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THE EFFECT OF OUABAIN ON THE ELECTROLYTE AND WATER TRANSPORT IN KIDNEY CORTEX AND LIVER SLICES

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It has been firmly established by a number of investigators (e.g. Krebs, Eggleston & Terner, 1951; Mudge, 1951*a*; Aebi, 1953; Whittam & Davies, 1953; Leaf, 1956; Cort & Kleinzeller, 1956; van Rossum, 1963) that on immersion of fresh animal tissues in an ice-cold isotonic solution of sodium chloride or a balanced physiological saline (leaching procedure), the cells swell, taking up water, Na^+ and Cl^- and losing K^+. On subsequent aerobic incubation of the tissues thus loaded with H_2O, Na^+ and Cl^-, many cells (e.g. kidney-cortex, liver, brain-cortex, etc.) are capable of extruding H_2O, Na^+ and Cl^-, and re-accumulating K^+ against the respective osmotic and electrochemical gradients. This latter movement of electrolytes (and water) is taken to be due to an active extrusion of Na^+ (or, according to some views, to the function of a sodium–potassium exchange 'pump'; for review see, for example, Ussing, 1960) and is inhibited by a number of metabolic inhibitors. In kidney cortex and liver slices, where these phenomena have been studied in some detail, the loss of cell water from slices previously leached at 0° C has hitherto always been found to be associated with a simultaneous extrusion of cell Na^+ and Cl^-, and re-accumulation of K^+, the only exception being an extrusion of a NaCl solution from kidney-cortex slices in a K^+-free saline (Kleinzeller, 1961), where the loss of cell water and Na was not associated with a re-accumulation of K^+. The *in vitro* extrusion of electrolytes by renal cells occurs as a slightly hypotonic solution of approximately 125 mM (Kleinzeller, 1961), an increase of the bulk cellular cations ($Na^+ + K^+$) occurring at the same time (Whittam & Davies, 1953); the ratio of extruded Na^+ to accumulated K^+ averages in this tissue about 2.

Since the cardiac glycoside ouabain, and its aglycone, strophanthidin, have been demonstrated to be fairly selective inhibitors of the active cation transport process (Schatzmann, 1953; see also Weatherall, 1962)

it would be reasonable to expect that these substances should block the aerobic extrusion from the leached cells of H_2O, Na^+ and Cl^-, and also prevent the re-accumulation of K^+. In fact, it has been observed by Burg & Orloff (1962) that, as compared with controls, concentrations as low as 0·03 mM strophanthidin depressed the tissue content of K^+ and increased that of Na^+ in rabbit-kidney cortex slices; unfortunately, these data were expressed per wet weight of the tissue, thus preventing an assessment of the net cation and water transport. Whittam & Willis (1963) also found that the addition of ouabain to aerobically incubated kidney-cortex slices produced a decrease of both oxygen uptake and tissue potassium.

However, when investigating the effect of ouabain on the steady-state efflux of ^{24}Na from kidney cortex slices (see Results) it was systematically observed that, as compared with controls, 0·3 mM ouabain hardly affected the steady-state level of tissue water, while producing a considerable increase of tissue Na and a decrease of tissue K^+. Thus, in this case high levels of tissue Na and a low content of K^+ were not associated with a swelling of the tissue. On the other hand, an ouabain-insensitive extrusion of Li^+ and water from Li-loaded kidney-cortex slices was found, although previously no transport of Li^+ against its concentration gradient could be demonstrated (Cort & Kleinzeller, 1957). A closer analysis of the effect of ouabain on the water and electrolyte transport appeared therefore to be of interest.

In this communication results of experiments are presented concerning the relation between water and electrolyte transport from lithium- and sodium-loaded cells, and the effect of ouabain on it, in rabbit-kidney cortex and liver slices. The possible mechanism of the ouabain-insensitive extrusion of electrolytes and water will then be considered.

A preliminary report on some of the findings has been presented (Kleinzeller & Knotková, 1963*a*).

METHODS

The experiments described here were carried out with slices of kidney cortex and liver of adult rabbits, and isolated kidney-cortex cells.

Preparation of tissues and cells. Slices of rabbit-kidney cortex and liver were prepared by the method of Deutsch (1936). Isolated rabbit-kidney cortex cells were obtained by the procedure described by Bosáčková (1962).

Experimental procedures. The net transport of electrolytes and water by the kidney and liver slices was studied with the technique of Mudge (1951*a*): The tissue preparations were first leached for 2·5 hr in ice-cold isotonic saline, exchanging the saline repeatedly in order to assure a balanced state of tissue components. Subsequently, groups of slices (approx. 150 mg wet weight) were incubated at 25 or 37° C aerobically (gas phase: O_2, or 5% CO_2 + 95% O_2, where bicarbonate salines were employed) for 60 min in 3 ml. of a balanced physiological saline of the Krebs–Ringer type, containing 4 mM-α-oxoglutarate as sub-

strate, without (control) or with 0·3–1 mM-ouabain. The incubation time was usually sufficient to bring about a steady-state level of tissue solutes. The incubation of the kidney cortex slices was mostly carried out at 25°; the observation of Mudge (1951*a*) was confirmed that at this temperature the steady-state distribution of electrolytes in this tissue appears to be more favourable than at 37°, higher apparent Donnan ratios of potassium and chloride being observed at the former temperature (Kleinzeller & Knotková, 1963*b*), in full agreement with the recent data of Whittam & Willis (1963).

In some experiments the effect of ouabain on the transport of electrolytes and water was also investigated by the reverse procedure: the slices were prepared without moistening the blade with saline, thus preventing major changes of cellular electrolytes during slicing. Immediately after preparing groups of slices (approx. 150 mg wet weight) these were either used after blotting for the estimation of the initial values of tissue water and electrolytes, or were incubated aerobically in 3 ml. Krebs–Ringer phosphate saline, containing 4 mM. α-oxoglutarate without (control) or with 0·3 mM-ouabain. Under these experimental conditions metabolic inhibitors produce a swelling of the tissue and changes of the electrolyte distribution (Robinson, 1950; Whittam & Davies, 1953) while in the controls the tissue solutes do not change appreciably.

After leaching and incubation, groups of slices were removed and in the blotted tissue the contents of water and ions were estimated. In some cases the amount of soluble protein lost by the slices into the incubating saline was also estimated: a portion of the medium was centrifuged at 10,000 × g for 10 min to remove washed out cells and cellular debris, and protein was analysed in a portion of the supernatant.

In isolated kidney-cortex cells the volume changes during aerobic incubation and the electrolyte distribution were followed as described by Bosáčková (1963).

The kinetics of ^{24}Na efflux from kidney cortex slices, and the effect of ouabain on the efflux, were studied by the washing-out technique reported earlier (Kleinzeller & Janáček, 1962), and from the data the steady-state rate constants of the Na efflux were calculated.

