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EFFECTS OF TEMPERATURE, MEDIUM K^+, OUABAIN AND ETHACRYNIC ACID ON TRANSPORT OF ELECTROLYTES AND WATER BY SEPARATED RENAL TUBULES

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SUMMARY

1. Isolated tubules from rabbit renal cortex are loaded with Na^+, Cl^-, water and leached of their K^+ by incubation at 0.5 °C in a K^+-free medium. Three aliquot fractions of the preparation are incubated: at 0.5 °C in a K^+-free medium, at 28 °C in a K^+-free medium, at 28 °C with 5 mM $[K^+]_0$, respectively. The incubation at 0.5 °C is used as a control. The tubules incubated at 28 °C, K^+-free, lose an isotonic NaCl solution and maintain nearly their whole K^+. Those incubated at 28 °C with 5 mM $[K^+]_0$, gain K^+ and lose water, Na^+ and Cl^- in such a way that the increase of the K^+ intracellular concentration is exactly balanced by the decrease of the Na^+ *plus* Cl^- intracellular concentrations.

2. Both extrusion of Na^+ (with Cl^- and water) from leached tubules, incubated at 28 °C in a K^+-free medium, and exchange of Na^+ for K^+ in leached tubules rewarmed with 5 mM $[K^+]_0$, are completely inhibited by ouabain and ethacrynic acid, which simultaneously induce an important K^+ tissular depletion.

3. When leached tubules are incubated at 0.5 °C or 28 °C in K^+-free medium, small amounts of this ion appear in the incubation medium. Tubular leakage is likely to account for these traces of K^+. Incubation of leached tubules in a K^+-free medium (except for tracer ^{42}KCl) induces a rapid exchange of tissular K^+ for medium K^+ at 28 °C, and a much smaller one at 0.5 °C.

4. When recapture of the escaped K^+ is partially prevented by dialysis of tubules suspension against a K^+-free medium, the K^+ tissular content is significantly reduced as well as the extrusion of Na^+, Cl^- and water.

5. It is concluded that both the net efflux of Na^+ (with Cl^- and water) induced by the rewarming of leached tubules into nominally K^+-free solutions, as well as the Na^+–K^+ exchange, demand the simultaneous movement of K^+ into the cells. It is further concluded that ions and water transport by renal tubular cells is governed in some manner by cell electrolyte composition.

INTRODUCTION

The active movements of Na^+ and K^+ in most mammalian tissues have been identified on the basis of the inhibitory effects of cardiotonic steroids. It has been

demonstrated that the active extrusion of Na^+ from the cells is coupled to the uptake of extracellular K^+ $[K^+]_0$[1-6], and that the energy for these movements of monovalent ions comes from the hydrolysis of adenosine triphosphate (ATP). The ouabain-sensitive transport system is closely related to a Na^+- and K^+-stimulated ATPase [(Na^+–K^+)-ATPase] which catalyses ATP hydrolysis in the presence of both Na^+ and K^+ and which is specifically inhibited by cardiotonic steroids such as ouabain[7-13].

However, the existence of an Na^+ extrusion together with Cl^- and water, independent from $[K^+]_0$[14-22] and insensitive to ouabain[15-18,20-22] has been recently reported from studies carried out on kidney cortex slices loaded with Na^+, Cl^- and water and leached of their K^+ by cooling. Whittembury and Proverbio[22] observed that ouabain inhibits the Na^+–K^+ exchange of Na^+-loaded guinea pig kidney cortex slices, but has no more than a marginal inhibitory effect on Na^+ extrusion (together with Cl^- and water) that occurs in a K^+-free medium. On the other hand ethacrynic acid completely inhibits Na^+ extrusion (together with Cl^- and water) but has very little effect on Na^+–K^+ exchange. Whittembury and Proverbio[22] asserted the existence, in the kidney tissue, of two distinct Na^+ pumps: "Pump A" that extrudes Na^+ (together with Cl^- and water) in a K^+-free medium, and that would thereby an important mechanism of cellular volume regulation, and "Pump B" that exchanges cellular Na^+ with extracellular K^+. "Pump A" also differs from "Pump B" in that its energetic source would be independent from (Na^+–K^+)-ATPase activity[23]. Willis[24], however, observes that ouabain completely inhibits Na^+ extrusion from kidney slices in a K^+-free medium but not completely in a medium with K^+. From these results, he suggests that in a K^+-free medium, Na^+–K^+ exchange can persist by means of K^+ trapped in the extracellular spaces within the kidney slices. He also holds that the partial effect of ouabain in a K^+-medium would result from a competition between this cation and ouabain for the binding of the transport sites.

