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OUABAIN AND REGULATION OF CELLULAR VOLUME IN SLICES OF MAMMALIAN RENAL CORTEX

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SUMMARY

1. The effect of ouabain on cellular volume recovery in rabbit, guinea-pig and rat renal cortical slices was studied. A concentration of ouabain that is maximally effective in inhibiting slice potassium accumulation was determined for each species. Slices from each species were either freshly prepared and then incubated, or leached and then incubated, or preincubated in oxygenated ordinary medium (equilibrated), leached and then reincubated in media with and without this concentration of ouabain. All incubations were at 25 °C.

2. Potassium loss produced by ouabain was greater in rabbit and guinea-pig slices than in rat slices.

3. With slices that were freshly prepared and then incubated, and with slices that were leached and then incubated, cellular volume recovery was inhibited by ouabain in rabbit and guinea-pig slices, but not in rat slices.

4. After equilibration, swelling during leaching was less, especially in rabbit and guinea-pig slices. However, on subsequent reincubation, significant differences in tissue water and cation contents that were consistent with inhibition of cellular volume recovery by ouabain, were seen in slices from these two species, but not in rat slices.

5. Slices from all three species, when incubated with concentrations of ouabain that were maximally effective in inhibiting potassium accumulation, appeared to approach a steady-state tissue potassium content that was greatest in rat slices and least in rabbit slices. Rat slices, previously depleted of potassium, reaccumulated potassium in the presence of 10 mM-ouabain to reach this steady-state potassium content.

6. Despite superficial appearance to the contrary (especially in the case of rat slices) these results are consistent with a major role for the conventional pump in controlling cortical cell volume. They do not provide evidence for the postulate that renal cortical cells possess a separate, ouabain-insensitive mechanism regulating cell volume.

INTRODUCTION

Evidence has been presented for an independent, ouabain-insensitive mechanism regulating cellular volume in mammalian renal cortex (Kleinzeller & Knotková, 1964*a*; Whittembury, 1968; Macknight, 1968*a*, *b*; Hughes & Macknight, 1976),

diaphragm (Kleinzeller & Knotková, 1964*b*), uterine smooth muscle (Rangachari, Daniel & Paton, 1973) and liver (Macknight, Pilgrim & Robinson, 1974). In contrast, ouabain-insensitive mechanisms were not important in the cellular volume recovery observed on incubation of freshly prepared rabbit renal cortical slices (Cooke, 1978*a*), a finding consistent with earlier observations (Whittam & Willis, 1963).

Different animal species differ markedly in their sensitivities to ouabain (Detweiler, 1967). As cellular volume recovery was inhibited by ouabain in freshly prepared rabbit renal cortical slices (Cooke, 1978*a*) but not inhibited in equilibrated, leached and reincubated rat renal cortical slices (Macknight, 1968*a*, *b*), species and procedural differences may mask the effect of ouabain on cellular volume regulation. Accordingly, this paper presents a comparison of the effect of ouabain on the rate of cellular volume recovery after metabolic inhibition in rabbit, guinea-pig and rat kidney slices. The results show that ouabain inhibits cellular volume recovery in slices from species in which the $(Na^+ + K^+)$-ATPase is more sensitive to ouabain but does not inhibit cellular volume recovery in rat slices. These results are interpreted as indicating that cellular volume is regulated by the conventional sodium pump, with the degree of inhibition by ouabain varying from species to species.

Preliminary accounts of part of this work have appeared elsewhere (Cooke, 1975, 1978*b*).

METHODS

Adult, male, New Zealand Black and White rats, New Zealand White rabbits, or guinea-pigs of an inbred strain maintained at the University of Otago, were killed by a blow on the back of the head. The kidneys were removed and placed in chilled ordinary medium until slices, approximately 0·2 mm thick, were prepared as described by Cohen (1945). Slices with any suspicion of medulla were rejected.

Ordinary medium (a modification of the medium of McIver & Raine, (1972) contained (mM) Na^+130 K^+5, Ca^{2+}2·5, Mg^{2+}1, Cl^-133, SO_4^{2-}1, phosphate 4 and glucose 20 at pH 7·2 with O_2 or N_2 as the gas phase. Previous experiments (Cooke, 1976) had revealed that the absence of CO_2 and bicarbonate from the incubation medium does not affect the rate of net sodium extrusion or potassium uptake in metabolizing rat renal cortical slices. Ouabain octahydrate (Sigma Chemical Co.) of appropriate mass was dissolved in media immediately before use. In potassium-free media, 5 mM-sodium chloride replaced 5 mM-potassium chloride, and in 40 mM-potassium medium, 40 mM-potassium chloride replaced 40 mM-sodium chloride. In these media, the other medium constituents were as described for ordinary medium. After cutting, slices were initially treated in one of three ways. In some experiments, they were distributed among 30 ml. aliquots of air-equilibrated medium at room temperature, where they remained until all slices were cut, and for 5 min thereafter, with occasional gentle shaking to free loose matter from the slices (Robinson, 1949). After a few slices from each medium had been taken for analysis (*freshly prepared* slices), the remaining slices were transferred to similar, but oxygenated, media at 25 °C and incubated for varying periods of time. Though the ionic composition of freshly prepared slices is not steady state with respect to time, the changes in ionic composition that occur during preparation of slices have slowed sufficiently for freshly prepared slices to provide a reproducible pool of swollen, sodium-rich and potassium-depleted slices (Cooke, 1978*a*) without the need for prolonged incubation under adverse conditions. In other experiments, slices were *equilibrated*, i.e. incubated in oxygenated ordinary medium at 25 °C without inhibitors for 15–40 min (Macknight, 1968*a*), and then distributed among different media for further incubation or leaching. Leaching was carried out at 0–2 °C in nitrogenated medium. Finally, in some experiments slices were leached without prior equilibration.