The measurement of O_2 uptake by the tissue was carried out by the conventional Warburg technique.

Salines. Ca-free isotonic salines of the Krebs–Ringer type, buffered with 0·1 vol. of 0·1 M phosphate or tris-buffer, pH 7·4, or simply 0·154 M-NaCl were used for the teaching process. Ca was omitted from the salines since it affects the swelling of the tissue, the electrolyte distribution and the passive permeability of the cell membrane (Kleinzeller & Cort, 1960). For the incubation, salines of the Krebs–Ringer type were employed. For further details, see Results.

Analytical methods. Details of the analytical methods used were described earlier (e.g. Kleinzeller, 1961). Tissue contents of Na^+, K^+, and Li^+ were estimated by flame photometry, Cl^- by potentiometric titration (Sanderson, 1952). Tissue water was assessed from the difference between wet and dry weight of the tissue. Soluble tissue protein in the saline was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

Presentation of results. Tissue water is expressed in kg H_2O/kg tissue solids (DS), electrolytes in m-equiv/kg DS. The apparent concentration of bulk cations in the tissue (i.e. $(Na^+ + K^+)$, or $(Na^+ + K^+ + Li^+)$) is then given per kg tissue water.

From the data obtained the values of apparent intracellular ionic concentrations and Donnan ratios were computed, taking the previously found figures of the extracellular (inulin) space in kidney cortex slices (Kleinzeller, 1961), i.e. 200 ml. extracellular water in slices leached at 0 and 250 ml. after aerobic incubation of the tissue at 25 or 37° C. The indices *i* and *o* refer to the intra- and extracellular space, respectively, assuming the solute concentration in the latter to be identical with that in the saline.

The rate of oxygen uptake by the tissue is expressed by the conventional quotient Q_{O_2} (μl. O_2/mg DS.hr).

For the designation of the rate constants of ^{24}Na efflux see Kleinzeller & Janáček (1962).

The results given are either typical experiments, each point being the mean of at least two analyses, or represent arithmetic means of values ± S.E. Where required, the statistical significance of the results was calculated with the Student's *t* test.

RESULTS

The transport of electrolytes and water by Li-loaded kidney-cortex slices and the effect of ouabain

Evidence has been presented earlier (Cort & Kleinzeller, 1957) that Li^+ readily penetrates into kidney-cortex cells during leaching in an isotonic Li-saline at 0° C. However, as opposed to Na^+, these cells were found not to be capable of aerobically extruding Li^+ against a variety of concentration gradients of this ion. It was now found that when kidney-cortex slices were first leached in a Na-free Li-saline and then aerobically incubated in a Li-saline, a transport of Li^+ and water took place in this tissue.

Time curve of Li^+ and H_2O transport. Groups of slices of rabbit-kidney cortex were first leached for 2·5 hr in a Ca-free Li-saline of the Krebs–Ringer type, in which NaCl was equivalently replaced by LiCl; in view of the low solubility of lithium phosphate, 0·1 M-tris-buffer, pH 7·4, was used instead of the 0·1 M-phosphate in the Krebs–Ringer phosphate-saline. A portion of the slices was then aerobically incubated (gas phase O_2) for varying time intervals at 25° C in the same saline, containing 2·5mM-Ca^{2+} and 4 mM-Li-α-oxoglutarate as substrate. The action and water contents of the slices were analysed after leaching and after incubation. As controls, Li-loaded slices were also incubated in the corresponding Na-saline.

The results show that in the Na-saline, the Li-loaded slices rapidly lost water and Li^+, accumulated K^+ and also took up Na^+ to a level found when Na-loaded tissue is incubated under these conditions. Leaching in the Li-saline thus did not markedly affect the cation transport system in kidney cortex slices. These observations are consistent with the following events: At first, Li^+ passively diffuses out of the cells. On the passive entry of Na^+ into the cells, the sodium pump starts operating with an extrusion of Na^+ (also Cl^- and H_2O) and re-accumulation of K^+, until a steady-state level distribution of tissue solutes between the cells and the saline is established.

During incubation in the Li-saline, a marked, though slower, loss of water and Li^+ also occurred with a simultaneous small loss of Na^+ and K^+; the initial rate of this Li^+ extrusion was of the order of 2 m-equiv/kg DS.min, as compared with the rate of 22 m-equiv Na^+/kg DS.min from Na-loaded slices in Na-saline under comparable conditions.

Character of the Li^+ transport in Li-saline and the effect of ouabain. A more detailed analysis of the distribution of water and electrolytes after

leaching and 60 min incubation at 36° C, and the effect of 0·3 mM-ouabain, is shown in Table 1.

It will be seen that, during leaching, Li^+ replaced most of the tissue Na^+. The amount of 75 m-equiv Na^+/kg DS remaining in the slices represents the 'non-removable' Na^+ (see Kleinzeller & Janáček, 1962); it has been

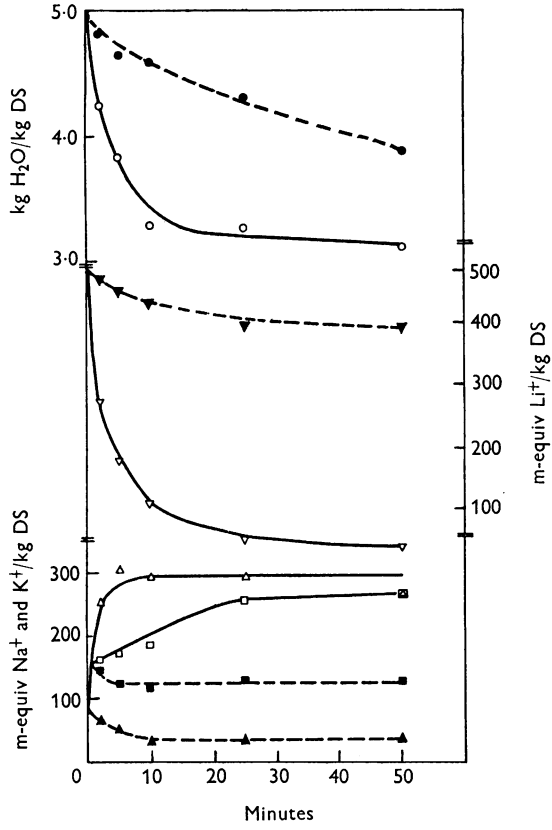

Fig. 1. Transport of electrolytes and water from Li-loaded slices: Time curve. Slices first leached for 2·5 hr in Li-saline (composition, mM: Li^+, 130; K^+, 10; Mg^{2+}, 1·3; $tris^+$, 8·8; $H_2PO_4^-$, 1·3; HCO_3^-, 5; Cl^-, 141). Subsequently, each group of slices (approx. 150 mg wet weight) incubated at 25° C aerobically (gas phase O_2) in 3 ml. saline of the same type, containing in addition 4 mM-α-oxoglutarate and 2·5 mM-Ca^{2+} (interrupted lines). As controls, Li-loaded slices were incubated in a Na-saline of the same type, Na^+ equivalently replacing Li^+ (full lines). Symbols: ○, H_2O kg/kg DS; ▽, Li^+; △, Na^+; □, K^+; all in m-equiv/kg DS. Means of two analyses.

reported elsewhere that this Na-fraction corresponds to a very slowly exchangeable component of cellular Na^+ (Kleinzeller & Knotková, 1963*b*) with a half-time of about 45 min.

