90/540, $0{\cdot}1 > P > 0{\cdot}05$). In media containing 90 mM-Cl both $[Cl]_i$ and Cl content were slightly and consistently elevated, although this rise was significant only for $[Cl]_i$ in medium 90/540 (46·9 ± 2·4 mM (8) – cf. control value (Table 1) = 36·2 ± 4·1 mM (7); $P < 0{\cdot}05$). No significant increases or decreases in $[Cl]_i$ or Cl content were observed in media containing > 90 mM-Cl.

Intracellular Na

Fig. 3*A* and *B* show respectively $[Na]_i$ and Na content as a function of $[Cl]_o$ in media of 540 and 1055 m-osmole/kg H_2O. From Fig. 3*A* it may be seen that in both media $[Na]_i$ increased as $[Cl]_o$ was raised, and was consistently slightly higher in media of 540 m-osmole/kg H_2O than in media of 1055 m-osmole/kg H_2O. This discrepancy is accentuated when Na content is represented (Fig. 3*B*), indicative of a relatively greater net loss of cellular Na during shrinkage in medium of the higher

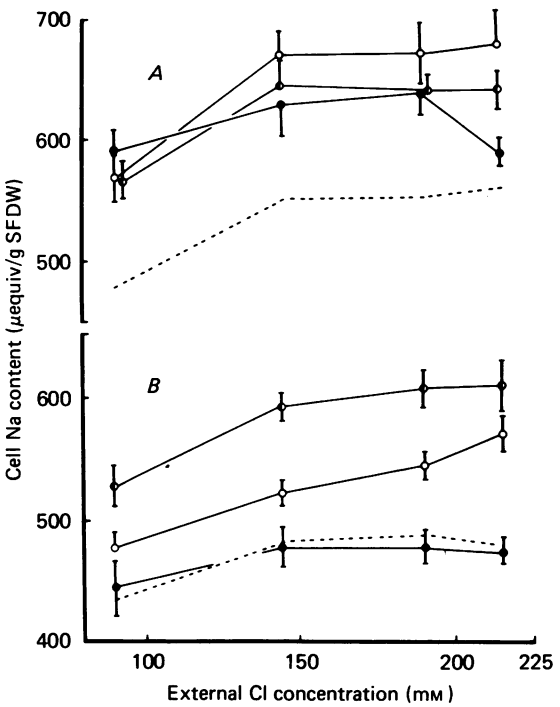

Fig. 4*A*, *B*. The effects of EA-Cys (●), ouabain (○) and EA-Cys plus ouabain (◐) (all 1 mM) on outer medullary intracellular Na content (μequiv/g SFDW) following incubation in media of 540 m-osmole/kg H_2O (Fig. 4*A*) and 1055 m-osmole/kg (Fig. 5*B*) containing 90, 144, 189 and 215 mM-Cl and 180 mM-Na. The dashed lines represent control values (from Fig. 3*B*). Points show mean ± S.E. of mean (+ or − S.E. of mean where error bars would otherwise overlap) ($16 \geqslant n \geqslant 7$).

osmolality. Na content, unlike $[Na]_i$, did not show any consistent dependency upon $[Cl]_o$ (Fig. 3*B*), but in media of both osmolalities was somewhat depressed in the presence of 90 mM-Cl. This might have been due to the presence of gluconate ions, which penetrate cells less readily than Cl.

The effects of EA-Cys, ouabain and EA-Cys plus ouabain on cellular Na content in media of 540 and 1055 m-osmole/kg are shown in Fig. 4*A* and *B*, respectively. The chief features of these may be summarized as follows. In media containing 540 m-osmole/kg H_2O (Fig. 4*A*), EA-Cys, ouabain and EA-Cys plus ouabain all significantly raised cellular Na content above control levels in the presence of 90,

144 and 189 mM-Cl. In medium 215/540 (i.e. the medium associated with the greatest shrinkage) Na content in the presence of EA-Cys (588 ± 18 μequiv/g SFDW (8)) did not differ significantly from the control value (561 ± 13 μequiv/g SFDW (12)). In this medium, unlike those containing lower Cl concentrations, the Na content in the presence of EA-Cys was highly significantly lower than in medium containing ouabain (675 ± 28 μequiv/g SFDW (8), $P < 0{\cdot}001$) or EA-Cys plus ouabain (631 ± 14 μequiv/g SFDW (8), $P < 0{\cdot}005$).