Slices required for analysis were removed from the medium, blotted and transferred to tared borosilicate glass tubes which were reweighed and then oven dried. After at least 2 hr at 105–110 °C,

tubes were removed from the oven, stoppered, and left to equilibrate with the room temperature and humidity. To compensate for the effects of temperature and humidity on the weight of the glass tubes, one in five tubes was a blank that was carried right through the analysis routine. Tissue water content was determined by loss of weight after drying (Little, 1964). Ions were extracted from the tissue in 0·1 M nitric acid for at least 2 hr (Little, 1964). The sodium and potassium concentrations in the acid extract were measured with an EEL flame photometer.

Results are presented as contents in terms of tissue dry weight rather than as concentrations in the tissue or cellular water. Values presented in graphs and tables are means ±S.E. of mean. Standard errors of means are not illustrated in graphs where twice the S.E. of mean is less than or equal to the vertical length of the graphical symbol. The significance of differences between means was assessed with Student's *t* test.

RESULTS

Effect of ouabain on freshly prepared rat and guinea-pig slices

In a preceding study (Cooke, 1978*a*), 1 mM-ouabain markedly inhibited cellular volume recovery in freshly prepared rabbit renal cortical slices. As considerable evidence in favour of a ouabain-insensitive mechanism regulating cellular volume has come from experiments with rat kidney slices (Macknight, 1968*a*, *b*), it was important to determine if ouabain could inhibit cellular volume recovery in freshly prepared rat slices.

The conventional ouabain-sensitive sodium pump not only extrudes sodium but is also believed to be responsible for the cellular accumulation of potassium. Therefore, one index of the degree of inhibition of this pump is provided by the extent of loss of cellular potassium. The concentration of ouabain producing maximum net potassium loss from rat renal cortical slices was assessed by incubating previously equilibrated slices at 25 °C in oxygenated ordinary medium containing 0 to 10 mM-ouabain, for 1 hr. The results in Fig. 1*A* show that 5, 7·5 and 10 mM-ouabain produced similar net potassium losses and sodium uptakes within the limits of experimental error. These values are in agreement with those reported for rat kidney slices at 38 °C by Allison (1975) who also found that 15 mM-ouabain produced similar potassium loss and depression of oxygen consumption to 10 mM-ouabain. In subsequent experiments with rat slices, 10 mM-ouabain was assumed to produce maximum or near maximum inhibition of the conventional sodium pump.

Freshly prepared rat renal cortical slices were swollen (Fig. 2*A*). On incubation in oxygenated media at 25 °C they recovered to a normal water content within 4 min (Fig. 2*A*). Though potassium accumulation was inhibited by 10 mM-ouabain, there was no detectable reduction in the rate of recovery of cellular volume in the presence of 10 mM-ouabain. The rates at which changes in tissue water and ions occurred and the lack of an effect of ouabain on cellular volume recovery are in agreement with the data of Macknight (1968*a*, *b*) even though he used a different incubation procedure. After 60 min of incubation, the tissue potassium content of slices was 270±2 (8) m-mole/kg dry wt. in ordinary medium and 152±4 (8) m-mole/kg dry wt. in 10 mM-ouabain medium. The difference in these values is considerably less than the corresponding difference in tissue potassium content observed during incubation of freshly prepared rabbit slices with 1 mM-ouabain (229±7 m-mole/kg dry wt., 18 degrees of freedom) where 1 mM-ouabain produced a 30-fold slowing of the rate of cellular volume recovery (Cooke, 1978*a*). As there seemed to be an inter-species

difference in the effect of ouabain on cellular volume recovery in freshly prepared slices, similar experiments were carried out with slices of guinea-pig renal cortex as these have been used extensively to investigate cellular volume regulation (Whittembury, 1968; Whittembury & Proverbio, 1970; Proverbio & Whittembury, 1975).