The data in Table 1 show that during incubation a marked net loss (per kg DS) of water (−1·21 kg), Li^+ (−119 m-equiv) and Cl^- (−148 m-equiv), and also of some Na^+ and K^+ took place, while the apparent intracellular concentrations of these ions were hardly affected. Thus, an extrusion of a practically isotonic solution of the electrolytes from the

TABLE 1. Ion and water transport by Li-loaded kidney-cortex slices and the effect of ouabain

	After leaching	After incubation	
		Control	0·3 mM-ouabain
Tissue contents:			
H_2O, kg/kg DS	4·83 ± 0·05	3·62 ± 0·04	3·77 ± 0·05
Na^+, m-equiv/kg DS	75 ± 6	44 ± 4	49 ± 3
K^+, m-equiv/kg DS	174 ± 2	140 ± 2	139 ± 4
Li^+, m-equiv/kg DS	497 ± 14	384 ± 5	401 ± 5
Cl^-, m-equiv/kg DS	455 ± 11	307 ± 15	353 ± 9
Apparent cation concn.:			
m-equiv $(Na^+ + K^+ + Li^+)$/kg tissue H_2O, mM	155 ± 3	157 ± 1	156 ± 1
Apparent intracellular concn. (mM):			
$[Na^+]_i$	20 ± 2	17 ± 2	18 ± 1
$[K^+]_i$	44 ± 1	50 ± 1	48 ± 1
$[Li^+]_i$	95 ± 3	96 ± 1	97 ± 1
$[Cl^-]_i$	79 ± 3	72 ± 5	75 ± 3
Apparent Donnan ratios:			
$[K^+]_i/[K^+]_o$	4·28 ± 0·09	5·2 ± 0·07	4·6 ± 0·11
$[Li^+]_i/[Li^+]_o$	0·72 ± 0·03	0·75 ± 0·01	0·75 ± 0·005
$[Cl^-]_i/[Cl^-]_o$	1·78 ± 0·07	1·93 ± 0·08	1·88 ± 0·07

Slices first leached for 2·5 hr in the following saline (mM): Li^+, 130; K^+, 10·0; Mg^{2+}, 1·3; $tris^+$, 8·8; $H_2PO_4^-$, 1·3; HCO_3^-, 5·0; Cl^-, 141. Subsequently, groups of slices were incubated aerobically for 60 min at 37° C (gas phase O_2) in the same type of saline, containing in addition 4 mM-α-oxoglutarate and 2·5 mM-Ca^{2+}. Values ± S.E., n = 9 (three animals).

cells occurred, as indicated by the following calculation: per kg DS, a total of 179 ± 33 m-equiv $(Na^+ + K^+ + Li^+)$, and simultaneously 1·21 ± 0·09 kg H_2O were lost from the tissue; therefore, these cations moved as a 179/1·21 = 148 ± 30 mM solution (the S.E. calculated here according to the rules for combining errors). The corresponding values for Cl^- are somewhat lower, suggesting that another anion (α-oxoglutarate and/or bicarbonate ?, see Whittam & Davies, 1953; Anderson & Mudge, 1955) participated in the process. This transport of electrolytes occurred at a constant electrochemical gradient, as indicated by the fact that within the limits of experimental error no marked changes of the Donnan ratios of K^+, Cl^- and Li^+ took place during incubation. A comparison of Fig. 1 with Table 1 shows that the same type of result was also obtained at 25°.

The distribution of tissue solutes after incubation in the presence of 0·3 mM-ouabain did not markedly differ from that of the control without inhibitor; thus, the transport of electrolytes and water from Li-loaded cells appears to be ouabain-insensitive.

The pattern of the electrolyte transport by the Li-loaded tissue in Li-saline differs from that accepted for an active electrolyte transport in kidney-cortex slices (see e.g. Mudge, 1951*a*; Krebs *et al.* 1951; Aebi, 1953; Whittam & Davies, 1953; Leaf, 1956; Kleinzeller, 1961) in that: (*a*) no re-accumulation of K^+ occurred; (*b*) the Donnan ratios of K^+ and Cl^- did not change; (*c*) ouabain had no effect. It should be pointed out that 0·3 mM-ouabain also did not markedly affect the respiration of Li-loaded slices (Table 2), the values of Q_{O_2} agreeing well with those found by Whittam & Willis (1963) for kidney-cortex slices incubated in Na-free choline saline.

TABLE 2. The effect of ouabain on the respiration of Li-loaded and Na-loaded kidney cortex slices

Slices were first leached for 2·5 hr at 0° C in 0·154 M-NaCl (Na-loading) or LiCl (Li-loading). Then the tissue was incubated either in Li-saline (see Table 1) or the corresponding Na-saline, without (control) or with 0·3 mM-ouabain. Gas phase; O_2; 60 min. Mean of two measurements.

	Incubating saline	Q_{O_2}	
		Control	0·3 mM-ouabain
Li-loaded slices	Li-saline	8·4	7·0
	Na-saline	14·0	7·6
Na-loaded slices	Na-saline	16·9	8·6

TABLE 3. The effect of DNP on ion and water transport by Li-loaded kidney cortex slices

	After leaching	After incubation	
		Control	0·1 mM-DNP
Tissue contents:			
H_2O, kg/kg DS	4·49 ± 0·08	3·81 ± 0·04	4·75 ± 0·02
Na^+, m-equiv/kg DS	155 ± 7	45 ± 8	47 ± 4
K^+, m-equiv/kg DS	148 ± 3	127 ± 1	128 ± 2
Li^+, m-equiv/kg DS	537 ± 17	493 ± 4	594 ± 5

For conditions of leaching and incubation see Table 1. Values ± S.E., $n = 6$.

Effect of 2,4,-dinitrophenol (DNP) on the transport of electrolytes by Li-loaded slices. As shown in Table 3, the transport of electrolytes by Li-loaded slices was completely inhibited by 0·1 mM-DNP and thus appears to be dependent on a supply of metabolic energy. Experiments not given here in detail showed that the described Li^+ transport was not affected by varying $[K^+]_o$ (0–30 mM) or $[Ca^{2+}]_o$ (0–5 mM) in the saline.