The complex structure of kidney slices and particularly the existence in this preparation of an undetected recycling of K^+ and Na^+ between the extracellular spaces within the slices and cells[25] may have complicated the interpretation of these results. These drawbacks have been prevented in the present studies by using a suspension of isolated tubules from rabbit renal cortex. It has been found in consideration of extracellular K^+, ouabain and ethacrynic acid effects on water and ions transport, that the efflux of Na^+ into originally K^+-free solutions does not represent a mode of behaviour of the transport mechanism distinct from the coupled Na^+–K^+ pump.

METHODS

The technique of preparation of the isolated kidney tubules is similar to that of Burg and Orloff[26]. White male rabbits weighing 2–3 kg, are anesthetized by an intravenous injection of sodium pentobarbital. The kidneys are removed and perfused with a Ringer–phosphate solution. A collagenase solution (Nutritional Biochemicals) is injected in the renal artery, after clamping the vein, until the capsule ruptures. The cortex of both kidneys is cut into fragments of 2–5 mm. The cortex fragments are immersed in 100 ml of collagenase solution. This suspension is continuously stirred for 60–90 min. The tubules are washed three times by means of centrifugation at 50 × *g* for 2 min, in order to eliminate collagenase. The medium chosen for the experimental incubation is then added and the suspension is filtered on a cotton gauze in

a special incubation flask, similar to that described by Burg and Orloff[26]. During the different stages of the preparation of the tubules, the solutions are continuously saturated with O_2, except during centrifugations in hermetically stoppered tubes.

The Ringer–phosphate solutions which are used contain: (1), standard medium: 115 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM sodium acetate and 5 mM glucose, buffered by 10 mM phosphate to pH 7.35. (2), K^+-free medium: similar to (1), except NaCl substituted for KCl. Collagenase was dissolved at a concentration of 0.4 % (w/v) in one of the two solutions defined above. The pH of the media including ethacrynic acid was adjusted to pH 7.35 by addition of 1 M NaOH.

Leaching

The tubules are prepared in Solution 2 in order to induce a K^+ tissular depletion. The suspension is stored at 0.5 °C for 60 min. The oxygenation of the suspension is interrupted for 5 min every 15 min in order to replace the supernatant of the tubules suspension by Solution 2 stored at 0.5 °C.

Incubation procedure

The leached tubules suspension, stored at 0.5 °C in Solution 2, is divided into 3–5 equal parts according to the experiments. One aliquot is incubated in a range of incubation flasks filled with Solution 2. During the same time, the other aliquots of the suspension are incubated at 28 °C in Solution 1 or 2, including or excluding ouabain (Sigma Chemical Corp.) or ethacrynic acid (Merck Sharp and Dohme). Each range included 3–8 separate experiments. The solutions are saturated with O_2. All incubations are made (except for dialysis experiments) in special incubation flasks similar to those described by Burg and Orloff[26]. Dialyses were performed in cellophane bags (Visking Nojax Casing, 18 mm diameter). Cannulas are inserted tightly at one end of each bag in order to oxygenate the preparation. Before utilization, the cellophane bags have been raised to ebullition three times with distilled water, then they have been rinsed with the K^+-free medium. The final concentration of tubules in the incubation media ranged from 1 to 5 % (wet w/v). [^{3}H]methoxy inulin (New England Nuclear Corp.) is used to determine the amount of medium trapped for each sample. Labeled inulin is added to the suspension before centrifugation. After the lapse of time chosen for incubation has passed, the suspension is taken out into special tubes (Fig. 1) kept at 0.5 °C. The samples are immediately centrifugated at 0 °C for 5 min, at 15000 × g. The supernatant is decanted and the superficial layer of cells is removed by suction.

