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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

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SUMMARY

1. The volume of distribution of [^{14}C]carboxyl inulin has been studied in slices of outer and inner medulla from rat kidney incubated in Krebs phosphate-bicarbonate Ringer, modified to render it iso-osmolal with the tissue fluids in these zones, under three conditions, (*a*) aerobically at 37° C (control), (*b*) anoxically at 37° C, and (*c*) aerobically at 0° C.

2. Under control conditions near steady-state volumes of approximately 24 and 42 μl./100 mg wet weight slice were obtained for outer and inner medulla respectively during the period 10–30 min from the start of incubation. In the outer medulla the volumes of distribution in anoxic and hypothermic slices exceeded that in control slices during this time, but control values increased from 30 to 100 min so that after 100 min the distribution volumes were approximately 30 μl./100 mg under each set of conditions.

3. In the inner medulla control and anoxic slices had inulin distribution volumes of approximately 42 μl./100 mg during 10–30 min, rising to over 50 μl./100 mg by 100 min. Slices incubated hypothermically reached a steady-state value of approximately 40 μl./100 mg by 30 min, which did not increase further for up to 100 min.

4. All slices lost about 10 % of their initial weight during the first 3 min of incubation. Thereafter control slices maintained weight constancy for at least 30 min (outer medulla) or 100 min (inner medulla); slices incubated anoxically or hypothermically gained weight, the gains being greatest in anoxic outer and hypothermic inner medulla.

5. The K concentration within control slices (both zones), hypothermic outer and anoxic inner medulla attained equilibrium when slice [K] was approximately 8 × medium [K] (5·9 mM). In anoxic outer and hypothermic inner medullary slices [K] fell to a significantly greater extent,

but interpretation of these findings in terms of slice K loss is subject to modification in respect of the increases in slice weight (water content) accompanying the [K] decreases.

6. There was a transient (1–3 min) rise in [Na] in all slices. This was followed by a [Na] decrease, which was most apparent in control slices, and finally a gradual increase towards medium [Na] (141 and 180 mM for outer and inner medulla respectively).

INTRODUCTION

The ability of cells to regulate their volume and intracellular solute concentrations assumes a special significance in the case of the renal medulla, since the cells of this region are subject to considerable fluctuation in the osmotic character of their environment. Several investigators have examined the effects of variable extracellular composition upon the metabolic properties of medullary cells (Kean, Adams, Winters & Davis, 1961; Lowenstein, Smith & Segal, 1968; Alexander & Lee, 1970; Abodeely & Lee, 1971; Gutman, Wald & Czaczkes, 1973) but the importance of the osmotic environment has generally been overlooked when not implicit in the nature of the investigation (Lee, Vance & Cahill, 1962; Gans, Bailie & Biggs, 1966; Lowenstein & Hagopian, 1969; De Jairala, Vieyra & MacLaughlin, 1972; De Jairala, Vieyra, Garcia & Rasia, 1973).

This paper reports the results of an investigation into certain characteristics of renal outer and inner medullary slices incubated aerobically at 37° C in Krebs phosphate-bicarbonate Ringer modified by the addition of urea until iso-osmolal with the fluids of these regions as determined by fresh tissue analysis. Three features in particular have been regarded as general indices of cell volume and integrity, viz. slice weight changes, slice K concentration, and inulin space. Inulin has been regarded as a satisfactory indicator of the extracellular volume in a variety of rat tissue including renal cortex (Rosenberg, Downing & Segal, 1962; Macknight, 1968), medulla (Gans *et al.* 1966), diaphragm (Randle & Smith, 1958) and skeletal muscle (Law & Phelps, 1966). In addition the effects of metabolic inhibitors (anoxia and hypothermia) have been investigated; these have been interpreted in the light of the presumed metabolic status of these regions, and in relation to the findings in slices incubated aerobically at 37° C.

A brief account of some of these findings has been published previously (Law, 1974).

METHODS

Experiments were performed using the kidneys from normal hydrated adult rats (200–350 g). Rats were killed by stunning and bleeding via the carotid arteries. Both kidneys were exposed by a mid-line incision, and rapidly excised. One kidney was stored on ice while slices were prepared from the contralateral kidney (a process requiring 4–8 min, including subsequent handling as detailed below). After careful decapsulation slices were cut freehand with a razor from the outer (red) and inner (white) medulla. The outer medullary slice was cut normal to the major axis of Henle's loop, at a level just above the junction with the inner medulla; subcortical tissue was excluded. It did not prove possible to obtain manipulable slices of inner medulla in the normal plane; accordingly these were cut parallel to the major axis of Henle's loop, from the junction with the outer medulla to the base of the papillary tip, and would be expected to include fluids of graded osmolality across their width. Outer and inner medulla corresponded approximately to zones 4 and 3 referred to by Atherton, Hai & Thomas (1968); for details of the tubular elements found in these zones see Fourman & Moffat (1971). The thickness of the slices was 0·3–0·4 mm, and their weights ranged from 3 to 10 mg. With practice it was found possible to cut slices rapidly and with a good degree of consistency. Each kidney provided a single slice from each zone.

Slices were blotted firmly but carefully on hard filter paper (Whatman No. 542) and weighed to the nearest 0·1 mg on a torsion balance (the error incurred in weighing thus being not greater than $\pm 1{\cdot}5\,\%$).

With minimal delay each slice was transferred to the appropriate incubation medium (3 ml.), in a hard glass microbeaker, containing [^{14}C]-carboxyl inulin (see below for details of incubation media). Beakers were sealed with parafilm containing a single pin-hole, and gassing continued throughout incubation (see below). Fluid loss due to evaporation during incubation was not more than 3 % in 100 min, as estimated (*a*) gravimetrically, and (*b*) by increase in inulin concentration. Incubation was continued for 1, 3, 10, 30 or 100 min; slice [Na] was additionally estimated after 17 min. At the end of this time slices were removed from the incubation medium, briefly rinsed in medium from which inulin had been omitted, blotted and reweighed. Paradise & Morrow (1972) and McIver & Macknight (1974) have stressed the errors which may arise through variability of blotting technique, and every effort was made to keep the blotting procedure as uniform as possible. Reweighed slices were transferred to fresh microbeakers and leached overnight (18 hr) in 2 ml. distilled water at 4° C. Preliminary experiments indicated that on such small pieces of tissue distilled water extracted solutes as efficiently as 0·1 N-HNO_3 (Little, 1964) (recovery of Na, K, urea and inulin $\geqslant 97\,\%$, checked by re-extraction). The leaching fluid was analysed for Na, K, urea and inulin.