The effect of ouabain on the steady-state efflux of ^{24}Na from kidney-cortex slices

From the data of Burg & Orloff (1962) it followed that 0·3 mM-strophanthidin in the incubating saline practically completely blocked the active transport of electrolytes in slices of rabbit-kidney cortex, as is

shown by the fact that under these conditions slices previously leached in NaCl even lost a portion of tissue K^+. Also the data of Whittam & Willis (1963) suggest that this concentration is sufficient to produce a minimum

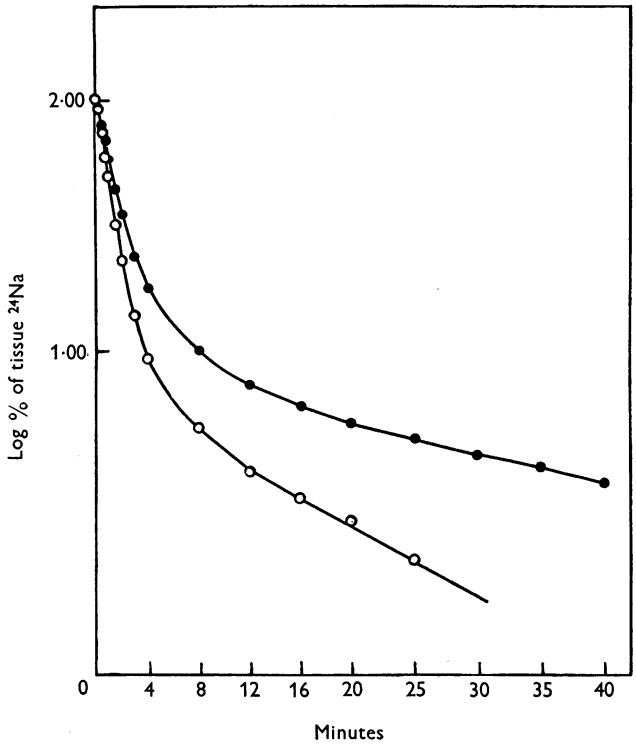

Fig. 2. The effect of 0·3 mM-ouabain on the kinetics of steady-state ^{24}Na efflux from kidney-cortex slices. Groups of slices were first incubated aerobically for 45 min. at 25° C in labelled Krebs–Ringer phosphate saline (4 mM-α-oxoglutarate as substrate) without (control) or with ouabain, then blotted and the efflux followed by the washing-out technique, with identical salines for the further incubation. ○, control; ●, ouabain. Typical experiment.

TABLE 4. The effect of ouabain on the steady-state rate constants of ^{24}Na efflux and tissue solutes

The values of the constants, computed from the kinetics of ^{24}Na efflux, are the mean of three experiments; values of tissue components are the mean ± S.E. of duplicate analyses before and after the washing-out procedure.

	Steady-state rate constants			Tissue components		
	k_2 (min^{-1})	k_2' (min^{-1})	k_2'' (min^{-1})	H_2O (kg/kg DS)	Na^+ (m-equiv/kg DS)	K^+
Control	0·052	0·504	1·298	2·99 ± 0·03	280 ± 4	338 ± 3
0·3 mM-ouabain	0·025	0·318	1·316	3·09 ± 0·08	468 ± 13	134 ± 2

level of tissue potassium and respiration. It was therefore of interest to examine the effect of 0·3 mM-ouabain on the steady-state efflux of ^{24}Na. After completion of the present investigation, Burg & Orloff (1963) presented evidence that 0·3 mM strophanthidin decreased the steady-state efflux of $^{42}K^+$. Figure 2 shows the result of a typical experiment; in Table 4 the values of the rate constants of ^{24}Na efflux are presented.

It will be seen that, as compared with the control without inhibitor, 0·3 mM-ouabain markedly decreased the rate constants k_2 and k_2', without affecting the fastest component k_2'' (which has been assigned to the diffusion of Na^+ from the extracellular compartment of the slices, see Kleinzeller & Janáček, 1962). The values of the rate constants k_2 and k_2' in the presence of ouabain are very similar to those obtained in the presence of 0·1 mM-DNP (Kleinzeller & Knotková, unpublished), suggesting that they approach those for a passive efflux of ^{24}Na across cellular diffusion barriers.

It will also be noted from Table 1 that, while the steady-state values of the tissue water in the presence of 0·3 mM-ouabain hardly differed from those of the control, there were very considerable differences in the composition of the tissue cations Na^+ and K^+.

The effect of ouabain on the transport of electrolytes and water in kidney cortex slices

The data shown in Table 4 indicate a discrepancy between the water and cation content of the slices incubated in the presence of ouabain since in previous experiments (see Mudge, 1951*a*, *b*; Aebi, 1953; Whittam & Davies, 1953; Leaf, 1956) high tissue Na^+ and low K^+ were always found to be associated with a swelling of the kidney cortex tissue. An attempt was therefore made to study the effect of ouabain on the relation between water and ion transport in this tissue in further detail.

Net transport of water and electrolytes. In these experiments slices were leached and incubated in salines of similar $[K^+]_o$, thus enabling a comparison not only of the net transport of electrolytes and water but also of the Donnan K^+ ratio after leaching and after subsequent incubation. The composition of the salines was also very similar to those used to follow the transport by Li-loaded slices (see above). The results of these experiments are presented in Table 5.

It will be noticed from the data that the concentration of 0·3 mM-ouabain blocked completely the net aerobic re-accumulation of K^+, the tissue K^+ being as much as 13 m-equiv/kg DS lower than in the leached slices. However, under these experimental conditions a highly significant loss of tissue water ($-0·84$ kg/kg DS) and electrolytes (-120 m-equiv Na^+ and -88 m-equiv Cl^-/kg DS) took place. On the other hand, although

marked changes in the intracellular concentrations of Na^+, K^+ and Cl^- occurred in the control experiments, in the presence of ouabain the apparent intracellular concentrations were unaffected. This last obser-

TABLE 5. The effect of ouabain on the transport of electrolytes and water in Na-loaded kidney cortex slices