Analytical methods

The weights of tissular samples are determined before and after overnight dessication in an oven at 90 °C. The cellular residues are extracted by 3 % (w/v) trichloroacetic acid with a sonifier (Sonifier B-12 Branson, Conn., U.S.A.). The concentration of Na^+ and K^+ in the media and previously centrifugated tissular extracts are determined by flame photometry. Cl^- has been estimated, using the method of Schales[27]. ^{3}H is determined by means of a liquid scintillation spectrometer (Nuclear Chicago Mark 1) after the dissolution in Insta-gel (Packard) of the lipid supernatant of tissue extracts in 3 % trichloroacetic acid and of the 1/31 diluted media by 3 % trichloroacetic acid. ^{42}K (^{42}KCl, from C.E.A., Saclay, France) is determined in the

media and tissular extracts by means of a scintillation spectrometer (Nuclear gamma). When ^{3}H and ^{42}K are present simultaneously, at least 15 half-life times of ^{42}K were allowed to elapse before counting ^{3}H. Radioactive samples were counted a time sufficient to obtain less than 5 % counting error. The soluble proteins present in the incubation media are estimated by means the method of Lowry *et al.*[28].

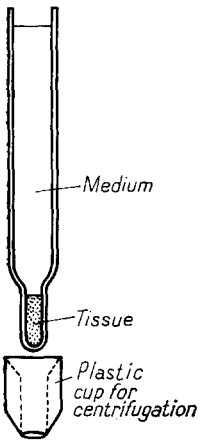

Fig. 1. Centrifuge tube for separation of tubules from suspension. The determination of the wet and dry weights, as well as the extraction of the cellular residue, are performed in these tubes previously weighed.

Results presentation

The following expressions have been used to compute the principal results. The cellular water content, $CW = WT - (DT + TM)/DT$ (kg cellular water/kg dry wt). The cellular ion content, $[I]_T = T_I - TM\ [I]_0/DT$ (mequiv/kg dry wt). The concentration of ions in cellular water $[I]_c = [I]_T/CW$ (mequiv/kg cellular water). For a given sample, WT is the wet weight, DT the dry weight, TM the weight of extracellular fluid. I is an ion, $[I]_0$ its concentration in the incubation media (mequiv/l) and T_I the whole amount of this ion (mequiv) in the sample. The rate of K^+ exchange is expressed as relative specific activity, *i.e.* specific activity of the tissue divided by the specific activity of the medium. The K^+ influx $[IMK^+]$ is given by the unsteady state formula[29]: $IMK^+ = ACW/DT \times SAm$, in which IMK^+ is K^+ influx (mequiv/kg dry wt). ACW is the ^{42}K radioactivity in the cellular water of the sample (cpm) and SAm the specific activity of the medium (cpm/mequiv K^+). The results given in the text, the tables and the figure show the mean $\pm$ S.D. of experimental values. The statistical significance of the differences between the groups has been evaluated with Student's *t* test.

RESULTS

Effects of temperature and medium K^+ on cell water and ion content of leached tubules (Table I)

The rewarming of leached tubules in a K^+-free medium induces a net loss of Na^+, Cl^- and water, whereas no net movement of K^+ is observed (column D, upper

part). The simultaneous increase of the temperature and $[K^+]_0$ induces a more important extrusion of Na^+, Cl^- and water and a net gain of K^+ (column E). The effect, only due to the increase of K^+ in the medium (column F), can be computed by abstracting from E value the effect due to the temperature increase in a K^+-free medium. The ratio of Na^+ extrusion to K^+ accumulation does not differ significantly from unity ($111.76 \pm 18.48/98.26 \pm 7.23$) $= 1.087 \pm 0.089$; simultaneously, the tubules lose 0.25 kg of water and 62 mequiv of Cl^-.