In media of 1055 m-osmole/kg H_2O cellular Na contents under control conditions and in the presence of EA-Cys were similar for all levels of $[Cl]_o$ (Fig. 4*B*). Ouabain increased Na content by comparison with control levels, although this was significant only in media of high $[Cl]_o$ (in 189 mM-Cl, 490 ± 18 (8) *vs.* 541 ± 12 (8), $P < 0{\cdot}025$; in 215 mM-Cl, 482 ± 17 (8) *vs.* 568 ± 16 (10), $P < 0{\cdot}005$; values are in μequiv/g SFDW, control values being quoted first). EA-Cys plus ouabain raised cellular Na contents still further (P *vs.* ouabain alone being $< 0{\cdot}025$ in all media).

Predictably, reduction of external Na to 100 mM led to very marked reduction in $[Na]_i$ under all conditions. Concentrations fell within the range 60–70 mM; individual values have here been omitted.

Intracellular K

The effects of EA-Cys, ouabain and EA-Cys plus ouabain on $[K]_i$ and K content are summarized in Table 2. Under control conditions, increases in $[K]_i$ resulted solely from decrease in cell volume. Neither EA-Cys nor EA-Cys plus ouabain significantly affected K content by comparison with control levels, although the associated impairment of cell shrinkage led to significant decreases in $[K]_i$. Results for EA-Cys alone have been omitted from Table 2 as they were not significantly different from those obtained using EA-Cys plus ouabain. Ouabain alone significantly lowered $[K]_i$ to an extent which could not be accounted for simply on the basis of cell swelling or impaired shrinkage, cellular K content also being significantly reduced (except in media 144/1055 and 215/1055).

DISCUSSION

There are four main questions to be considered in evaluating the physiological significance of the present findings. First, is the apparent effect of external Cl ions on cell volume simply an osmotic phenomenon, or is it an effect of this anion *per se*, operating through related variations in the intracellular concentration? Secondly, if the latter is wholly or partly the case, is such control exerted directly (e.g. through the operation of a Cl-extrusion mechanism) or indirectly (e.g. by the constraining effect of Cl upon a cation extrusion mechanism)? Thirdly, are cell shrinkage and net cell Cl loss quantitatively related? Lastly, what information regarding the roles of Cl and cations in mediating cell shrinkage, and the possible source of energy for this occurrence, can be obtained from studying the effects of inhibitors of ion transport, ouabain and EA-Cys?

Urea makes a major contribution to medullary fluid hyperosmolality *in vivo*. However, it freely permeates outer medullary cells. Interstitial urea thus exerts little osmotic influence upon cells in this region, and alterations in concentration

TABLE 2. The effect of extracellular Cl concentration ($[Cl]_o$) on outer medullary intracellular K concentration ($[K]_i$) and content in media of 540 and 1055 m-osmole/kg H_2O under control conditions and in the presence of ouabain (1 mM) and ouabain plus EA-Cys (1 mM). Values are mean ± S.E. of mean (n). In all cases n for concentration and content are identical. The significance of differences between values in the presence of ouabain or ouabain plus EA-Cys and those under control conditions are represented thus: * $P < 0.05$; ** $P < 0.025$; *** $P < 0.001$

		Control		Ouabain		Ethacrynic acid-cysteine plus ouabain	
$[Cl]_o$ (mM)	Osmolality (m-osmole/kg H_2O)	$[K]_i$ (mM)	K content (μequiv/g SFDW)	$[K]_i$ (mM)	K content (μequiv/g SFDW)	$[K]_i$ (mM)	K content (μequiv/g SFDW)
90	540	54·1 ± 4·8 (7)	289 ± 19	39·0 ± 0·8 (8)	236 ± 9*	40·8 ± 1·3 (8)*	251 ± 11
	1055	68·4 ± 1·4 (14)	287 ± 7	35·9 ± 0·9 (8)***	204 ± 7***	46·3 ± 1·5 (8)***	310 ± 13
144	540	59·9 ± 1·8 (8)	301 ± 12	44·1 ± 3·0 (8)***	236 ± 13**	47·9 ± 2·1 (7)***	293 ± 15
	1055	56·1 ± 1·6 (8)	245 ± 11	45·2 ± 1·0 (8)***	224 ± 4	45·8 ± 0·9 (8)***	271 ± 8
189	540	66·4 ± 1·2 (8)	300 ± 13	51·7 ± 2·2 (7)***	239 ± 9**	53·6 ± 2·3 (7)***	264 ± 12
	1055	65·2 ± 2·4 (8)	264 ± 12	44·1 ± 2·6 (8)***	212 ± 14*	55·0 ± 2·3 (6)*	251 ± 7
215	540	68·2 ± 1·4 (14)	280 ± 6	53·0 ± 2·6 (8)***	235 ± 10***	58·7 ± 1·5 (8)***	268 ± 10
	1055	74·7 ± 2·8 (16)	280 ± 6	65·1 ± 2·3 (8)*	294 ± 9	54·5 ± 1·7 (7)***	274 ± 7