	Expt. 1		
		Incubation at 25°	
	After leaching	Control	0·3 mM-ouabain
Tissue contents:			
H_2O, kg/kg DS	4·57 ± 0·07	2·97 ± 0·04	3·73 ± 0·08
Na^+, m-equiv/kg DS	651 ± 13	279 ± 5	531 ± 7
K^+, m-equiv/kg DS	148 ± 3	344 ± 2	135 ± 2
Cl^-, m-equiv/kg DS	524 ± 13	252 ± 5	436 ± 10
Apparent cation concn.:			
m-equiv (Na^+K^+)/kg tissue H_2O, mM	175 ± 2	210 ± 2	179 ± 1
Apparent intracellular concn. (mM):			
$[Na^+]_i$	139 ± 2	77 ± 2	149 ± 1
$[K^+]_i$	41 ± 1	172 ± 1	48 ± 1
$[Cl^-]_i$	108 ± 2	59 ± 2	110 ± 3
Apparent Donnan ratios:			
$[K^+]_i/[K^+]_0$	7·4 ± 0·2	23·5 ± 0·2	6·6 ± 0·1
$[Cl^-]_0/[Cl^-]_i$	1·27 ± 0·06	2·33 ± 0·1	1·23 ± 0·03
	Expt. 2		
		Incubation at 25°	
	After leaching	Control	1 mM-ouabain
Tissue contents:			
H_2O, kg/kg DS	4·41 ± 0·02	2·79 ± 0·02	4·07 ± 0·03
Na^+, m-equiv/kg DS	627 ± 5	283 ± 5	698 ± 7
K^+, m-equiv/kg DS	143 ± 5	271 ± 3	78 ± 1
Cl^-, m-equiv/kg DS	480 ± 2	230 ± 2	447 ± 6
Apparent cation concn.:			
m-equiv $(Na^+ + K^+)$/kg tissue H_2O, mM	175 ± 3	200 ± 2	191 ± 2
Apparent intracellular concn. (mM):			
$[Na^+]_i$	140 ± 1	78 ± 3	180 ± 2
$[K^+]_i$	41 ± 2	144 ± 2	24 ± 2
$[Cl^-]_i$	99 ± 1	58 ± 2	102 ± 1
Apparent Donnan ratios:			
$[K^+]_i/[K^+]_0$	5·8 ± 0·03	22·2 ± 0·02	3·7 ± 0·02
$[Cl^-]_0/[Cl^-]_i$	1·41 ± 0·01	2·36 ± 0·09	1·32 ± 0·06

Slices leached for 2·5 hr at 0° C in a saline containing (mM): Na^+, 152; K^+, 5·6; Cl^-, 136·6; phosphate, 8·1, pH 7·4. Aerobic incubation (gas phase O_2) at 25° C for 60 min in a Na-saline of the Krebs–Ringer type, containing 0·1 vol. 0·1 M-tris-buffer, pH 7·4 (instead of phosphate buffer) and 4 mM-α-oxoglutarate as substrate ($[Na^+]_0$, 127·2 mM; $[K^+]_0$, 6·3 mM; $[Cl^-]_0$, 135·8 mM), without (control) and with 0·3 or 1 mM-ouabain. Values ± S.E., n = 6–8 (two animals).

vation is also borne out by the apparent Donnan ratios: while in the control the Donnan ratios of both K^+ and Cl^- greatly increased on incubation of the tissue, indicating a transport of electrolytes against their

electrochemical gradients, in the presence of ouabain the respective ratios were, within the limits of experimental error, identical with the values for the leached slices. Finally, it may be calculated from the data that the accumulation of total bulk cations in the cells which is known to occur when leached slices are incubated aerobically in a balanced saline (Aebi, 1953, Whittam & Davies, 1953) was decreased by the presence of 0·3 mM-ouabain.

The argument might be raised that 0·3 mM-ouabain was not sufficient to block completely the ion transport, the extrusion of water and NaCl from the cells thus being due to a residual function of the cation pump. Although one would then have to explain the unusual pattern of electrolyte transport, a further experiment was carried out in which the concentration of ouabain was raised to 1 mM. From Table 5, Expt. 2, it will be seen that even under these conditions a highly significant loss of water occurred ($P < 0·01$), while a net movement of Na^+ into the cells took place and K^+ was lost.

The possibility was envisaged that ouabain might produce a damage of the tissue cells associated with a loss of cell protein, electrolytes and water. To test this, the loss of soluble protein from the slices into the incubating saline was examined. No effect of 0·3 mM-ouabain was found, the appropriate values (means of 3 analyses) being: control, 28·1 g protein/kg DS; ouabain, 29·7 g/kg DS.

These results therefore suggest that under conditions where ouabain produced a complete blockage of the aerobic re-accumulation of K^+, the tissue cells extruded a practically isotonic solution of electrolytes (with 0·3 mM-ouabain this was essentially NaCl) at a constant or, with 1 mM-ouabain, a decreased electrochemical gradient.

It will be noted that the pattern of electrolyte and water changes during incubation in the presence of ouabain is essentially identical with that found for the transport of electrolytes and water from Li-loaded cells (see Table 1). Similarly this ouabain-insensitive extrusion of water and Na^+ was found to be inhibited by 0·1 mM-DNP and hence dependent on metabolic energy.

The effect of ouabain on fresh tissue. Since Robinson (1950) and Mudge (1951*b*) demonstrated that metabolic inhibitors produced a swelling of kidney-cortex tissue with a concomitant increase of tissue Na^+ (and Cl^- and loss of K^+, it has been taken that an inhibition of active cation transport is associated with a swelling of the cells. The effect of 0·3 mM-ouabain on the capability of kidney-cortex slices to maintain *in vitro* the *in vivo* ionic concentration gradients was therefore examined by studying the distribution of water and electrolytes during incubation of freshly prepared slices. The results are shown in Fig. 3.

It will be seen that in the controls the water, Na^+ and Cl^- contents did not change appreciably during incubation, while the K^+ content somewhat increased; thus, an accumulation of bulk cations in the tissue took place. In the presence of ouabain, the water content slightly increased in the first 10 min of incubation and subsequently fell, reaching a value practically identical with that of the control; on the other hand, within 10 min

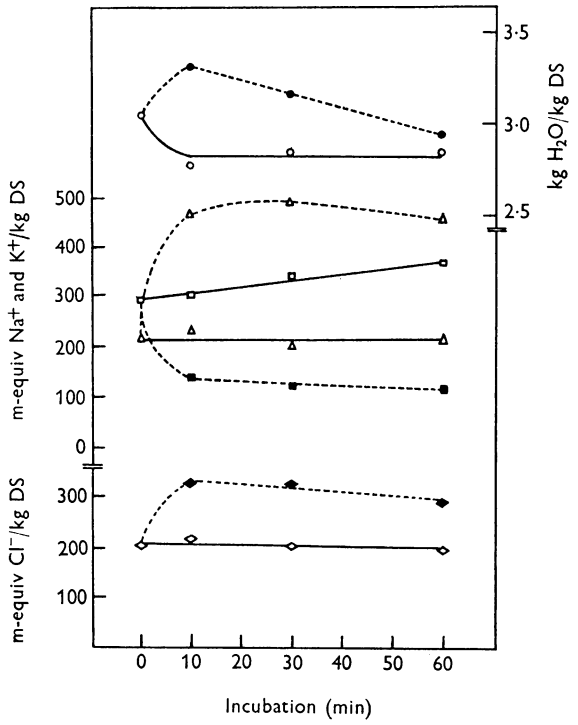

Fig. 3. The effect of 0·3 mM-ouabain on tissue water and electrolytes in kidney-cortex slices. Groups of fresh slices incubated aerobically (gas phase O_2) in 3 ml. Krebs–Ringer phosphate saline, containing 4 mM-α-oxoglutarate without (control; full lines) and with 0·3 mM-ouabain (interrupted lines). Symbols: ○, H_2O kg/kg DS; △, Na^+; □, K^+; ◇, Cl^-; all in m-equiv/kg DS. All values are the mean of three analyses.

the ionic distribution corresponded to that usually associated with a considerable swelling of the tissue, i.e. high Na^+ and low K^+, Cl^- being intermediate. These results are thus fully consonant with those described above, with a different experimental approach.