As shown by the results summarized in the lower part of Table I, the extrusion of water, Na^+ and Cl^- in a K^+-free medium does not change significantly the intracellular concentration of these two ions. Accordingly, the NaCl solution lost by tubules is isotonic to cellular fluid. On the other hand, tubules retain K^+ and the intracellular concentration of this cation increases in proportion to the decrease of cellular volume. This increase in $[K^+]_c$ is small and $[Na^+]_c + [K^+]_c + [Cl^-]_c$ is not significantly different at 0.5 °C or 28 °C. The increase in $[K^+]_c$ at 28 °C solely related to the uptake of external K^+ (column F) amounts to 69.57 ± 3.05 mequiv and is exactly balanced by the decrease of $[Na^+]_c + [Cl^-]_c$ amounting to $50.23 + 22.42 = 72.66 \pm 7.48$ mequiv/kg cellular water.

Accordingly, the extrusion of Na^+ (with Cl^- and water) observed in a K^+-free medium and the exchange of Na^+ for external K^+ occurred so that $[Na^+]_c + [K^+]_c + [Cl^-]_c$ remains constant.

Na^+ efflux (together with Cl^- and water) into nominally K^+-free medium

The previous results with isolated kidney tubules are fundamentally in concordance with those previously reported from studies carried out on kidney cortex slices[15,19,20,22]. Two types of Na^+ extrusion by the leached tubules are observed (Table I); one consists chiefly of an exchange with K^+ of the medium (column F); the other occurs independently of any net movement of this cation (column D). Nevertheless, this latter type of Na^+ extrusion, so-called K^+-independent, occurs in fact in media containing small amounts of this cation.

In the present studies carried out on isolated kidney tubules, it was never possible to keep the incubation media free from K^+, notwithstanding the fact that K^+ cellular contents of the leached tubules are similar to those reported in the studies carried out on leached kidney slices[19,20,22]. The results of Table I show that after 50 min incubation in an initially K^+-free medium, $[K^+]_0$ for the leached tubules incubated at 0.5 °C is of 0.140 mM, and of 0.191 mM for those incubated at 28 °C. This difference in $[K^+]_0$ is highly significant ($P < 0.01$) and thus shows that the tubules incubated at 28 °C have lost more K^+ than those incubated at 0.5 °C.

In spite of this fact, K^+ contents are identical in these two situations. This is probably due to the fact that the cellular destruction is more important at 28 °C than at 0.5 °C. The tubules incubated at 0.5 °C lose in the medium 37.798 ± 4.290 g of soluble proteins/kg dry wt ($n = 6$) whereas at 28 °C the loss amounts to 49.59 ± 6.65 ($n = 5$). This difference is significant ($P < 0.01$) and the ratio $[K^+]_0$/lost proteins is of 0.037 ± 0.004 at 0.5 °C; which is not significantly different from the ratio of 0.038 ± 0.004 obtained at 28 °C. In a nominally K^+-free medium, the concentration gradient of this cation between the tubules and the medium is 415.24 ± 16.52 (58.4/0.140) at 0.5 °C and 409.03 ± 20.97 (78.08/0.191) at 28 °C (Table I). The difference is not significant.

TABLE I

EFFECT OF REWARMING AND EXTERNAL K^+ ON K^+ UPTAKE AND Na^+, Cl^-, WATER EXTRUSION IN SEPARATED RENAL TUBULES

Tubules were leached for 60 min at 0.5 °C in K^+-free medium, then incubated for 50 min in Conditions A, B and C. Each value represents the mean ± S.D. of 6 experiments.