cause only small changes in cell volume (Law, 1975*b*). Does the relationship between cell volume and $[Cl]_o$ under control conditions (Fig. 1) thus merely illustrate the consequence of altering effective external osmotic pressure by replacing urea with relatively less permeant Cl ions?

There is no reason to suppose that a simple osmotic effect does not occur. As such, it would influence the relationships illustrated in Figs. 1 and 2. But there are grounds for inferring that cell shrinkage is also influenced by $[Cl]_o$ *per se*. If cell shrinkage were simply dependent upon effective external osmotic pressure it would be expected that incubation in hyperosmolal media should lead to loss of cell water alone, with a consequent rise in $[Cl]_i$ relative to $[Cl]_o$. In fact, as may be seen from Table 1, the ratio $[Cl]_i/[Cl]_o$ remained approximately constant for all levels of osmolality and $[Cl]_o$, indicating that net cellular Cl and water loss occur *pari passu*.

Furthermore, in the preparation of media containing 90 mM-Cl, Na gluconate was substituted for NaCl such that the sum of Cl and gluconate concentrations was 144 mM. Most cells are highly impermeable to gluconate ions, yet for both osmolalities the volumes of cells in 90 mM-Cl exceeded those in 144 mM-Cl media, from which gluconate was excluded (Fig. 1). Unless outer medullary cells are abnormally permeable to gluconate, the volumes observed cannot be explained on purely osmotic grounds.

The findings presented in Fig. 2 relate to the question of whether the influence of Cl ions on cell volume is direct or is due to their constraining effect upon cation extrusion. KCl extrusion has been proposed as a volume-regulatory mechanism in cortical cells in hypotonic media (Dellasega & Grantham, 1973), but the present results do not suggest any regulatory role for K ions under control conditions (Table 2), and consideration will be given here only to the possibility that Na extrusion might be Cl-dependent. If Na extrusion is constrained by the availability of intracellular Cl, then raising $[Cl]_o$ would be expected progressively to reduce such restraint (through associated increase in $[Cl]_i$) and lead to increased shrinkage such as has been observed in these experiments.

The marked decrease in $[Na]_i$ caused by reducing $[Na]_o$ from 180 to 100 mM should lead to increased availability of intracellular Cl relative to intracellular Na. Despite the associated change in the volume/$[Cl]_o$ relationship from rectilinear to curvilinear (Fig. 2), in only a single case (medium 90/1055) are cell volumes significantly different in media of differing Na concentration. It seems unlikely that the trivial increases in $[Cl]_i$ and Cl content in this medium are causally related to the observed enhancement of cell shrinkage. The enhancement does not reach a high level of significance, and were it to be interpreted in terms of intracellular restraint of one extrusible ion species by another would more readily be accountable in terms of restraint of Cl ions by Na ions than the reverse. Any more elaborate explanation would require knowledge of the concentrations or activities of these ions at the sites of extrusion. This cannot be assessed by considering the intracellular compartment as homogeneous with respect to solute distribution. Compartmentalization of Cl occurs in renal cortical cells (Kleinzeller, Nedvidkova & Knotkova, 1967), and studies on washout of ^{36}Cl suggest that this may also occur in medullary cells (Law, 1977).