It should be mentioned that at 37° C the pattern of water and cation distribution within the first 10 min of incubation in the presence of ouabain did not differ from that found at 25° C. Subsequently, the tissue con-

tinued to swell slowly, the water content after 60 min being 3·6 kg/kg DS i.e. identical with that given by Whittam & Willis (1963) for practically the same experimental conditions. No explanation can be offered at present for this discrepancy between the ouabain effects at 25 and 37° C.

The effect of ouabain on cell volume changes. A loss of an electrolyte solution from the cells would be expected to produce corresponding

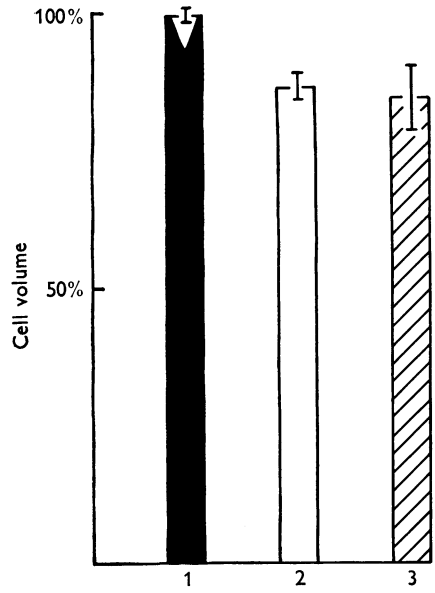

Fig. 4. Effect of ouabain on cell volume changes during incubation. Isolated kidney cortex cells were first maintained for 2·5 hr in a balanced saline at 0° C, then incubated aerobically (O_2) for 60 min at 25° C in the same saline, containing 4 mM-α-oxoglutarate as substrate. For composition of the saline see Bosáčková (1963). The cell volume is expressed in percentage of the volume at 0° C and represents the mean ± S.E. of four analyses in each of two experiments. Columns: 1, after leaching; 2, after incubation (control); 3, after incubation (0·3 mM-ouabain). Significance: volume changes during incubation of control: $P < 0{\cdot}01$; in the presence of ouabain: $0{\cdot}02 < P < 0{\cdot}05$.

volume changes. For the investigation of this aspect, isolated cells of rabbit-kidney cortex proved to be a convenient preparation (Bosáčková, 1963). The results of a study of the ouabain effect on the volume changes taking place during aerobic incubation of cells previously loaded with water, Na^+ and Cl^- (and impoverished of K^+) at 0° C are shown in Fig. 4. It will be seen that in the presence of 0·3 mM-ouabain the volume changes did not significantly differ from those in the control without inhibitor. The pattern of electrolyte distribution in the cell water was identical with

that found in the slices (Table 5) and the relevant data are not given here in detail.

It may thus be concluded that under conditions where 0·3 mM-ouabain blocks completely the active cation transport, an extrusion of an electrolyte solution with concomitant cell volume changes takes place.

The effect of $[K^+]_0$. It has been shown previously (Kleinzeller, 1961) that when slices previously leached in isotonic NaCl are incubated in a K-free saline, a simultaneous extrusion of H_2O, Na^+, Cl^- and some loss of

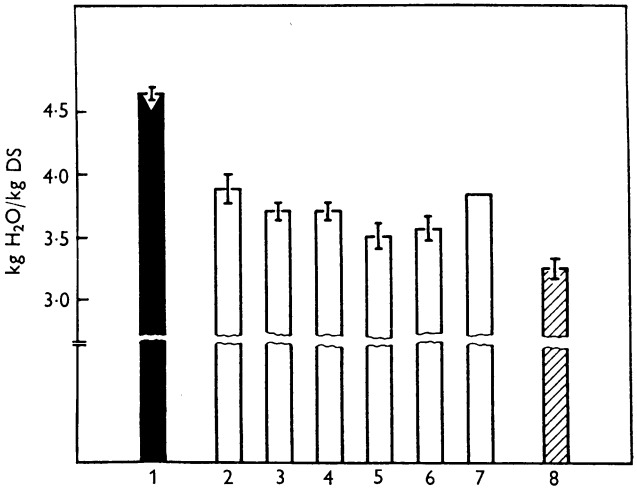

Fig. 5. Effect of $[K^+]_0$ on tissue-water changes during incubation of Na-loaded slices in the presence of 0·3 mM-ouabain. Slices leached 2·5 hr in 0·154 M-NaCl, then incubated aerobically at 25° C for 60 min in salines of varying $[K^+]_0$ (equivalent replacement of Na^+ for K^+) with 0·3 mM-ouabain. Mean values ± S.E. of tissue H_2O (kg/kg DS) only where four or more analyses were carried out. Columns: 1, after leaching; 2–7, after incubation in varying $[K^+]_0$; 8, control without ouabain, $[K^+]_0 = 5$.

K^+ occurs. Thus the electrolyte changes taking place in the K-free saline are very similar to those described above for the incubation of Na-loaded slices in a balanced saline containing ouabain. It was therefore of interest to examine the effect of $[K^+]_0$ on the transport of water and electrolytes, insensitive to 0·3 mM-ouabain.

Figure 5 shows that, although a significant net transport of water occurs even in the absence of K^+ from the incubating saline (−0·74 kg/kg DS), the water and electrolyte extrusion was further enhanced by the presence of 1·5–5 mM-K^+; at 20 mM-K^+, the loss of water was significantly smaller, evidently due to the swelling effect of this ion (see Kleinzeller, 1961). The pattern of ionic distribution again followed that described

above and is not given in detail; at higher $[K^+]_o$ (10–20 mM) the tissue K^+ increased in accordance with the previous observation that the cellular K^+ increases in proportion to the $[K^+]_o$ (Cort & Kleinzeller, 1956).

Thus, a definite effect of $[K^+]_o$ on the ouabain-insensitive transport of water and electrolytes from Na-loaded slices could be demonstrated, as opposed to Li-loaded cells.

Varying $[Ca^{2+}]_o$ in the saline (0–7·5 mM) had no effect on the ouabain-insensitive extrusion of water and electrolytes.

The effect of ouabain on the transport of electrolytes and water in rabbit liver slices

The above results raised the question whether the ouabain-insensitive transport of electrolytes and water might also be found in liver tissue; Na-loaded liver slices are known to be capable of transporting cations and water against their respective concentration gradients (see e.g. Aebi, 1953; van Rossum, 1963).

Here data on the effect of ouabain on ion and water movements in rat liver slices are reported.