	Conditions					
	A *0.5 °C* ($[K^+]_0 = 0$)	*B* *28 °C* ($[K^+]_0 = 0$)	*C* *28 °C* ($[K^+]_0 = 5$ *mequiv/l*)	*D* (*B*−*A*)	*E* (*C*−*A*)	*F* (*C*−*B*)
CW (kg water/kg dry wt)	2.57 ± 0.10	1.94 ± 0.14	1.69 ± 0.10	− 0.63 ± 0.10	− 0.88 ± 0.10	− 0.25 ± 0.14
$[Na^+]_T$ (mequiv/kg dry wt)	285.44 ± 5.77	207.54 ± 18.48	95.78 ± 10.80	−78.40 ± 5.39	−190.16 ± 5.39	−111.76 ± 18.48
$[K^+]_T$ (mequiv/kg dry wt)	150.45 ± 2.91	151.51 ± 7.23	249.77 ± 4.64	2.12 ± 2.09	99.31 ± 2.91	98.26 ± 7.23
$[Cl^-]_T$ (mequiv/kg dry wt)	290.25 ± 8.67	204.47 ± 16.34	141.71 ± 9.38	−85.86 ± 8.62	−148.62 ± 8.62	− 62.76 ± 16.34
$[Na^+]_c$ (mequiv/kg cellular water)	110.81 ± 3.31	106.71 ± 4.17	56.48 ± 5.93	− 4.43 ± 2.77	− 54.33 ± 3.32	− 50.23 ± 4.17
$[K^+]_c$ (mequiv/kg cellular water)	58.40 ± 1.32	78.08 ± 3.04	147.65 ± 7.41	19.68 ± 1.32	89.25 ± 1.33	69.57 ± 3.05
$[Cl^-]_c$ (mequiv/kg cellular water)	112.28 ± 5.78	105.27 ± 6.08	82.84 ± 2.18	− 7.08 ± 5.67	− 29.43 ± 5.78	− 22.42 ± 6.08
$[Na^+]_c + [K^+]_c + [Cl^-]_c$ (mequiv/kg cellular water)	281.49 ± 9.71	290.06 ± 8.93	286.97 ± 7.21			
$[K^+]°$, final concn (mequiv/l)	0.140 ± 0.006	0.191 ± 0.003	4.880 ± 0.060			

Exchange of ^{42}K

The rate of exchange of tissular K^+ with that passively lost by the leached tubules incubated at 0.5 °C and at 28 °C in originally K^+-free medium (except for a tracer amount of ^{42}K: 0.018 mM) has been studied.

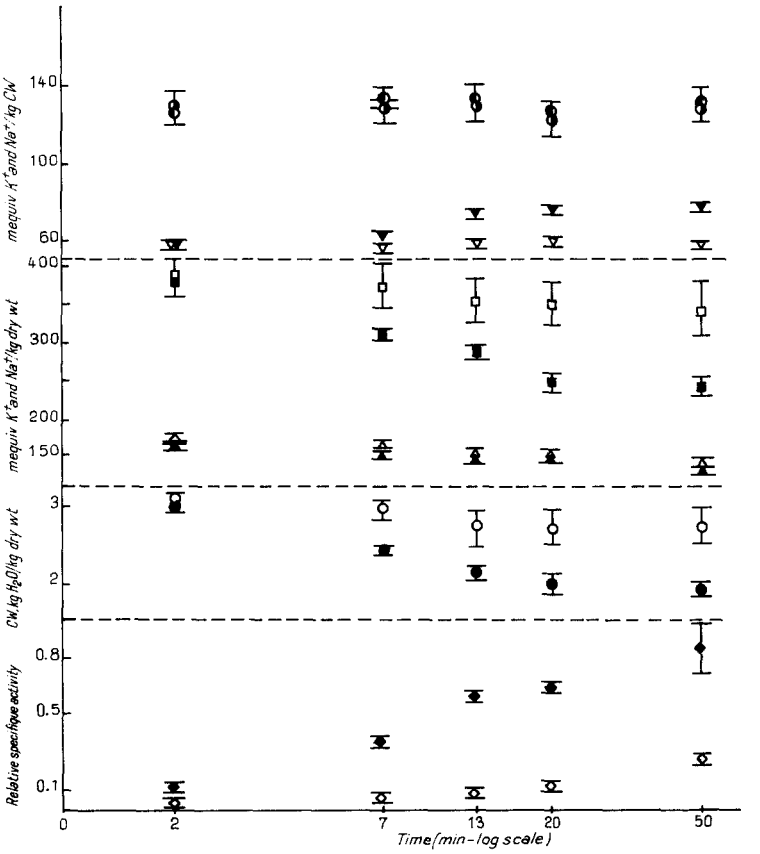

Fig. 2. Effect of temperature on ^{42}K exchange and electrolyte and water composition of separated renal tubules. Leached tubules have been reincubated at 0.5 °C and at 28 °C in a K^+-free medium (except tracer amount of ^{42}KCl). Relative specific activity of the tissue was calculated for each sample as the ratio of the specific activity of the tissue to that of the medium. Each point is the mean ± S.D. of 3 experiments. Incubation at 0.5 °C: ◇, rel. spec. act.; ○, water; □, Na^+; △, K^+ (per kg dry wt); ◑, Na^+; ▽, K^+ (per kg cellular water). Incubation at 28 °C: ◆, rel. spec. act.; ●, H_2O; ■, Na^+; ▲, K^+ (per kg dry wt); ◐, Na^+; ▼, K^+ (per kg cellular water).