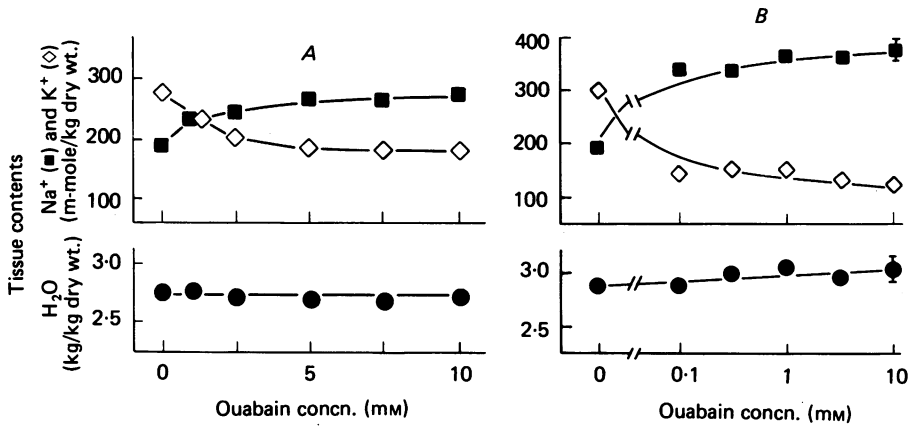

Fig. 1. The tissue water, sodium, and potassium contents of rat (*A*) and guinea-pig (*B*) renal cortical slices that were equilibrated and then incubated for 60 min in ordinary medium with different concentrations of ouabain. Values are means ±S.E. mean of eight observations.

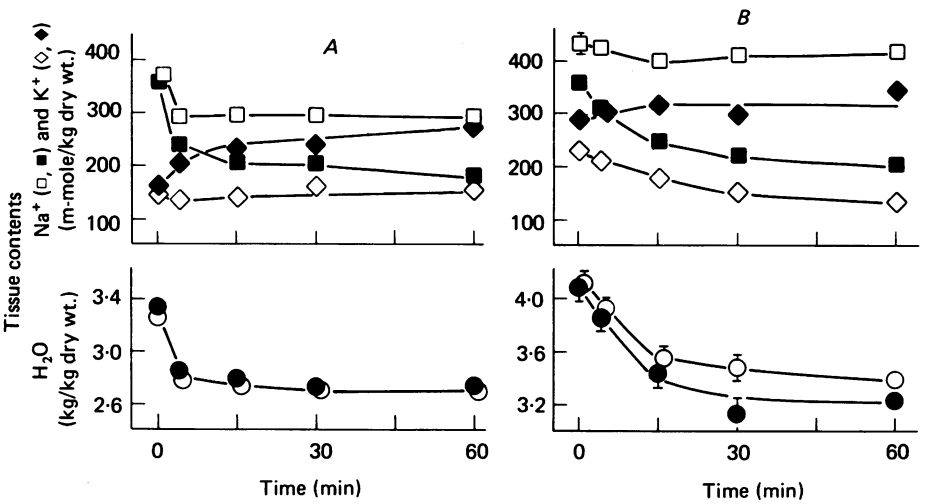

Fig. 2. The tissue water, sodium, and potassium contents of freshly prepared rat (*A*) and guinea-pig (*B*) renal cortical slices incubated in ordinary medium (filled symbols) or in ordinary medium with 10 mM-ouabain (open symbols) for up to 60 min. Values are means ±S.E. mean of eight observations.

The amount of potassium lost from guinea-pig slices incubated in medium with 3 mM-ouabain, was similar to that lost by slices in 10 mM-ouabain medium (Fig. 1*B*). In subsequent experiments with guinea-pig slices, 10 mM-ouabain was assumed to produce maximum or near maximum inhibition of the conventional sodium pump in this species. Freshly prepared guinea-pig slices appeared to have a slower rate of cellular volume recovery than did rat or rabbit slices (Fig. 2*B*). There were significant

differences in the tissue water contents of freshly prepared slices incubated with and without 10 mM-ouabain after 30 ($P < 0.05$) and 60 ($P < 0.05$) min of incubation. These differences in tissue water content are small but do suggest that ouabain was inhibiting cellular volume restoration in these freshly prepared guinea-pig slices. The difference between the tissue potassium contents of slices incubated in ordinary medium was, at 60 min, 208 ± 9 m-mole/kg dry wt. (14 degrees of freedom).

Comparison of these results with those reported previously for rabbit slices (Cooke, 1978*a*) shows that, as judged by the concentration of ouabain required to produce maximum tissue potassium loss, rabbit slices are more sensitive to ouabain than are guinea-pig slices which in turn are more sensitive than rat slices. Also, at these high concentrations of ouabain there was greater potassium loss in rabbit and guinea-pig slices than in rat slices. With regard to inhibition of cellular volume recovery by ouabain in freshly prepared slices, a similar trend was evident, with marked inhibition in rabbit slices, slight inhibition in guinea-pig slices and no inhibition in rat slices. This correlation between the extent of potassium loss and the inhibition of volume recovery suggests that cellular volume recovery is mediated by the conventional sodium pump.