TABLE 6. Effect of ouabain on the transport of cations and water in rabbit liver slices

Slices leached in ice-cold 0·154 M-NaCl for 2·5 hr, then groups of slices (approx. 150 mg. wet weight) incubated aerobically at 37° C (95% O_2 + 5% CO_2) for 60 min in 3 ml. of medium I of Krebs (1950), without (control) and with 0·3 mM-ouabain. Values ± S.E., $n = 5$.

	After leaching	After incubation	
		Control	0·3 mM-ouabain
Tissue contents:			
H_2O, kg/kg DS	5·03 ± 0·12	3·68 ± 0·08	5·77 ± 0·08
Na^+, m-equiv/kg DS	902 ± 41	473 ± 32	841 ± 17
K^+, m-equiv/kg DS	74 ± 6	193 ± 10	66 ± 2
Apparent cation concn.:			
m-equiv $(Na^+ + K^+)$/kg tissue H_2O, mM	190 ± 1	181 ± 4	157 ± 3

The effect of ouabain on the net transport of cations and water. Rabbit liver slices were first leached at 0° C in 0·154 M-NaCl; as incubating medium, the saline serum substitute (medium I of Krebs, 1950) was employed since it has been shown by this author that the respiration rate of the tissue is highest in this saline.

It will be seen from the data in Table 6 that a marked transport of tissue cations and water took place during aerobic incubation at 37° C of Na-loaded rabbit liver slices; in contrast to kidney cortex slices, 0·3 mM-ouabain completely inhibited both electrolyte and water movement. The same type of result was also obtained when incubating the tissue at 25° C.

Liver tissue differs from kidney cortex also in that no water extrusion can be found when incubating Li-loaded tissue in a Li-saline; details of these experiments are not given here.

The effect of ouabain on fresh liver slices. Further confirmation of the above findings was sought using the reverse technique, i.e. by studying the effect of ouabain on the capacity of liver slices to maintain *in vitro* the concentration gradient of Na^+ and K^+ found *in vivo*. The results of one such experiment are shown in Fig. 6.

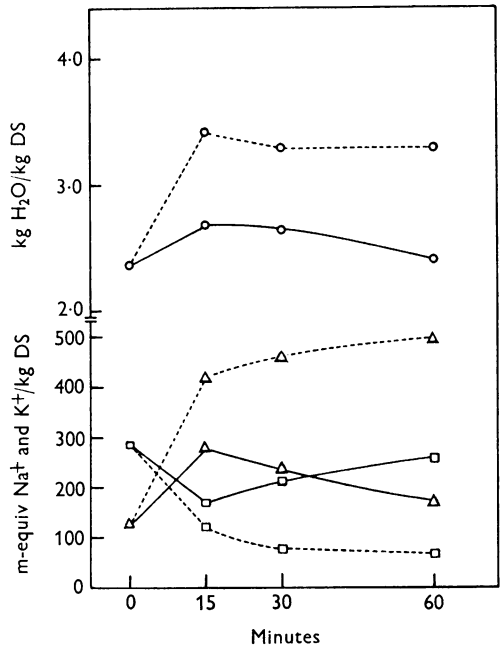

Fig. 6. The effect of 0·3 mM-ouabain on tissue water and electrolytes in liver slices. Groups of fresh slices incubated aerobically (gas phase 95 % O_2 + 5 % CO_2) in 3 ml. medium I of Krebs (1950) without (control; full lines) and with 0·3 mM-ouabain (interrupted lines). Symbols: ○, H_2O, kg/kg DS; △, Na^+; □, K^+; both in m-equiv/kg DS. All values are the mean of three analyses.

It will be noticed that under these conditions the level of tissue water and cations in the control slices returns to the values in the fresh tissue within about 60 min (see also Krebs *et al.* 1951) but that 0·3 mM-ouabain produced a marked swelling of the slices, increased tissue Na^+ and lowered K^+. Liver tissue thus differs from kidney-cortex slices (Fig. 3) in that here high Na^+ and low K^+ is associated with the swelling. However, a comparison of the values for water in Fig. 6 and Table 6 shows that the swelling of the liver tissue in the presence of ouabain was considerably smaller than that found in the leached slices. Thus, the possibility cannot

be excluded that liver tissue is also capable of extruding some Na^+ and water in the presence of the inhibitor though only to a small extent. It should also be mentioned that when incubating previously Na-loaded liver slices of adult animals, the level of tissue solutes does not return to that found in fresh tissue (van Rossum, 1963); this is in contrast to the results obtained on incubation of fresh tissue. This difference may be due to a damaging effect of the leaching procedure on the transport process.

The effect of ouabain on the respiration of liver slices. 0·3 mM-ouabain inhibited the respiration rate of Na-loaded rabbit liver slices but did not affect the Q_{O_2} of Li-loaded slices in Li-saline. It should be pointed out that the Q_{O_2} of Na-loaded slices in Na-saline was only about 60 % of that of fresh tissue in the same medium. Thus, the leaching procedure appears to damage both the cation transport (see above) and the respiratory systems.

TABLE 7. The effect of ouabain on the respiration of rabbit liver slices

The tissue was incubated immediately after slicing (fresh tissue) or after preliminary leaching for 2·5 hr at 0° in 0·154 M-NaCl (Na-loading) or LiCl (Li-loading) Incubation of the tissue in the appropriate Li- or Na-saline of the Krebs-Ringer phosphate type, containing as substrates those suggested by Krebs (1950) for medium I. Gas phase: O_2; 60 min. Values ± S.E., $n = 5$.

Treatment of tissue	Incubating saline	Q_{O_2}	
		Control	0·3 mM-ouabain
Fresh tissue	Na-saline	8·0 ± 1·2	6·4 ± 1
Na-loading	Na-saline	5·3 ± 0·3	4·4 ± 0·3
Li-loading	Li-saline	3·9 ± 0·5	3·7 ± 0·4

DISCUSSION

The reported results demonstrated in kidney-cortex slices a metabolically-dependent, but ouabain-insensitive, extrusion of a practically isotonic solution of electrolytes. Evidence will be presented elsewhere that the same phenomenon occurs also in intact rat diaphragm. On the other hand, no such ouabain-insensitive transport was found in liver slices. The possible mechanism of the ouabain-insensitive extrusion process will be considered here.