As is shown on Fig. 2, during the whole experimental period, at 0.5 °C as well as at 28 °C, the variation of tissular K^+ content did not exceed 20%. The rise in temperature in the incubation medium from 0.5 °C to 28 °C induces an important increase of the K^+ exchange velocity. The increase of K^+ influx at 28 °C is coupled to a net loss of Na^+ and water. Neither the K^+ tissular content, nor Na^+ and K^+ concentration gradients, are significantly modified in concordance with the results previously obtained (Table I).

TABLE II

EFFECT OF DIALYSIS ON CELL WATER AND ION CONTENT OF SEPARATED RENAL TUBULES

The leached tubules have been incubated for 50 min in K^+-free medium at 0.5 °C (Column A), or in the same medium during the same time at 28 °C in cellophane bags, in the absence of medium outside the bags (Column B), or with an external circulation of K^+-free medium (Column C). Each value represents the mean ± S.D. of 6 experiments.

	Conditions					
	A 0.5 °C	*B* 28 °C	*C* 28 °C + *dialysis*	*D* (*B* − *A*)	*E* (*C* − *A*)	*F* (*C* − *B*)
CW (kg water/kg dry wt)	2.40 ± 0.07	1.75 ± 0.05	1.89 ± 0.04	− 0.64 ± 0.05	− 0.50 ± 0.05	0.13 ± 0.04
$[Na^+]_T$ (mequiv/kg dry wt)	280.37 ± 10.81	199.59 ± 5.65	245.32 ± 9.17	−80.79 ± 10.81	−35.16 ± 10.73	45.73 ± 5.65
$[K^+]_T$ (mequiv/kg dry wt)	155.12 ± 2.01	149.56 ± 1.03	123.99 ± 1.91	− 5.56 ± 1.97	−31.12 ± 1.98	−25.57 ± 0.99
$[Cl^-]_T$ (mequiv/kg dry wt)	247.48 ± 8.07	188.54 ± 14.05	206.09 ± 12.24	−60.66 ± 5.16	−41.39 ± 8.05	17.55 ± 12.23
$[Na^+]_c$ (mequiv/kg cellular water)	116.79 ± 4.05	113.66 ± 2.59	129.61 ± 4.77	− 3.84 ± 3.21	12.82 ± 4.03	15.94 ± 2.59
$[K^+]_c$ (mequiv/kg cellular water)	64.62 ± 1.07	85.26 ± 2.40	65.52 ± 1.87	20.64 ± 1.04	0.90 ± 1.03	−19.68 ± 2.47
$[Cl^-]_c$ (mequiv/kg cellular water)	103.11 ± 3.82	107.31 ± 6.87	108.86 ± 5.97	4.20 ± 3.81	5.75 ± 3.80	1.55 ± 6.86
$[K^+]_0$, final concn (mequiv/l)	0.254 ± 0.003	0.323 ± 0.003	0.221 ± 0.003			

Reduction of the medium K^+ concentration by dialysis of tubules suspensions

The great sensitivity of the K^+ exchange rate to temperature raises the question whether the net Na^+ efflux observed while rewarming the tubules in a nominally K^+-free medium represents an extrusion of Na^+ in exchange for K^+ traces present in the media. In order to test this hypothesis, the efflux of Na^+ has been determined while the recapture of K^+ passively lost by the leached tubules was partially prevented by dialysis of tubules suspensions against a K^+-free medium.