Composition of incubation media. Preliminary analysis of medullary tissue from eight rats was carried out by the technique described previously for rabbit renal tissue (Law, 1973). The following values were obtained (mean $\pm$ S.E., $n = 16$), for outer medulla, solute-free dry solids (SFDS) $10{\cdot}9 \pm 0{\cdot}4\,\%$ (w/w), Na $128 \pm 2{\cdot}8$ mM, K $93 \pm 1{\cdot}5$ mM, urea 122 ± 16 mM: for inner medulla, SFDS $7{\cdot}7 \pm 0{\cdot}3\,\%$ (w/w), Na $180 \pm 5{\cdot}5$ mM, K $66 \pm 1{\cdot}4$ mM, urea 251 ± 30 mM. These values were obtained on segments of tissue (10–30 mg), and it is relevant to inquire whether the figures obtained are applicable to the very much smaller slices used for incubation (3–10 mg). It did not prove possible to obtain consistent figures for SFDS of slices, but analysis of Na, K and urea in un-incubated slices, and conversion of the values obtained into slice concentrations (mM) using SFDS values obtained in larger segments, indicated that fresh slice composition was not significantly different from segment composition as

reported above. It therefore seems probable that percentage dry weights obtained in the larger segments may be applied also to slices. The time between slice sectioning and immersion in incubation medium was kept to a minimum (30–50 sec) in order to reduce the possibility of evaporative loss from the slice surfaces.

Summing these concentration as 2([Na]+[K])+[urea] (Schmidt-Nielsen & O'Dell, 1961) yielded approximate osmolalities of 565 and 740 m-osmole/kg H_2O for outer and inner medullary fluid respectively. These osmolalities were used as the basis for preparing incubation media iso-osmolal with the medullary fluids. The composition of segments, cut from the same levels as slices, was checked for all kidneys used in the course of subsequent experiments, in order to detect any pre-existing osmolal imbalance between individual slices and incubation media. A comparison between these findings and those made during the preliminary investigation of tissue composition (see above) is made in the Results section (see Table 1), in which the validity of the formula used to calculate osmolality is briefly assessed.

Incubation media were based upon Krebs phosphate-bicarbonate Ringer (1950), containing (mM) Na^+ 141, K^+ 5·9, Mg^{2+} 1·2, Ca^{2+} 2·6, Cl^- 104·8, $H_2PO_4^{2-}$ 2·2, HCO_3^- 24·9, SO_4^{2-} 1·2, glucose 10, pyruvate 4·8, glutamate 4·8. fumarate 5·3. All reagents were Analar grade where available. This solution contains metabolic substrate as glucose, believed to be the preferred medullary substrate (Abodeely & Lee, 1971; Holmes & DiScala, 1971), and as tricarboxylic acid cycle intermediates which may be aerobically metabolized *in vitro*, at least by the canine kidney (Mikulski, Angielski & Rogulski, 1972). Media were gassed to pH 7·4 with 95% O_2/5% CO_2.

The osmolality of this solution is approximately 310 m-osmole/kg H_2O. In order to raise the osmolality to levels compatible with those in the medullary fluids, the following additions were made: to the medium for outer medullary incubation, 255 m-mole urea/l.; to the medium for inner medullary incubation, 39 m-equiv NaCl/l. (thus raising medium [Na] and [Cl] to 180 and 144 mM respectively) plus 350 m-mole urea/l.

[^{14}C]carboxyl inulin (The Radiochemical Centre, Amersham) was added to both media in order to produce a final concentration of approximately 0·5 μc/ml., and the media were filtered before use. Slices were incubated in three groups.

(*a*) *Control* at 37° C gassed with 95% O_2/5% CO_2.

(*b*) *Anoxic* at 37° C gassed with 95% N_2/5% CO_2.

(*c*) *Hypothermic* at 0° C gassed with 95% O_2/5% CO_2.

Analytical methods. Na and K in leaching fluid from fresh and incubated tissue was estimated by flame photometry against 0·1 or 0·2 mM standards. Urea was estimated colorimetrically by a modification of the Berthelot reaction (Boehringer Mannheim). Results (as mM) were expressed in terms of the fluid content (tissue wet weight minus dry weight) of tissue at the time of analysis (i.e. at the end of incubation in the case of slices, or in fresh tissue in the case of unincubated segments). The activity of 1 ml. leaching fluid was determined using a Packard TriCarb Liquid Scintillation Spectrometer (the remaining 1 ml. thus being available for electrolyte and urea analysis) and compared with that of a 500 × dilution of incubation medium. The slice inulin space, as μl./100 mg wet wt. of tissue, was calculated as follows:

$$\text{Space} = \left(\frac{2 \times \text{counts per min in 1 ml. leachate}}{500 \times \text{counts per min in 1 ml. dil. incubation med.}}\right) \times \left(\frac{1 \times 10^5}{\text{Final wet wt. (mg)}}\right).$$

Assumptions. Other than those specifically referred to elsewhere in the text, the following assumptions were made.

(i) The specific gravity 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 slices and segments were negligible in relation to incubation and leaching fluid volumes.

(iv) Paired kidneys from an individual rat are identical in composition. No evidence was obtained in the present work to suggest that this is not so. It was previously noted (Law, 1974) that inner medullary solutes were slightly diluted during storage but this finding does not bear significantly upon any of the results reported in this paper.