It has been pointed out in Results (*a*) that $[Cl]_i$ at the start of experimental incubations was approximately 80 mM, and (*b*) that the ratio $[Cl]_i/[Cl]_o$ = *ca.* 0·41

was maintained independent of $[Cl]_o$ and total osmolality. Clearly, therefore, net Cl loss was greatest from cells incubated in media containing the lowest Cl concentration, namely those in which least shrinkage occurred. Since it is unlikely that maximal Cl loss and minimal shrinkage are causally linked, even indirectly, it is worth while to determine whether net Cl loss can be resolved into two components, one associated only with maintenance of the ratio $[Cl]_i/[Cl]_o$, the other with cell volume decrease. In Table 3 an attempt has been made to quantify the latter component. This shows the difference (loss or gain) between the observed cellular Cl content (as in Table 1)

TABLE 3. Net gain (+) or loss (−) of Cl from medullary cells incubated in media of 540 and 1055 m-osmole/kg H_2O and containing 90, 144, 189 and 215 mM-Cl. As explained in the accompanying Discussion these values represent the difference between the observed cellular Cl contents (as shown in Table 1) and the contents which would have been found had no alteration in cell volume occurred and the ratio $[Cl]_o/[Cl]_i = 0{\cdot}41$ been adhered to for each level of $[Cl]_o$

$[Cl]_o$ (mM)	Osmolality (m-osmole/kg H_2O)	Net gain (+) or loss (−) of cellular Cl (μequiv/g SFDW)		
		Control	EA-Cys	Ouabain
90	540	−2	+76	−9
	1055	+9	+66	−9
144	540	−46	+12	−76
	1055	−40	+2	−55
189	540	−74	−7	−111
	1055	−80	−18	−123
215	540	−104	−36	−154
	1055	−119	−73	−117

and the content which would have been expected had the maintenance of the ratio $[Cl]_i/[Cl]_o$ not been accompanied by alteration in cell volume. Although the quantitative accuracy of the values derived from so small a sample must clearly be treated with caution, the overall trend of the findings is clear. Under all conditions studied, alteration of cell volume was related not to external osmolality but to the level of $[Cl]_o$ and hence $[Cl]_i$, although causality of relationship cannot here be established.

The effects of EA-Cys on Cl transport in the medulla are imperfectly understood. Burg & Green (1973*b*) have shown that in the diluting segment cellular entry of Cl across the luminar membrane is inhibited, an effect which might be supposed to depress cell Cl content. However, the specificity of EA-Cys is questionable (Macknight, 1969; Epstein, 1972; Law, 1976), and more than one transport process may be affected. In the present experiments EA-Cys caused net retention of intracellular Cl amounting to 40–80 μequiv/g SFDW at all levels of $[Cl]_o$ (Table 3). By analogy with the effects of EA-Cys at cortical level (Whittembury, 1968; Whittembury & Proverbio, 1970) this might be interpreted as inhibition of a volume-regulatory NaCl extrusion pump. Such inhibition should lead to retention of Na as well as Cl, while leaving intracellular K content unaffected. While these predictions are confirmed for Cl (Table 3) and K (Table 2), there is net retention of Na (by comparison with controls) only in media 90/540, 144/540 and 189/540, i.e. media causing relatively little cell shrinkage (Fig. 4*A* and *B*). It has been pointed out in the Intro-

duction that increased external osmolality may be associated with enhanced Na–K–ATPase activity and it is possible that in the present experiments such enhancement was sufficient to prevent cellular retention of Na in the presence of EA-Cys only when a certain level of effective external osmolality was achieved (as in 215/540 and all media of 1055 m-osmole/kg H_2O).

The effect of EA-Cys on $[Cl]_i$ reported here (Table 1) differs from that in an earlier study (Law, 1977) in which a small but significant decrease was found. This cannot be explained on the basis of minor technical differences. Final Cl concentrations in the presence of EA-Cys (present, $79{\cdot}3 \pm 4{\cdot}0$ (8); previous, $70{\cdot}4 \pm 3{\cdot}9$ (10)) are not significantly different at the level $P < 0{\cdot}05$, and the principal reason for failure to observe a decrease in the present study is the consistently slightly lower initial values of $[Cl]_i$ encountered.