The effect of dialysis on $[K^+]_0$, cell water and ions content of the tubules, are summarized in Table II. The leached tubules incubated at 28 °C in dialysis bags (without any medium outside) lose 0.64 kg water, 80 mequiv Na^+, 60 mequiv Cl^- and 5.56 mequiv K^+/kg dry wt (column D). The intracellular K^+ concentration (mequiv/kg cellular water) increases by 32 % ($P < 0.001$) whereas those of Na^+ and Cl^- are not significantly affected. Save the low K^+ loss that is observed, the previous results are worthy of comparison with those obtained when the tubules were immersed in special incubation flasks (compare columns D in Tables I and II).

When dialysis bags containing the tubules are continually flowed by the K^+-free medium, the final $[K^+]_0$ is reduced by about 30 % and the tubules lose 0.50 kg of water, 35 mequiv of Na^+, 41 mequiv of Cl^- and 31 mequiv of K^+ (column E). Column F shows the results presented as differences between the dialysed tubules and control tubules run in parallel. These results indicate that the dialysis induced a tissular K^+ depletion of 25 mequiv ($P < 0.001$) and a significant gain of 0.13 kg water, 45 mequiv of Na^+ and 17 mequiv of Cl^- ($P < 0.01$, $P < 0.0005$, $P < 0.01$ respectively). The dialysis inhibited more the efflux of Na^+ (56 %) than that of Cl^- (29 %) or of water (20 %).

It was previously observed (Table I, column F, lower part) that $[K^+]_0$ increases at a given temperature, and consequently the increase of $[K^+]_c$ induced inverse changes for $[Na^+]_c$ and $[Cl^-]_c$ concentrations in such a way that the sum $[Na^+]_c + [K^+]_c + [Cl^-]_c$ remained constant. The dialysis induces the same phenomenon but in an opposite direction; the reduction of $[K^+]_c$ is 19.68 ± 2.67 mequiv, and this decrease is essentially balanced by the increase of $[Na^+]_c$ which is 15.94 ± 2.59 mequiv (column F, lower part).

These results show that at least a fraction of the extrusion of Na^+, Cl^- and water observed while rewarming the leached tubules in a nominally K^+-free medium needs a movement of K^+ into the cells.

Action of ouabain and ethacrynic acid

In order to know whether the extrusion of Na^+, partially persisting when $[K^+]_0$ and $[K^+]_T$ are reduced by dialysis, can nevertheless be due to an expulsion of Na^+ in exchange of external K^+, it was necessary to measure the efflux of Na^+ by the leached tubules incubated at 28 °C when medium K^+, whatever its concentration, is not available for the tubules. These experiments are allowed by inhibitors of (Na^+–K^+)-ATPase, such as ouabain and ethacrynic acid. They must prevent the recapture of the escaped K^+, and so induce an important K^+ loss. If the extrusion of Na^+ is in fact coupled to the reaccumulation of K^+, passively lost by the tubules, one must expect that ouabain and ethacrynic acid inhibit completely Na^+ extrusion.

The results (Table III) show that the tubules previously leached at 0.5 °C and incubated at 28 °C in an originally K^+-free medium with ouabain gain 127.92 ± 26.27

TABLE III

EFFECT OF INHIBITORS ON ELECTROLYTE AND WATER CONTENT OF SEPARATED RENAL TUBULES, REINCUBATED AT 28 °C IN A K^+-FREE MEDIUM

The leached tubules have been incubated for 50 min in the Conditions A, B, C and D undermentionned. Each value represents the mean ± S.D. of 6 experiments.