RESULTS

Weight changes

The weight changes (as a percentage of initial weight, 100) of slices of outer and inner medulla incubated under control, anoxic and hypothermic conditions are shown in Fig. 1*a* and *b*. All slices lost approximately 10% of their initial weight during the first minute of incubation. Thereafter no further weight changes occurred in control slices for at least 30 min, although by 100 min the mean weight of outer medullary slices had increased somewhat. Under anoxic conditions the weight of slices from both zones increased again by 3 min, but thereafter the weight of outer medullary slices continued to increase while that ofinner slices remained approximately constant (the decrease in weight during the period 10–100 min is not significant). By 30 and 100 min the mean weight of outer slices significantly exceeded that of inner slices (30 min, 107·1 ± 1·7% (16) *vs.* 99·7 ± 2·0% (16), $P < 0{\cdot}01$; 100 min, 107·4 ± 1·4% (16) *vs.* 98·6 ± 1·2 (16), $P < 0{\cdot}001$; values are mean ± S.E. (*n*)). Conversely, during hypothermic incubation, the weight of inner medullary slices increased to a markedly greater extent than that of outer slices, although this difference disappeared by 100 min.

[^{14}C] *carboxyl inulin space*

Inulin spaces, as μl./100 mg final wet weight of tissue (i.e. post-incubation), are shown in Fig. 2*a* and *b*. The spaces in outer medulla reached a steady state within approximately 10 min in control slices; the volume of distribution did not increase significantly within the next 20 min, the mean value over this period being 24·4 ± 0·9 μl./100 mg (%) $n = 17$. The space rose to 31·6 ± 1·7% (8) after 100 min incubation. In anoxic conditions the inulin space also appeared to reach equilibrium in the period 10–30 min but at a significantly higher value (28·7 ± 0·8% (17), $P < 0{\cdot}001$). There was no further significant increase in distribution volume between 30 and 100 min. The curve for outer medullary hypothermic incubation presented a somewhat different appearance; an equilibrium value was not established

until nearly 30 min (30·8 ± 1·7% (10)), there then being no further increase for up to 100 min.

Under all conditions the inner medullary inulin space considerably exceeded that in the outer medulla. Unlike outer medulla, inner medullary space under control conditions increased slightly during the period 10–30 min, but this increase was not significant (40·3 ± 0·5% (9) *vs.* 44·0 ± 1·6% (11), $0{\cdot}1 > P > 0{\cdot}05$) and for the purposes of comparison

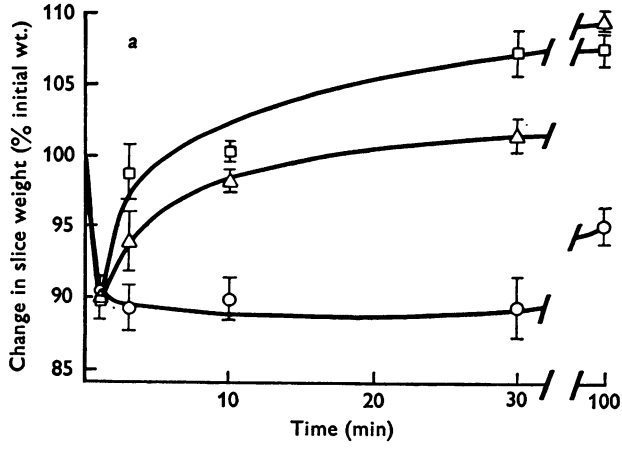

Fig. 1*a*. Weight (as % of initial weight, 100) of slices of renal outer medulla incubated under control (○), anoxic (□) and hypothermic (△) conditions. Values are mean ± 2 S.E. (n = 16–22).

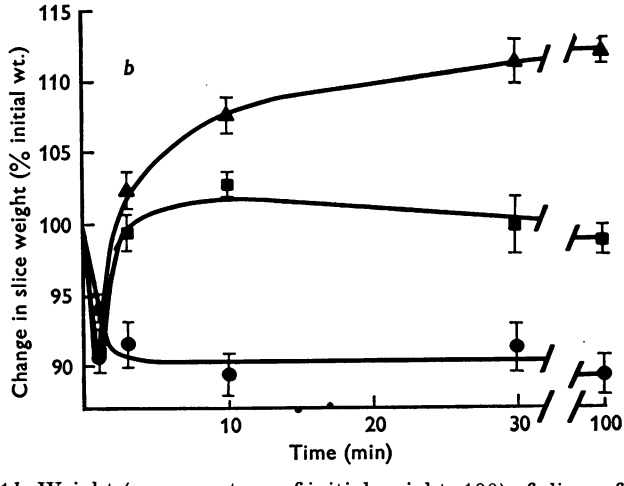

Fig. 1*b*. Weight (as percentage of initial weight, 100) of slices of renal inner medulla incubated under control (●), anoxic (■) and hypothermic (▲) conditions. Values are mean ± 2 S.E. (n = 16–22).

with the outer medulla an approximate value of 42% may be assigned to the slices during this period. During the same period the space increased significantly in anoxic slices ($39{\cdot}3 \pm 1{\cdot}1$% (10) *vs.* $45{\cdot}3 \pm 1{\cdot}7$% (7), $P < 0{\cdot}01$). The inulin spaces of both control and anoxic slices increased to slightly over 50% by 100 min; hypothermic inner medullary slices, like those in the outer medulla, reached an apparent equilibrium value by

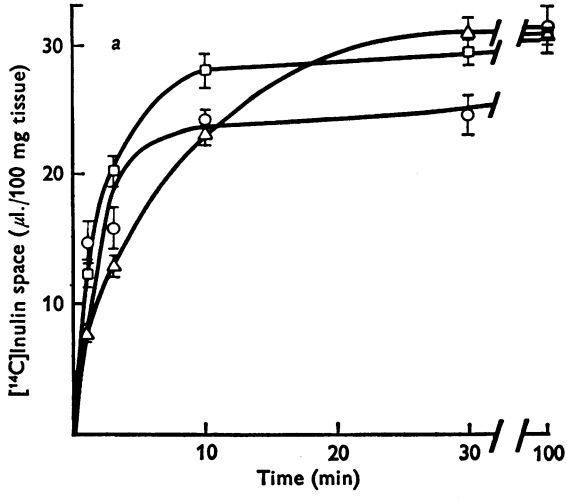

Fig. 2*a*. The inulin space as a function of time in slices of renal outer medulla incubated under control (○), anoxic (□) and hypothermic (△) conditions. Values are mean ± 2 S.E. (n = 8–10).