Effect of ouabain on leached and reincubated slices

Much of the original evidence favouring the existence of a separate ouabain-insensitive mechanism regulating cellular volume was obtained from slices that were leached, and then incubated in warm oxygenated media (Kleinzeller & Knotková, 1964*a*; Whittembury, 1968).

When rabbit renal cortical slices were treated in this way, they were swollen and sodium-rich at the end of leaching (Fig. 3*A*). During reincubation in oxygenated ordinary medium at 25 °C, a relatively normal water content was obtained within 4 min (Fig. 3*A*). In the presence of 1 mM-ouabain cellular volume recovery was severely inhibited and was not complete at the end of 60 min of incubation. The difference between the tissue potassium contents of slices reincubated in 1 mM-ouabain medium and those in ordinary medium was, after 60 min, 199 ± 5 m-mole/kg dry wt. (12 degrees of freedom).

Leached and reincubated guinea-pig renal cortical slices showed a similar, though possibly smaller, effect of ouabain on cellular volume recovery (Fig. 3*B*). The tissue water contents of slices incubated in medium with 10 mM-ouabain were significantly different from slices in ordinary medium after 15 ($P < 0.001$), 30 ($P < 0.02$) and 60 min ($P < 0.005$). The difference in tissue potassium content after reincubation for 60 min was 191 ± 7 m-mole/kg dry wt. (14 degrees of freedom).

Rat renal cortical slices leached and reincubated in ordinary medium or in 10 mM-ouabain medium both showed the same rate of cellular volume recovery and maintained similar final tissue water contents (Fig. 3*C*). Though 10 mM-ouabain did not inhibit cell volume recovery, potassium accumulation was inhibited, with the difference in tissue potassium content at the end of incubation being 112 ± 8 m-mole/kg dry wt. (10 degrees of freedom).

Thus, similar species differences were observed in both freshly prepared slices and in leached and incubated slices. With this latter procedure cell volume recovery was grossly inhibited in rabbit slices but there was still some reduction in tissue water

content after 60 min incubation as was noted by Kleinzeller & Knotková (1964*a*). Without the intervening points, the dramatic difference between the rates of volume recovery with and without ouabain could easily be overlooked. Ouabain inhibited cell volume recovery in guinea-pig slices but not in rat slices. The difference in tissue potassium content of slices incubated with and without ouabain was again greater in rabbit and guinea-pig slices than in rat slices.

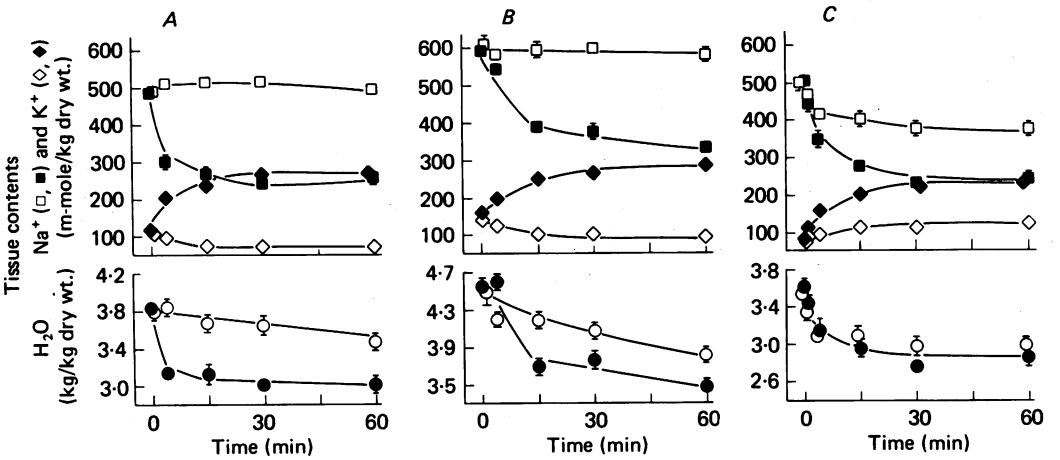

Fig. 3. The tissue water, sodium, and potassium contents of rabbit (*A*), guinea-pig (*B*) and rat (*C*) renal cortical slices leached in ordinary medium (filled symbols) or in ordinary medium with 1, 10 or 10 mM-ouabain respectively (open symbols) for 2·5 hr and then reincubated in similar, but oxygenated media, at 25 °C for up to 60 min. Values are means ± S.E. mean of six to eight observations.

Effect of ouabain on equilibrated, leached and reincubated slices

Macknight & Leaf (1977) suggested that results obtained by incubation procedures that lack an initial incubation in oxygenated medium without inhibitors, may not reflect a specific effect of ouabain on a volume regulating mechanism, but may be secondary to the incubation procedure. Unfortunately, following a preliminary incubation in oxygenated medium, leaching produces less swelling (cf. Figs. 3 and 4), making investigation of the effects of ouabain on cellular volume using this protocol more difficult.