Character of the electrolyte and water transport from Li-loaded cells in Li-saline. It has been previously shown (Cort & Kleinzeller, 1957) that kidney-cortex slices are not capable of transporting Li^+ against its concentration gradient. The observation of net Li^+ transport in this tissue does not appear to invalidate the earlier conclusion. From the following observations it is suggested that this extrusion does not conform to the pattern of an active Li^+ transport: (*a*) the transport of Li^+ did not take place against its concentration gradient, the net Li^+ extrusion from the cells not bringing about a decrease of its (apparent) intracellular con-

centration; (*b*) the Li^+ transport was not associated with a countermovement of K^+ into the cells; (*c*) the apparent Donnan ratios of Li^+, K^+ and Cl^- did not change during the transport process, indicating that the Li^+ transport occurred at a constant electrochemical gradient; this point would require, however, further corroboration by direct measurement of the membrane potential, especially in view of the widely differing values of the Donnan ratios for the individual ions. It should also be mentioned that only in frog skin a small active Li^+-transport under specified conditions (Zerahn, 1955) was demonstrated while no extrusion of Li^+ against its concentration gradient could be found in frog muscle (Keynes & Swan, 1959) and erythrocytes (Maizels, 1961). Thus, most animal cells appear to be clearly discriminating between Na and Li ions.

Since active Li^+ transport appears to be excluded, an alternative mechanism for this metabolically dependent extrusion of an electrolyte solution should be sought and this will be discussed below.

The ouabain-insensitive transport of electrolytes and water from Na-loaded kidney-cortex cells. The pattern of changes of tissue electrolytes and water on incubation of Na-loaded kidney-cortex slices in the presence of 0·3 and 1 mM-ouabain (Table 2) is essentially identical with that found for the (ouabain-insensitive) extrusion of Li^+ and water from Li-loaded cells (Table 1) and also with that found for the extrusion of Na^+ and water from Na-loaded cells in a K-free saline (Kleinzeller, 1961). The arguments raised above against an active transport of Li^+ may also be appropriate for the ouabain-insensitive extrusion of Na^+ and water by this tissue. The possible objection that the extrusion of Na^+ (and water) from the slices in the presence of ouabain may be due to an incomplete inhibition of the sodium pump (and thus be active transport in the sense used by Ussing, 1960) appears to be invalidated by: (*a*) the fact that an extrusion of water took place even in the presence of 1 mM-ouabain, although much lower concentrations of this cardiac glucoside (0·1 mM) have been shown to be sufficient for complete blocking of the extrusion of Na^+ and re-accumulation of K^+ (see Burg & Orloff, 1962; Whittam & Willis, 1963) in this tissue; (*b*) the differences observed between kidney-cortex and liver slices; it is difficult to visualize that 0·3 mM-ouabain would completely block the sodium pump in one tissue but be insufficient to inhibit the extrusion of water from kidney cortex slices while completely blocking the re-accumulation of K^+. It thus appears reasonable to suggest that both the ouabain-insensitive extrusion of Na^+ from Na-loaded kidney-cortex cells and that of Li^+ from Li-loaded cells, are brought about by the same mechanism. It may be appropriate to point out here that the Q_{O_2} of kidney-cortex slices in a Na-saline, insensitive to 0·3 mM-ouabain, was identical with that found for Li-loaded tissue in Li-saline in the presence

and absence of ouabain (Table 2). This finding also points to identical mechanisms of the Li^+ and ouabain-insensitive Na^+ extrusions. Furthermore, the fact that the pattern of Na^+ (and water) extrusion from Na-loaded kidney cortex slices in a K-free saline is essentially identical with that of the ouabain-insensitive extrusion of Na^+ (and water), the latter being stimulated by external K^+, may suggest a common mechanism also for this phenomenon.

If the view is acceptable that the described ouabain-insensitive extrusion of electrolytes from kidney cortex cells is not brought about by the cation pump, possible alternative mechanisms should be considered. One such alternative may be suggested, i.e. that the ouabain-insensitive electrolyte and water extrusion is due to mechanical (and metabolically dependent) forces, such as the squeezing of an electrolyte solution from the cells. Such a mechanism would be consonant with the observed facts, i.e. no change of the intracellular ion concentration, and the net extrusion of electrolytes and water would bring about a decrease in cell volume, while the Donnan ratios of the electrolytes would remain practically constant. It may be recalled that a contractile mechanism has been indicated for the observed volume changes in mitochondria (see Lehninger, 1962) and also for some cells (see Wohlfarth-Bottermann, 1963). The differences between the kidney cortex (and also rat diaphragm) and liver tissue may thus be due to the absence of the contractile mechanism in the latter tissue, or its damage by the slicing and leaching procedure. It may be relevant to mention here that by light-scattering techniques conformational changes could be observed on addition of ATP to the $Na^+ + K^+$-activated ATPase from the microsomal fraction of kidney-cortex cells; these conformational changes were also found to be ouabain-insensitive (to be published).

No suggestion can at present be offered as to the possible functional role of the observed ouabain-insensitive extrusion of electrolytes and water.

SUMMARY

1. Rabbit kidney-cortex slices, previously loaded at 0° C with Li^+, were incubated aerobically in a balanced Li-saline. It was found that:

(*a*) the slices extruded cations (mainly Li^+, but also some Na^+ and K^+) and Cl^- as a practically isotonic solution at a constant Donnan ratio of potassium and chloride;

(*b*) this electrolyte and water extrusion was found to be insensitive to 0·3 mM-ouabain; the tissue respiration was also not markedly affected by this inhibitor;

(*c*) 0·1 mM-dinitrophenol completely inhibited the ion and water transport by Li-loaded tissue.

2. The steady-state rate constants of ^{24}Na efflux from the cellular compartments of kidney-cortex slices were found to be decreased by 0·3 mM-ouabain by about one-half.

3. The effect of ouabain on the ion and water transport by Na-loaded kidney cortex slices was studied. It was found that:

(*a*) in the presence of 0·3 and 1 mM-ouabain a significant extrusion of cations (Na^+ and K^+) and Cl^- as a practically isotonic solution at a constant Donnan ratio of K^+ and Cl^- occurred;

(*b*) 0·3 mM-ouabain did not produce a marked swelling of fresh slices during aerobic incubation at 25° C;

(*c*) 0·3 mM-ouabain did not affect the volume shrinkage of Na-loaded isolated kidney cortex cells during aerobic incubation at 25°;

(*d*) the ouabain-insensitive extrusion of water and electrolytes was found to be affected by the external concentration of K^+, being maximal at 5–10 mM-K^+;

(*e*) the ouabain-insensitive extrusion of water and electrolytes was completely inhibited by 0·1 mM-dinitrophenol.

4. The aerobic transport of water and electrolytes by rabbit-liver slices at 37° C was studied. It was found that:

(*a*) 0·3 mM-ouabain completely inhibited the extrusion of electrolytes and water from Na-loaded slices;

(*b*) 0·3 mM-ouabain produced a marked swelling of fresh slices during aerobic incubation of the tissue;

(*c*) no extrusion of Li^+ from Li-loaded liver slices in Na-saline was observed;

(*d*) ouabain inhibited the Q_{O_2} of Na-loaded slices but did not affect that of Li-loaded slices in a Li-saline.

5. The results are discussed. It is suggested that the ouabain-insensitive, metabolically dependent extrusion of water and electrolytes from kidney cortex cells is not due to an active process of ion transport and an alternative mechanism is considered.

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