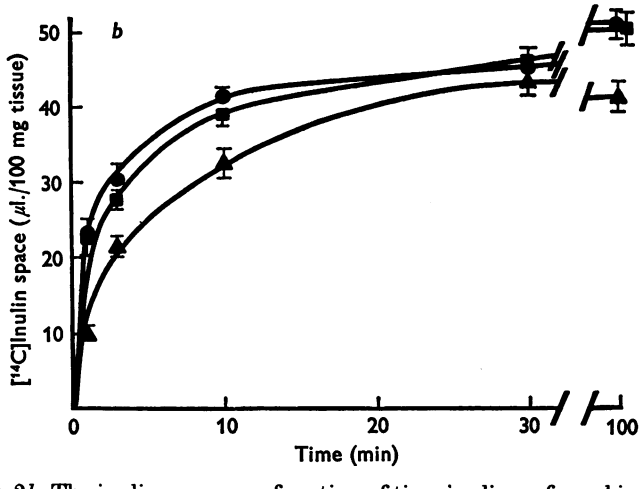

Fig. 2*b*. The inulin space as a function of time in slices of renal inner medulla incubated under control (●), anoxic (■) and hypothermic (▲) conditions. Values are mean ± 2 S.E. (n = 8–10).

30 min (40·4 ± 1·2 % (8)) which did not alter significantly for up to 100 min.

The physiological significance of these figures in terms of true extracellular space representation is examined in the Discussion, particularly in relation to intracellular penetration of inulin. It is concluded there that values of approximately 24 % and 42 % may be reasonable estimates of the extracellular space of incubated outer and inner slices under the control conditions of these experiments and during the time interval specified (10–30 min).

Potassium

The ability of slices, and therefore presumably predominantly of the constituent cells, to maintain [K] in excess of incubation medium [K] (5·9 mM) has been regarded here as one indication of cellular metabolic integrity. Fig. 3*a* and *b* show the extent to which this was achieved. The K concentration of all slices fell by 20–30 % during the first minute of incubation. After 10 min and 3 min incubation, outer and inner medullary slices respectively showed no further fluctuation of [K] for up to 100 min, maintaining a slice: medium ratio of approximately 8:1. In anoxic outer medullary slices [K] fell slowly for up to 100 min, at the end of which time the slice: medium ratio was approximately 5:1. However, since these slices were simultaneously gaining weight, a process presumably involving cellular swelling and dilution of cytoplasmic K, the loss of K was probably less than concentration values alone indicate. The probable disparity between inulin space and extracellular space under these conditions (see Discussion) precludes the possibility of accurately quantifying this loss in terms of cellular K leakage. The same consideration applies to hypothermic inner medullary slices, in which the slice: medium ratio fell to about 4:1 by 100 min while slice weight increased. Conversely, hypothermic outer and anoxic inner medullary slices maintained [K] close to control levels; since there was moderate weight increase in these slices during incubation, it may be concluded that here intracellular [K] was maintained by means of uptake of K from the incubation medium.

Note that under control conditions, and other conditions in which slice [K] was maintained at approximately control values, the proportionate fall in [K] was greater in outer than in inner medulla (~ 45 % *vs.* ~ 30 %, as percentage of [K] in fresh tissue).

Sodium

The Na concentration of slices fluctuated as shown in Figs. 4*a*, 4*b* and 4*c*. The basic pattern of fluctuation was common to all slices, consisting of an initial phase (up to 3 min) during which [Na] rose, followed by a

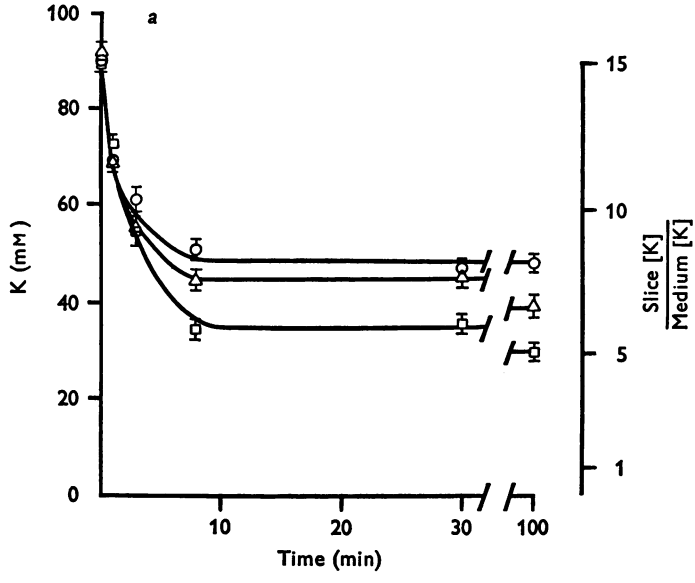

Fig. 3*a*. K concentration in slices of renal outer medulla incubated under control (○), anoxic (□) and hypothermic (△) conditions. The right-hand ordinate indicates the ratio between slice and incubation medium concentrations. Values are mean ± 2 S.E. (n = 32–38 for initial concentrations, six to nine after incubation).