In equilibrated, leached and reincubated rabbit renal cortical slices there was slight swelling during leaching followed by a return to the usual water content. As differences are slight it is important to realize that the weighings were carried out in four batches (0 and 4, 15, 30, 60 min samples) which were internally balanced and included a number of blanks. The average of the changes in the weights of the blank tubes provided a correction factor for the small changes in the weight of the glass tubes that occur with changes of relative humidity and temperature. However, comparisons within each batch are more reliable than comparisons between batches when small changes are being assessed, because the tubes are ordered within each batch so that time-dependent effects should cancel out. Thus the variation from 4 to 15 min of incubation is probably of less significance than the small but statistically significant ($P < 0{\cdot}02$) difference in tissue water content between slices in ordinary

medium and those in 1 mM-ouabain medium after 4 min of incubation (Fig. 4*A*). Guinea-pig slices leached in ouabain media after preliminary equilibration had significantly lower tissue water contents ($P < 0.05$) than slices leached in ordinary medium (Fig. 4*B*). A similar reduction in swelling with ouabain was noted when guinea-pig slices were incubated anaerobically at 25 °C after preliminary equilibration

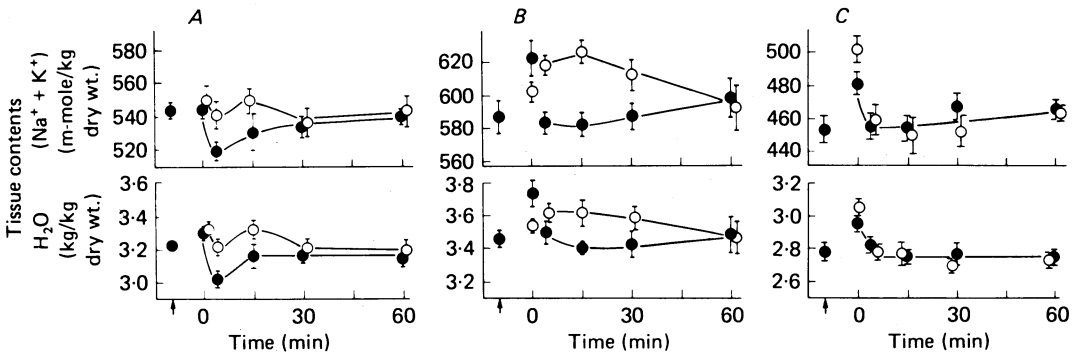

Fig. 4. The tissue water and sodium plus potassium contents of rabbit (*A*), guinea-pig (*B*) and rat (*C*) renal cortical slices that were equilibrated (arrowed), leached for 2·5 hr either in ordinary medium (filled symbols) or in ordinary medium with 1, 10 or 10 mM-ouabain respectively (open symbols), and then reincubated in similar, but oxygenated, media at 25 °C for up to 60 min. Values are means ± S.E. mean of six to eight observations.

(K. R. Cooke, unpublished observations). Despite this effect, after 15 min of reincubation in 10 mM-ouabain medium, guinea-pig slices had significantly greater tissue water contents ($P < 0.05$) than slices in ordinary medium (Fig. 4*C*). In these experiments with rabbit and guinea-pig slices the sum of tissue sodium and potassium contents changed in parallel with the tissue water content showing an inhibitory effect of ouabain on the recovery of tissue combined sodium and potassium ions content (Figs. 3*A*, *B*). Volume recovery in equilibrated, leached and reincubated rat renal cortical slices was not inhibited by 10 mM-ouabain (Fig. 4*C* and Macknight, 1968*a*). Such information as can be obtained from these small differences is in agreement with the data presented in Figs. 2, 3 showing that ouabain inhibits cell volume recovery in kidney slices from some species (rabbit, guinea-pig) but not in those from the rat which is known from studies of the $(Na^+ + K^+)$-ATPase to have a sodium pump that is more resistant to the effect of cardiac glycosides.

Effect of ouabain on inhibition of potassium accumulation

Rabbit slices are more sensitive to ouabain than guinea-pig slices, which in turn are more sensitive than rat slices, as judged from the concentration of ouabain required to produce maximum potassium loss. In addition, rabbit slices lost more potassium on incubation with ouabain than did rat slices and during the incubation of freshly prepared slices appeared to reach a steady-state tissue potassium content (Cooke, 1978*a*). As shown in Table 1, the pooled values for the tissue potassium contents of rabbit, rat and guinea pig slices incubated with ouabain in the experiments shown in Figs. 2–4 and in a preceding paper (Cooke, 1978*a*) suggest that slices tend towards a steady-state tissue potassium content which is least in rabbit slices and

greatest in rat slices. Assuming that there is in the different species a similar balance between potassium permeability and potassium movement associated with the sodium pump, then these differences in steady-state tissue potassium content are consistent with less complete inhibition of the sodium pump by ouabain in rat slices.