Ouabain is thought to inhibit active Cl extrusion across the peritubular membrane of cells in the diluting segment (Burg & Green, 1973*a*; Rocha & Kokko, 1973), and this would be expected to lead to intracellular accumulation of Cl. As shown by Law (1977) ouabain lowers $[Cl]_i$ in medullary slices. In the present study $[Cl]_i$ and Cl content were significantly reduced only in medium 215/540, although minor reductions occurred in most other media (Table 1). There was no evidence of significant Cl accumulation, but this may reflect dissimilar effects of ouabain on distinct parts of the cell population. In regard to accompanying inhibition of shrinkage (Fig. 1) the influence of ouabain may be exerted by means of a reduction in Na/K exchange-dependent cell metabolism. Whittembury & Grantham (1976) have postulated that in the cortex the energy derived from hydrolysis of ATP is utilized both for Na/K exchange and for volume-regulatory electrogenic NaCl extrusion. If Cl extrusion in the outer medulla is in part dependent upon energy derived from this source, Cl-related regulation of cell volume should be modified in the presence of ouabain. That ouabain interferes with Na/K exchange may be seen from the fact that it decreases intracellular K content (except in medium 215/1055) (Table 2) and increases Na content (Fig. 4*A* and *B*). (The additive effects of EA-Cys and ouabain on cell Na content in media of 1055 m-osmole/kg H_2O (Fig. 4*B*) are consistent with inhibition of separate Na extrusion mechanisms, in accordance with the dual pump hypothesis as reviewed by Giebisch, Boulpaep & Whittembury (1971).) It is not clear why the effects are not additive in media of 540 m-osmole/kg H_2O (Fig. 2*A*). It is widely accepted that ouabain interferes with Na/K exchange principally by inhibition of Na–K-activated ATPase. The activity of this enzyme may be increased by raising extracellular osmolality (Alexander & Lee, 1970). The present results support the view that ouabain-sensitive processes play an increasingly important part in the regulation of cell volume as external [Cl] and osmolality are increased. This is evident from the marked departure from rectilinearity of the relation between cell volume and $[Cl]_o$ when ouabain was added to media 189/1055 and 215/1055, and would be consistent with enhanced importance of this enzyme in effecting cell shrinkage in these media. Increased enzymic activity with shrinkage could also partly explain why, for all levels of $[Cl]_o$, $[Na]_i$ and cell Na content were lower in the medium of higher osmolality (Fig. 3*A* and *B*).

In conclusion, there is reason to believe that Cl ions exert an influence upon outer medullary cell volume which cannot be accounted for solely in terms of effective extracellular osmotic pressure. Cell shrinkage in hyperosmolal media *in vitro* is

accompanied by a component of net Cl loss which increases in magnitude as shrinkage becomes more pronounced. The cortico-medullary Cl concentration gradient becomes steeper during hydropenia in the intact kidney of the rat (personal observation) and the dog (Ruiz-Guiñazú, Arrizurieta & Yelinek, 1964). It may be, therefore, that medullary cells *in vivo* (or a part of the cell population) shrink to a greater extent when medullary interstitial osmolality is high (the concentrating kidney) than when it is low (the diluting kidney), and that it is the concomitant fluctuation in interstitial [Cl] which effects this. The active or passive nature of Cl extrusion during shrinkage cannot be determined from the present findings, and thus it cannot be decided whether or not this loss is in part a manifestation of the Cl-driven hyperosmotic NaCl transport known to occur in the diluting segment. The observed effects of the known inhibitors of ion transport are not, however, inconsistent with the involvement of active processes.

The findings provide a basis upon which corresponding investigations might profitably be carried out on isolated perfused tubular segments, in particular those from the diluting segment.

REFERENCES

ALEXANDER, J. C. & LEE, J. B. (1970). Effect of osmolality on Na^+-K^+-ATPase in outer renal medulla. *Am. J. Physiol.* **219**, 1742–1745.

BULLIVANT, M. (1978). Volume changes in cells of the outer medulla during perfusion of the rat kidney. *J. Physiol.* **280**, 125–139.

BURG, M. B. & GREEN, N. (1973*a*). Function of the thick ascending limb of Henle's loop. *Am. J. Physiol.* **224**, 659–668.

BURG, M. B. & GREEN, N. (1973*b*). Effect of ethacrynic acid on the thick ascending limb of Henle's loop. *Kidney int.* **4**, 301–308.

DELLASEGA, M. & GRANTHAM, J. J. (1973). Regulation of renal tubule cell volume in hypotonic media. *Am. J. Physiol.* **224**, 1288–1294.