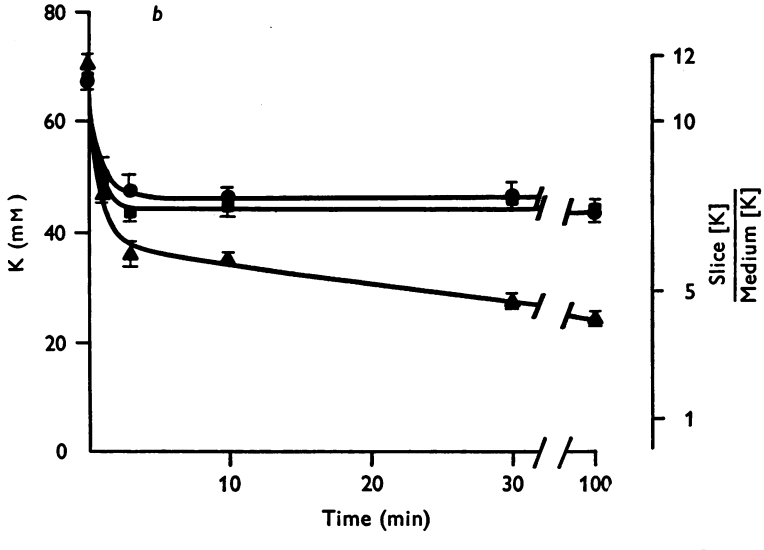

Fig. 3*b*. K concentration in slices of renal inner medulla incubated under control (●), anoxic (■) and hypothermic (▲) conditions. The right-hand ordinate indicates the ratio between slice and incubation medium concentrations. Values are mean ± 2 S.E. (n = 37–46 for initial concentrations, seven to ten after incubation).

variable decrease and finally a slow increase towards medium [Na] values. Comparison with Fig. 1 shows that these fluctuations do not appear to be directly related to changes in slice weight (water content). They were most marked in control slices, notably as regards the phase of decreasing [Na]. In the outer medulla [Na] fell by a mean of 29 mM between the third and tenth minute of incubation ($138 \pm 6{\cdot}6$ (10) to $109 \pm 5{\cdot}0$ (9), $P < 0{\cdot}005$). The inner medullary [Na] decrease over the period 1–10 min was from $209 \pm 4{\cdot}9$ to $147 \pm 4{\cdot}7$ mM, $P < 0{\cdot}001$. Slice [Na] during anoxia and hypothermic approximated to medium [Na] in both zones after 30 min incubation, indicating widespread cellular penetration. In control slices [Na]

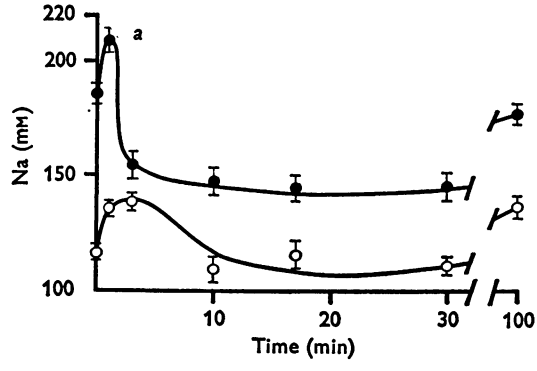

Fig. 4*a*. Na concentration in slices of renal outer (○) and inner (●) medulla incubated under control conditions. The Na concentrations in the incubation media were 141 mM and 180 mM respectively. Values are mean ± 2 S.E. (n = 41 for initial concentrations, eight or nine after incubation).

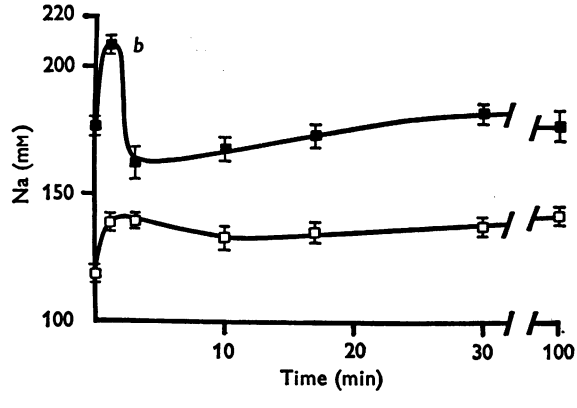

Fig. 4*b*. Na concentration in slices of renal outer (□) and inner (■) medulla incubated under anoxic conditions. The Na concentrations in the incubation media were 141 mM and 180 mM respectively. Values are mean ± 2 S.E. (n = 43 for initial concentrations, eight or nine after incubation).

remained constant from 10 to 30 min (outer medulla) and from 3 to 30 min (inner medulla) at mean values of approximately 112 and 148 mM respectively. By 100 min [Na] in these slices also approximated to medium [Na].

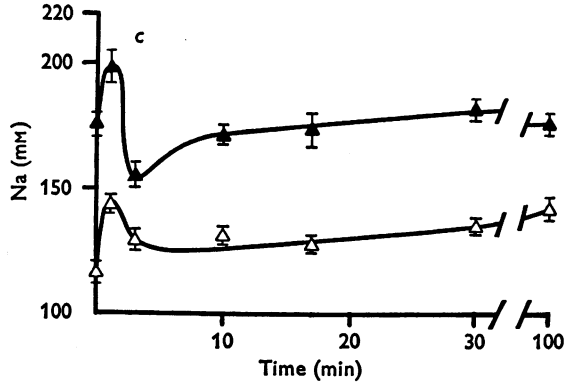

Fig. 4*c*. Na concentration in slices of renal outer (△) and inner (▲) medulla incubated at 0° C. The Na concentrations in the incubation media were 141 and 180 mM respectively. Values are mean ± 2 S.E. (n = 44 for initial concentrations, eight or nine after incubation).

Urea and osmolality

Urea, presumed to be freely permeant, equilibrated at media concentrations within 3 min in all slices, and thereafter did not fluctuate significantly. Urea concentrations were not estimated after 100 min incubations. The equilibration curves present no features of special interest and are omitted here. Osmolal equibration curves showed small but statistically insignificant fluctuations during the period 1–10 min, due presumably to the variations in slice [Na]. As shown in Table 1, slice osmolalities did not vary significantly from 10 to 30 min. Note that the mean value for osmolality over this period somewhat exceeds that found in fresh tissue, and that in the outer medulla this discrepancy is statistically significant. However, the formula 2([Na] + [K]) + [urea] although widely used for approximate estimates of osmolality, involves too many simplifications for relatively small quantitative discrepancies to be regarded as necessarily physiologically significant. Not only are minor ions (e.g. NH_4^+, Ca^{2+}) disregarded, but dissociation constants of unity, and the monovalency of anions, are assumed. Note also that whereas estimates of fresh tissue osmolality appear amply to vindicate the choice (based on preliminary analyses) of 740 m-osmole/kg H_2O for the inner medullary incubation medium, a mean osmotic imbalance of 36 m-osmole/kg H_2O was apparently imposed upon outer medullary slices (Table 1, columns *a* and *b*). But even assuming that such an imbalance actually existed – rather

than being merely a consequence of the approximation used to estimate tissue osmolality – it is clear from the succeeding paper (Law, 1975) that its magnitude would have been insufficient to cause appreciable osmotic change in cell volume.