TABLE 1. Potassium loss in ouabain medium

	Tissue potassium contents (m-mole/kg dry wt).				
Incubation time...	15 min		30 min		60 min
Rabbit	122±11 (24)		107±10 (23)		100±9 (23)
		$P > 0.10$		$P > 0.60$	
Guinea-pig	151±9 (23)		139±8 (23)		126±6 (23)
		$P > 0.05$		$P > 0.20$	
Rat	139±6 (19)		148±6 (20)		152±6 (20)
		$P > 0.10$		$P > 0.60$	

The data were collected from the experiments described in Figs. 1–4, and from an experiment with freshly prepared rabbit slices that was similar to the experiments in Fig. 2 (Cooke, 1978*a*). Ouabain was present at a concentration of 1 mM with rabbit slices and 10 mM for rat and guinea-pig slices. *P* values are for comparisons with the 60 min value for slices from the same species.

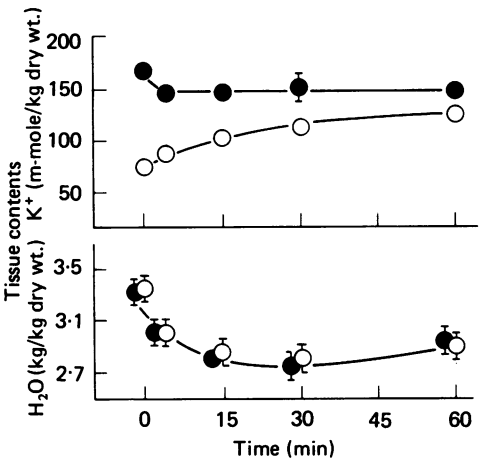

Fig. 5. The tissue water and potassium contents of rat renal cortical slices that were equilibrated, then leached, either for 5 hr in potassium-free medium plus 10 mM-ouabain (open circles) or for 2 hr in 40 mM-potassium medium and for 3 hr in ordinary medium with 10 mM-ouabain (filled circles), and then reincubated in oxygenated ordinary medium with 10 mM-ouabain. Values are means±S.E. mean of six to eight observations.

The existence of a steady-state tissue potassium content with a high concentration of ouabain might imply residual potassium accumulation. From the differences in the apparent steady-state levels in Table 1, it would be expected that more of the potassium uptake mechanism would be free of inhibition in rat slices. Thus, if the potassium content of rat slices were depressed below this steady-state value, then, on subsequent incubation in the presence of 10 mM-ouabain, there should still be potassium accumulation to the steady state value. Results from such an experiment as shown in Fig. 5. In this experiment rat renal cortical slices accumulated 44±12 m-mole potassium/kg dry wt. ($P < 0.001$) over 60 min incubation in the presence of

10 mM-ouabain. This uptake was calculated assuming an extracellular space of 26% of wet weight (McIver & Macknight, 1974). Potassium accumulation in rat slices in the presence of a high concentration of ouabain is also evident in Fig. 3*C* and in the data of Macknight (1969).

DISCUSSION

It is generally accepted that different animal species vary in their sensitivity to cardiac glycosides (Detweiler, 1967). The concentration of ouabain required for 50% inhibition of the $(Na^+ + K^+)$-ATPase isolated from cells of leaky epithelia shows a consistent trend with rat least sensitive, rabbit most sensitive and guinea-pig intermediate (Table 2). Thus the conventional sodium pump of rat epithelia is much more resistant to inhibition by ouabain than is that of guinea-pig or rabbit epithelia.

TABLE 2. Inter-species variation in the effect of ouabain on $(Na^+ + K^+)$-ATPase

	pI_{50}		
	Rabbit	Guinea-pig	Rat
Small intestine	6[a]	5·7[a]	4·0[a]
Gall-bladder	5·6[b]	—	—
Kidney	6[c]	5·4[c]	3·7[d]

Data are from Robinson, (1970); van Os & Slegers, (1970); Tobin & Brody, (1972); Györy, Brendel & Kinne, (1972). pI_{50} is the negative logarithm to the base 10 of the concentration of ouabain, in mole/l., that causes half-maximal inhibition of $(Na^+ + K^+)$-ATPase activity.