EPSTEIN, R. W. (1972). The effects of ethacrynic acid on active transport of sugars and ions and on other metabolic processes in rabbit kidney cortex. *Biochim. biophys. Acta* **274**, 128–139.

GIEBISCH, G., BOULPAEP, E. L. & WHITTEMBURY, G. (1971). Electrolyte transport in kidney tubule cells. *Phil. Trans. R. Soc.* B **262**, 175–196.

JACOBSON, H. R., GROSS, J. B., KAWAMURA, S., WATERS, J. D. & KOKKO, J. P. (1976). Electrophysiological study of isolated perfused human collecting ducts. *J. clin. Invest.* **58**, 1233–1239.

KLEINZELLER, A., NEDVIDKOVA, J. & KNOTKOVA, A. (1967). Effect of saline osmolarity on the steady-state level of water and electrolytes in kidney cortex cells. *Biochim. biophys. Acta* **135**, 286–299.

KNOX, W. H., SAX, J. A., WILSON, D. R. & SEN, A. K. (1977). Effect of osmolality on renal medullary Na-K-ATPase activity in the post-obstructive kidney. *Can. J. Physiol. Pharmacol.* **55**, 1112–1115.

LAW, R. O. (1975*a*) The inulin space, solute concentrations, and weight changes in rat renal medullary slices incubated in iso-osmolal media, and their modification during anoxia and hypothermia. *J. Physiol.* **247**, 37–54.

LAW, R. O. (1975*b*). Volume adjustment by renal medullary cells in hypo- and hyperosmolal solutions containing permeant and impermeant solutes. *J. Physiol.* **247**, 55–70.

LAW, R. O. (1976). 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. *J. Physiol.* **254**, 743–758.

LAW, R. O. (1977). Factors affecting the distribution of chloride ions in rat renal outer medulla. *J. Physiol.* **266**, 173–189.

LAW, R. O. (1978). The influence of external chloride concentration on the volume of renal medullary cells in hyperosmolal media. *J. Physiol.* **275**, 26–27*P*.

LEE, J. B., ALEXANDER, J. C. & ABODEELY, D. A. (1970). Regulation of the fuel of respiration by Na-K-ATPase. *J. clin. Invest.* **49**, 16a.

LINSHAW, M. A., STAPLETON, F. B., CUPPAGE, F. E. & GRANTHAM, J. J. (1977). Effect of basement membrane and colloidal osmotic pressure on renal tubule cell volume. *Am. J. Physiol.* **233**, F325–332.

MACKNIGHT, A.D. C. (1969). The effects of ethacrynic acid on the electrolyte and water contents of rat renal cortical slices. *Biochim. biophys. Acta* **173**, 223–233.

MACKNIGHT, A. D. C. & LEAF, A. (1977). Regulation of cellular volume. *Physiol. Rev.* **57**, 510–573.

MCIVER, D. J. L. & MACKNIGHT, A. D. C. (1974). Extracellular space in some isolated tissues. *J. Physiol.* **239**, 31–49.

ROCHA, A. S. & KOKKO, J. P. (1973). Sodium chloride and water transport in the medullary thick ascending limb: Evidence for active chloride transport. *J. clin. Invest.* **52**, 612–623.

RUIZ-GUIÑAZÚ, A., ARRIZURIETA, E. E. & YELINEK, L. (1964). Electrolyte, water and urea content in dog kidneys in different states of diuresis. *Am. J. Physiol.* **206**, 725–730.

WHITTEMBURY, G. (1968). Sodium and water transport in kidney proximal tubular cells. *J. gen. Physiol.* **51**, 303–314*s*.

WHITTEMBURY, G. & GRANTHAM, J. J. (1976). Cellular aspects of renal sodium transport and cell volume regulation. *Kidney int.* **9**, 103–120.

WHITTEMBURY, G. & PROVERBIO, F. (1970). Two modes of Na extrusion in cells from guinea-pig cortex slices. *Pflügers. Arch.* **316**, 1–25.

WILLIAMS, M. M. M., MOFFAT, D. B. & CREASEY, M. (1971). The effect of anti-diuretic hormone on the permeability of the vessels of the renal medulla of the rat during water diuresis. *Q. Jl exp. Physiol.* **56**, 250–256.