TABLE 1. A comparison between the osmolalities (m-osmole/kg H_2O, mean ± S.E. (n)) in (a) the incubation media, (b) fresh medullary tissue, (c) slices after 10 min incubation, (d) slices after 30 min incubation. Column (e) indicates the mean of the 10 and 30 min values which were not significantly different under any conditions. Values in columns (b)–(e) were calculated using the formula 2 ([Na]+[K])+[urea] (Schmidt-Nielsen & O'Dell, 1961). In column (f) the significance between (b) and (e) is shown

	a	*b*	*c*	*d*	*e*	*f*
Outer medulla	Medium	Fresh tissue	10 min incubation	30 min incubation	Mean	P (b *vs.* e)
Control	565	533 ± 12 (37)	582 ± 17 (9)	600 ± 23 (9)	591 ± 13 (18)	< 0·005
Anoxic	565	525 ± 9 (37)	577 ± 16 (7)	564 ± 11 (9)	571 ± 8 (16)	< 0·005
Hypothermic	565	530 ± 10 (33)	588 ± 18 (9)	580 ± 16 (9)	584 ± 11 (18)	< 0·005
Mean	565	529 ± 6 (107)	—	—	583 ± 6 (52)	< 0·001
Inner medulla						
Control	740	735 ± 25 (39)	780 ± 27 (9)	782 ± 30 (9)	781 ± 19 (18)	n.s.
Anoxic	740	721 ± 18 (37)	779 ± 15 (9)	757 ± 12 (7)	768 ± 9 (16)	n.s.
Hypothermic	740	765 ± 20 (30)	760 ± 10 (9)	745 ± 10 (9)	753 ± 7 (18)	n.s.
Mean	740	739 ± 12 (106)	—	—	767 ± 7 (52)	0·2 > P > 0·1

DISCUSSION

The characteristics of renal outer and inner medullary slices have been examined during incubation in iso-osmolal media under control conditions (oxygenation at 37° C) and under conditions, anoxia and hypothermia, likely to modify the metabolic behaviour of the constituent cells. Apparent extracellular space (inulin space), and thus by extension cellular volume, slice weight changes (indicating swelling or shrinkage), and K retention have been regarded as the chief readily observable indices of cellular integrity.

In these respects the rapid weight loss which all slices underwent at the commencement of incubation was probably not functionally significant. It was not due to osmolal inbalance between slices and media (see Table 1). The likely source of this loss is cytoplasm from severed cells, and possibly also tubular and vascular contents (particularly in the outer medulla, where these would have been cut in the transverse plane). The thickness of the slices used (0·3–0·4 mm) corresponds to twelve to fifteen cells; cytoplasmic loss from damaged cells at the edges and faces of the slices could thus account for considerable weight loss. That control slices there-

after maintained weight constancy may be taken as an indication, though not as conclusive evidence, of continuing cellular integrity under these conditions. During anoxic incubation outer medullary slices gained weight to a significantly greater extent than inner slices. The relative participation of cells and extracellular space in these weight increases cannot be accurately evaluated since the co-identity of inulin space and extracellular space is uncertain under these conditions (see below). That outer medullary cells should appear more sensitive than inner ones to anoxia, and thus swell to a greater extent, would, however, be compatible with predominantly oxidative metabolism in this region (as in the cortex above it), in contrast to the anaerobic glycolysis generally regarded as the major energy source in the inner medulla (Lee & Peter, 1969; Abodeely & Lee, 1971). Conversely, hypothermia caused greater swelling in the inner than the outer medulla, and may thus have a more adverse effect on cell volume regulating mechanisms in this region.

A crucial point in the investigation of extracellular dimensions within a tissue concerns the extent to which the space occupied by the marker molecule (in this case inulin) corresponds to the true extracellular space under the prevalent experimental conditions, and also how far the latter may relate to the extracellular space *in vivo* or at zero time of incubation. As pointed out in the Introduction, inulin has been widely regarded as a reliable delineator of extracellular space; it possesses the required characteristics of any fluid compartment marker which have been reviewed in many past papers. In regard to its suitability or otherwise as an extracellular space marker for slices of renal medulla *in vitro* the following points are pertinent. Penetration of inulin into slices is relatively rapid within the first 10 min of incubation (as judged from the steepness of the space/time curve). This phase may be assumed to represent initial penetration of inulin into all, or most of, the available extracellular space. Thereafter there is a slow and gradual increase of inulin space up to 100 min of incubation. The contrast between initial rapid and slow subsequent phases is less apparent in hypothermically incubated slices (particularly outer medulla) than under control or anoxic conditions; nor does the inulin space in the former increase during the 30–100 min period (see Figs. 2*a* and 2*b*). But it is unlikely that these differences of time course reflect any physiologically significant differences in the fundamental processes governing the rate of increase of inulin space. The phase of slow penetration most probably represents either the gradual cellular penetration of inulin, or a genuine increase in the extracellular space relative to the total weight of slice. Should the former be the case, then the suitability of inulin as an extracellular space marker under these conditions is called in question; if the latter, then the inulin space could still afford useful

information regarding extracellular and cellular dimensions under specific conditions and after a given period of incubation. In neither case could an accurate estimate of zero time extracellular space be made. Recent findings of McIver & Macknight (1974) suggest intracellular penetration as the more likely cause in increasing inulin space. The present findings indirectly support this view. Thus if the extracellular space, as indicated by inulin space, was in fact increasing in size, then in those slices in which slow weight increases (10–100 min) were accompanied by a gradual increase in extracellular space as a proportion of total slice weight (viz. hypothermic and anoxic outer medulla, hypothermic inner medulla) it would be necessary to postulate that the rate of extracellular space swelling exceeded the rate of cellular swelling. A disproportionate swelling of extracellular space has been reported during *in vitro* incubation of skeletal muscle, but only at a tissue depth of twelve or more cells (Law, 1967), and it is improbable that this would have occurred in the very thin sections used in the present experiments. Furthermore, if the inulin space is assumed to correspond to the true extracellular space, then since the extracellular (incubation fluid) osmolality is known, and since the whole slice osmolality can be calculated approximately by the method already described (see Methods), a simple calculation (incorporating appropriate allowance for outer and inner medullary percentage dry weight) allows an approximate estimate of intracellular fluid osmolality to be made.