Differences in the sensitivity of the $(Na^+ + K^+)$-ATPase to ouabain have been explained by differences in the stability of the ouabain-enzyme complex (Allen & Schwartz, 1969; Akera, Brody, So, Tobin & Baskin, 1974). The ouabain-enzyme interaction can be described by a single reversible equilibrium; $E + Ou = E - Ou$, with a second order association rate and first-order dissociation rate (Glynn & Karlish, 1975). The dissociation constant calculated from the quotient of the rate constants for association and dissociation is $1{\cdot}5 \times 10^{-5}$ M for guinea-pig kidney cortex (Erdmann & Schoner, 1973*a*). Assuming similar rates of association for the different species (Tobin, Henderson & Sen, 1972), and half-times for dissociation of 10 min for rabbit kidney, 3 min for guinea-pig kidney and 0·05 min for rat kidney (Allen & Schwartz, 1969; Tobin & Brody, 1972), dissociation constants for rabbit and rat kidney can be calculated. With an ouabain concentration of 10 mM the proportion of free enzyme to inhibited enzyme can then be calculated to be of the order of 0·1% in rat kidney, and 0·001% in rabbit and guinea-pig kidney preparations of $(Na^+ + K^+)$-ATPase incubated to steady-state inhibition.

However, Proverbio, Robinson & Whittembury (1970) showed that the concentration of ouabain required for 50% inhibition of the changes in tissue ion contents that occurred when leached guinea-pig renal cortical slices were rewarmed was 2–10 times greater than that required for 50% inhibition of the isolated $(Na^+ + K^+)$-ATPase. Whittam & Willis (1963) reported that 50% of the inhibition of cation exchange and oxygen consumption that could be produced by ouabain in rabbit renal cortical slices was obtained with 15 μM-ouabain, a concentration that is an order of magnitude greater than that required for 50% inhibition of rabbit kidney $(Na^+ + K^+)$-

ATPase activity (Tobin & Brody, 1972). In the present series of experiments, the order of the pI_{50} for ouabain can be crudely estimated as ~ 3 for rat slices (Fig. 1*A*, > 4 for guinea-pig (Fig. 1*B*) and ~ 5 (Cooke, 1978*a*) for rabbit slices. These values show the same inter-species trend that is evident in Table 2, and are in agreement with work cited above suggesting that the pI_{50} determined from electrolyte movements in slices is an order of magnitude greater than that obtained from studies of the isolated $(Na^+ + K^+)$-ATPase. A similar difference may be evident in the effect of ouabain on proximal tubular reabsorption in the rat (György, Brendel & Kinne, 1972).

Jorgensen (1977) suggested that ouabain bound slowly in intact tissue, and that the rate of binding was dependent on ion concentrations and the turnover rate of the pump sites. In the present series of experiments, ion concentrations in the medium were the same in all experiments. Low temperatures during leaching would lower the rate of binding, but, as the dissociation constant for the ouabain-enzyme complex is only slightly temperature-sensitive (Erdmann & Schoner, 1973*a*), if leaching is sufficiently prolonged, steady-state binding and inhibition by ouabain might still be achieved, provided there was a sufficient supply of ATP. In this regard, it is worth noting that the high affinity of the $(Na^+ + K^+)$-ATPase for ATP would allow even undetectable levels of ATP, 10^{-5} M, to drive the sodium pump at substantial rates (Erdmann & Schoner, 1973*b*; Jorgensen, 1977). Furthermore, the energy required for sodium extrusion in the kidney slice preparation, where transtubular transport seems to be much reduced (Whittembury & Grantham, 1976) is almost certainly a very small fraction of that which the cell is capable of producing during transtubular transport. So that, though markedly inhibited by chilling and/or nitrogen, the cell might still be able to produce sufficient ATP to maintain a reduced rate of pump turnover. Indeed, the relative absence of swelling and potassium loss when rabbit and guinea-pig slices were equilibrated and then either leached for 2·5 h, or incubated in nitrogen bubbled media at 25 °C (Cooke, 1977), strongly suggests some residual metabolism. Thus it is possible that a sufficient number of pump sites might have been active in the presence of high concentrations of ouabain, so as to produce substantial, if not maximum, binding of ouabain to the $(Na^+ + K^+)$-ATPase during leaching and prior to incubation. For example, following prolonged leaching of rabbit slices in ouabain media, cellular volume recovery was inhibited by ouabain from the onset of incubation (Fig. 3*A*). However, in guinea-pig slices, the initial loss of tissue water seen in the first 15 min of incubation of freshly prepared slices and in the first 4 min of incubation of leached slices may reflect a lag period until sufficient ouabain is bound to inhibit cellular volume recovery. Whether the lack of inhibition of cellular volume recovery in rat slices is partly due to binding delays is uncertain but it should be noted that in experiments with rat $(Na^+ + K^+)$-ATPase, ouabain-binding reached a steady-state much more rapidly than in more ouabain-sensitive preparations of $(Na^+ + K^+)$-ATPase (Akera & Brody, 1978).

When doubts over the extent of binding during reduced pump turnover, and the apparently greater pI_{50} in intact tissue are taken into consideration, the estimated proportion of pump sites free of ouabain that can be derived from $(Na^+ + K^+)$-ATPase studies seems likely to be a gross underestimate of the proportion of continued pump activity during the initial stages of reincubation. Any free pump sites would presumably turn over more rapidly than under control conditions as a result of the

increased intracellular sodium concentration (Glynn & Karlish, 1975; Spring & Giebisch, 1977). This possible residual sodium extrusion by the classical sodium pump must be balanced against the probable rate at which sodium enters the renal cortical cell.