Values for tissue composition and inulin space after 30 min incubation have been employed in making this calculation: this time was chosen since by 30 min intra- and extracellular osmolalities should be equal, any pre-existing imbalance having been dissipated (this is amply confirmed in the succeeding paper, Law 1975). On this basis the mean osmolalities of intracellular fluid in outer and inner medulla under control conditions become 585 and 765 m-osmole/kg H_2O respectively. These values only slightly exceed those of the incubation media (565 and 740 m-osmole/kg H_2O respectively). In contrast, the values so obtained for anoxic slices are 637 and 875 m-osmole/kg H_2O and for hypothermic slices 653 and 809 m-osmole/kg H_2O. Even allowing for the simplifications and assumptions implicit in this method of calculating osmolality, it may be seen that the presumed co-identity of inulin space and extracellular space leads to considerably greater discrepancy between intra- and extracellular fluid osmolalities during anoxia and hypothermia than occurs under control conditions. From this it follows that, at least after 30 min incubation, the inulin space during anoxia and hypothermia provides an overestimate of extracellular space, probably due to cellular penetration, and thus a derived underestimate of intracellular volume. Conversely, inulin spaces of 24 and 42 μl./100 mg tissue for outer and inner slices respectively (see

Results) appear to provide a reasonably good estimate of extracellular spaces under control conditions from 10 to 30 min, throughout which time slices appeared to be in steady state, or near steady state, conditions as regards composition (water, electrolytes, urea and osmolality). A similar calculation using values after 100 min incubation suggests that after this time intracellular penetration occurred even in control slices (658 and 868 m-osmole/kg H_2O).

Although it is not possible from the available evidence to assert that 24 and 42 % (v/w) inulin spaces represent complete demarcation of the extracellular space of the outer and inner medulla after 30 min incubation, still less that at zero time or *in vivo*, these figures are at least in reasonably good agreement with those obtained by other workers. The outer medullary value approximates to that reported for the cortex (Rosenberg *et al.* 1962; Gans *et al.* 1966; Macknight, 1968; De Jairala *et al.* 1973), while that for the inner medulla corresponds to that found in the whole medulla by Gans *et al.* (1966) and the inner medulla by De Jairala *et al.* (1973). In view of the different techniques employed by various workers, as well as the possible influence of plane of section (De Jairala *et al.* 1973), minor quantitative discrepancies are not unreasonable.

Slice [K] values are consistent with differential effects of anoxia and hypothermia on the two medullary zones studied. The significant, rapid loss of K from all slices during the early stages of incubation can partially be accounted for by cytoplasmic loss from damaged cells and perhaps also tubular fluid loss. Its extent was such, however, as to suggest a considerable component of loss from intact cells. Thereafter, slices considered (on the grounds of weight increase) to be in a metabolically deleterious condition (anoxic outer and hypothermic inner medullary slices) maintained their [K] at levels significantly lower than those in control slices. However, since this lowering of concentration was accompanied by weight increase, and therefore by fluid uptake, the K loss in terms of actual content was less than slice [K] figures alone would indicate. Indeed, a comparison of Figs. 1*a* and 3*a* suggests that, at least during the period 10–30 min, the decrease in K content of anoxically incubated outer medullary slices may have been very small. The fall in slice [K] in hypothermic inner medulla, however, would appear to represent definite K loss; after 30 min incubation the concentration was approximately two thirds that in control slices (Fig. 3*b*), which cannot be completely accountable in terms of slice water uptake (the mean slice weight at this time being approximately 20 % higher than that of control slices; Fig. 1*b*). In those slices (hypothermic outer and anoxic inner medulla) believed to be in reasonable metabolic condition the values for [K] were very close to control values; since there were moderate weight increases in these slices it must be assumed that in

these slices K content increased, presumably through cellular K uptake from the incubation media.

The Na concentrations (based upon whole tissue analysis) which were chosen for the incubation media were probably less than those surrounding the cells *in vivo*, since not only does such analysis include relatively Na-poor cells, but also the medullary interstitium is a region into which NaCl is actively extruded by the cells in the ascending limb of the loop of Henle (efflux may be passive in the thin segment (Imai & Kokko, 1974)). This likely deficit in extracellular [Na] coupled with a somewhat higher urea concentration than was found in fresh tissue (see Methods) might variously have affected cell electrolyte exchanges via Na-K-activated ATPase. Gutman *et al.* (1973) report that in rat kidney Na-K-activated ATPase activity was increased in the medulla ($\simeq$ outer medulla in the present work) but decreased in the papilla ($\simeq$ inner medulla) as the ambient [Na] was raised between 100 and 300 mM. Urea (100–900 mM) had a similar effect. Thus in regard to the present experiments any activating or inhibiting effect of low [Na] should to some extent have been compensated by the opposing effects of elevated urea concentration. Alexander & Lee (1970) have observed a positive correlation between [Na] and Na-K-activated ATPase in the rabbit kidney, but since the effect was mimicked by equi-osmolar addition of sucrose or choline chloride the enzyme may be more dependent upon osmolal concentration than upon [Na] *per se*, in which respect the media used in the present experiments should have been satisfactory for normal enzyme activity.

The same general observations relating to slice electrolyte content and concentrations apply to Na as to K. The chief interest here, however, lies not in any indications of differential metabolic effects of hypothermia or anoxia but in explanation of the fluctuations in slice [Na]. The marked rise in [Na] at the start of incubation probably resulted from loss of relatively Na-free cytoplasm from damaged cells. If the true interstitial [Na] was higher than the medium [Na], as suggested above, then the phase during which slice [Na] fell should be explainable in terms of equilibration between these two regions. Following this, the slow rise in [Na] may represent cellular penetration, which must have occurred in all slices in view of the 100 min [Na] values. Clearly these three suggested phases would to some extent overlap, and no single one can be regarded as representing an isolated occurrence.

REFERENCES

ABODEELY, D. A. & LEE, J. B. (1971). Fuel of respiration of outer renal medulla. *Am. J. Physiol.* **220**, 1693–1700.

ALEXANDER, J. C. & LEE, J. B. (1970). Effect of osmolality on Na^+-K^+-ATPase in outer renal medulla. *Am. J. Physiol.* **219**, 1745–1753.

ATHERTON, J. C., HAI, M. A. & THOMAS, S. (1968). The time course of changes in renal tissue composition during water diuresis in the rat. *J. Physiol.* **197**, 429–443.

DE JAIRALA, S. W., VIEYRA, A. & MACLAUGHLIN, M. (1972). Influence of ethacrynic acid and ouabain on the oxygen consumption and potassium and sodium content of the kidney external medulla of the dog. *Biochim. biophys. Acta* **279**, 320–330.

DE JAIRALA, S. W., VIEYRA, A., GARCIA, A. P. & RASIA, M. L. (1973). Kinetics of inulin penetration in cortex and medullary kidney slices. *Can. J. Physiol. Pharmacol.* **51**, 511–515.

FOURMAN, J. & MOFFAT, D. B. (1971). *The Blood Vessels of the Kidney*, 1st edn., pp. 5–6. Oxford, Edinburgh: Blackwell Scientific Publications.

GANS, J. H., BAILIE, M. D. & BIGGS, D. L. (1966). *In vitro* metabolism of C^{14}-labelled amino acids by sheep kidney cortex and medulla. *Am. J. Physiol.* **211**, 249–254.

GUTMAN, Y., WALD, H. & CZACZKES, W. (1973). Urea and sodium: effect on microsomal ATPase in different parts of the kidney. *Pflügers Arch. ges. Physiol.* **345**, 81–92.

HOLMES, E. W. & DISCALA, V. A. (1971). Oxygen consumption, glycolysis, and sodium reabsorption in the hypothyroid rat kidney. *Am. J. Physiol.* **221**, 839–843.

IMAI, M. & KOKKO, J. P. (1974). Sodium chloride, urea and water transport in the thin ascending limb of Henle. *J. clin. Invest.* **53**, 393–402.

KEAN, E. L., ADAMS, P. H., WINTERS, R. W. & DAVIS, R. E. (1961). Energy metabolism of the renal medulla. *Biochim. biophys. Acta*, **54**, 474–478.

KREBS, H. (1950). Body size and tissue respiration. *Biochim. biophys. Acta* **4**, 249–269.

LAW, R. O. (1967). The distribution of [^{14}C]sucrose within the skeletal muscle of the rat *in vitro*. *J. Physiol.* **190**, 71–79.

LAW, R. O. (1973). The effects of 11-deoxycorticosterone and antidiuretic hormone (pitressin) on fluid exchanges and electrolyte excretion by normal and starved polyuric-polydipsic rabbits. *Pflügers Arch. ges. Physiol.* **345**, 249–263.

LAW, R. O. (1974). [^{14}C] carboxyl inulin distribution and solute exchanges in incubated slices of rat renal medulla. *J. Physiol.* **238**, 32–34*P*.

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

LAW, R. O. & PHELPS, C. F. (1966). The size of the sucrose, raffinose, and inulin spaces in the gastrocnemius muscle of the rat. *J. Physiol.* **186**, 547–557.

LEE, J. B. & PETER, H. M. (1969). Effect of oxygen tension on glucose metabolism in rabbit kidney cortex and medulla. *Am. J. Physiol.* **217**, 1464–1471.

LEE, J. B., VANCE, V. K. & CAHILL, G. F. (1962). Metabolism of C^{14}-labelled substrates by rabbit kidney cortex and medulla. *Am. J. Physiol.* **203**, 27–36.

LITTLE, J. R. (1964). Determination of water and electrolytes in tissue slices. *Analyt. Biochem.* **7**, 87–95.

LOWENSTEIN, L. M. & HAGOPIAN, L. (1969). Amino acid transport in stored renal medulla. *Transplantation* **8**, 558–565.

LOWENSTEIN, L. M., SMITH, I. & SEGAL, S. (1968). Amino acid transport in the rat renal papilla. *Biochim. biophys. Acta* **150**, 73–81.

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

Macknight, A. D. C. (1968). The extracellular space in rat renal cortical slices incubated at 0·5° and 25°. *Biochim. biophys. Acta* **163**, 85–92.

Mikulski, P., Angielski, S. & Rogulski, J. (1972). Metabolism of tricarboxylic acid cycle in rat kidney medulla *in vitro*. *Am. J. Physiol.* **223**, 485–491.

Paradise, R. P. & Morrow, R. J. (1972). Constancy of intracellar water as a function of dry weight. *Proc. Soc. exp. Biol. Med.* **141**, 836–838.

Randle, P. J. & Smith, G. H. (1958). Regulation of glucose uptake by muscle. 2. The effects of insulin, anaerobiosis and cell poisons on the penetration of isolated rat diaphragm by sugars. *Biochem. J.* **70**, 501–508.

Rosenberg, L. E., Downing, S. J. & Segal, S. (1962). Extracellular space estimation in rat kidney slices using C^{14} saccharides and phlorizin. *Am. J. Physiol.* **202**, 800–804.

Schmidt-Nielsen, B. & O'Dell, R. (1961). Structure and concentrating mechanism in the mammalian kidney. *Am. J. Physiol.* **200**, 1119–1124.