In mammalian small intestine, Necturus proximal tubule and gall-bladder, the unidirectional flux of sodium from the interestitium to the cell across the basolateral membrane is insignificant when compared to influx across the luminal membrane (Schultz, Frizzell & Nellans, 1974; Kimura & Spring, 1979; Graf & Giebisch, 1979). Less information is available for mammalian proximal tubule but in agreement with observations in these other leaky epithelia there seems to be neutral saline co-transport and co-transport of sodium and organic solutes across the luminal membrane (Rector, Berry & Yee, 1977; Frömter, 1979) and a low conductance to sodium ions across the peritubular membrane (Boulpaep, 1976). Thus, *in vivo*, the proximal tubular cells that comprise the bulk of renal cortical slices (Linshaw & Stapleton, 1978), probably receive almost all incoming sodium across the luminal membrane, with the passive peritubular influx being very small in comparison. The lumina of tubules in kidney slices are collapsed (Bojesen & Leyssac, 1965; Maude, 1968), and exchanges of water and ions are believed to occur predominantly across the peritubular membranes (Whittembury & Grantham, 1976). Thus, in kidney slices bathed in ordinary medium, with a high peritubular membrane potential (Whittembury, 1965), the rate of sodium entry into the cell may be only a small fraction of that which would occur if unrestricted transtubular transport was proceeding.

For a ouabain-insensitive reduction in tissue water content on reincubation to be considered as evidence in favour of a separate ouabain-insensitive mechanism, it is essential that the classical sodium pump be completely inhibited. As argued in this Discussion it is possible that sufficient numbers of pump sites may still be available, at least in rat slices, to extrude sodium as fast as it enters the cells despite the presence of high concentrations of ouabain. Thus ouabain-insensitive cell volume recovery in rat slices (Figs. 1*A*, 3*C*, 4*C*, and Macknight, 1968) cannot be considered as evidence in favour of a separate ouabain-insensitive cell volume regulating mechanism without direct and overwhelming proof that ouabain is *completely* inhibiting all classical pump sites. This is particularly important in preparations, such as the kidney slice or non-perfused tubule (Linshaw & Stapleton, 1978), where the rate of sodium entry may be only a small fraction of the rate *in vivo*.

The simplest interpretation of the present results is that ouabain inhibited the classical sodium-potassium pump in rabbit slices sufficiently to reduce the rate at which sodium could be extruded from cells below that at which sodium could enter the cells. Eventually, a steady-state ionic composition was achieved with the rate of sodium entry reduced, through a fall in membrane potential and an increase in the intracellular sodium concentration, to that which could be extruded through the inhibited sodium pump whose turnover had increased with the increased intracellular sodium concentration. The more effective the inhibition by ouabain, the lower the steady-state intracellular potassium concentration would be – in keeping with the species differences shown in Table 1. However, in rat slices, due to the inherent insensitivity of the $(Na^+ + K^+)$-ATPase and possibly due to reduced binding during chilling and anoxia, the rate at which sodium could still be extruded was so much

greater than its rate of entry that cell volume recovery was not inhibited. The steady-state intracellular potassium concentration with high concentrations of ouabain was high enough to allow a demonstration of potassium accumulation and hence presumably of sodium extrusion despite the presence of ouabain.

Throughout this Discussion, the observed differences in tissue water and ion content changes have been assumed to reflect changes in intracellular composition. There is, *a prior*, no reason to expect that incubation with a high concentration of ouabain will produce changes in extracellular space, and ouabain at 10^{-2} M was without effect on the extracellular space in rat renal cortical slices (Macknight, 1968*b*). Unfortunately, inulin equilibrates slowly with the extracellular space (McIver & Macknight, 1974) so that inulin spaces are of no value where changes in water content occur over a few minutes as in most of these experiments. Thus, though the possibility that changes in extracellular space may contribute to the Results in this paper cannot be excluded, there is no evidence to support this view.

As a coherent explanation can be advanced to explain these results in terms of an incompletely inhibited sodium pump, and as ouabain has not been shown by any direct method to be completely inhibiting the classical sodium pump in this preparation, the data presented in this paper should not be interpreted as evidence for a ouabain-insensitive cellular volume regulating mechanism that is independent of the $(Na^+ + K^+)$-ATPase. Rather, the dramatic effect of ouabain on cellular volume recovery in rabbit slices, together with species differences in the effects of ouabain on cellular volume recovery and ionic composition that correlate with effects on the $(Na^+ + K^+)$-ATPase, indicate that the main, active mechanism, mediating cellular volume recovery in renal cortical slices is the classical sodium pump.

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