	Conditions						
	A 0.5 °C ($[K^+]_0 = 0$)	*B* 28 °C ($[K^+]_0 = 0$)	*C* 28 °C ($[K^+]_0 = 0$) + *ouabain* (2 *mM*)	*D* 28 °C ($[K^+]_0 = 0$) + *ethacrynic acid* (1 *mM*)	*E* (*B*−*A*)	*F* (*C*−*B*)	*G* (*D*−*B*)
CW (kg water/kg dry wt)	2.59 ± 0.12	1.99 ± 0.12	2.68 ± 0.08	5.07 ± 0.15	−0.60 ± 0.13	0.68 ± 0.12	3.07 ± 0.12
$[Na^+]_T$ (mequiv/kg dry wt)	284.27 ± 7.70	221.30 ± 19.17	412.19 ± 26.27	781.10 ± 23.03	−62.97 ± 7.70	190.88 ± 29.18	559.79 ± 29.18
$[K^+]_T$ (mequiv/kg dry wt)	150.40 ± 2.92	151.52 ± 7.20	27.90 ± 1.43	25.34 ± 1.84	2.13 ± 2.10	−123.62 ± 7.23	−126.18 ± 7.23
$[Cl^-]_T$ (mequiv/kg dry wt)	289.46 ± 8.76	210.64 ± 15.57	286.80 ± 12.39	568.34 ± 13.69	−78.82 ± 8.76	75.99 ± 15.37	363.53 ± 25.93
$[Na^+]_c$ (mequiv/kg cellular water)	109.72 ± 3.16	110.45 ± 9.05	153.48 ± 6.10	154.02 ± 2.97	2.30 ± 2.05	43.03 ± 9.05	43.57 ± 9.05
$[K^+]_c$ (mequiv/kg cellular water)	58.09 ± 2.14	75.89 ± 2.74	10.39 ± 0.21	4.99 ± 0.34	17.20 ± 2.14	− 65.50 ± 2.74	−70.90 ± 2.74
$[Cl^-]_c$ (mequiv/kg cellular water)	111.80 ± 5.90	105.45 ± 5.98	106.90 ± 4.72	112.15 ± 5.30	− 6.35 ± 5.90	4.90 ± 3.07	7.83 ± 4.01
$[Na^+]_c + [K^+]_c + [Cl^-]_c$ (mequiv/kg cellular water)	279.61 ± 10.17	291.79 ± 11.85	270.77 ± 7.31	271.16 ± 7.73			

mequiv of Na^+ and lose 122.50 ± 1.42 mequiv of K^+/kg dry wt, whereas no significant movement of water nor Cl^- occurs. The mean of the ratios Na^+ gain/K^+ loss in each individual experiment is not significantly different from unity. The effect of ouabain alone (column F) is calculated by subtracting the effect of rewarming from the action of the inhibitor at 28 °C. Such a calculation yields a gain of 0.68 kg of water and 75 mequiv of Cl^-/kg dry wt. These gains are not significantly different from the losses of water and Cl^- induced by rewarming (column E). Ouabain also induces a loss of 123 mequiv of K^+ and a gain of 190 mequiv of Na^+.

The tubules incubated at 28 °C with ethacrynic acid gain (per kg dry wt) 2.48 ± 0.13 kg of water, 496.90 ± 7.70 mequiv of Na^+, 278.86 ± 8.75 mequiv of Cl^- (Table III). The net loss of K^+ due to ethacrynic acid alone (column G) is of 126.18 ± 7.23 mequiv, and is not significantly different from that induced by ouabain, which is of 123.62 ± 7.23 mequiv. On the other hand, the inhibitory action of ethacrynic acid on the transport of Na^+, Cl^- and water is far beyond that of ouabain. Ethacrynic acid induces, in relation to ouabain (column D *minus* column C) a supplementary gain of 2.38 ± 0.12 kg of water, 368.90 ± 29.17 mequiv of Na^+ and 281.54 ± 15.37 mequiv of Cl^-.

The effects of ouabain and ethacrynic acid on the intracellular ion concentrations are summarized in the lower part of Table III. Ouabain and ethacrynic acid induce the same increase of intracellular Na^+ concentration (column F and G respectively) and abolishes the concentration gradients between tubules and medium. In the presence of ouabain and ethacrynic acid, $[K^+]_c$ decreases to 10.39 and 4.99 mequiv/kg cellular water respectively, with a final external K^+ concentration of 0.87 mM (ouabain) and 1.04 mM (ethacrynic acid). The $[Na^+]_c$ increase is smaller than the $[K^+]_c$ decrease, accordingly the sum $[Na^+]_c + [Cl^-]_c + [K^+]_c$ is significantly ($P < 0.05$) diminished by both inhibitors as compared to control values. This decrease, however, is small and amounts to 7.07 ± 4.43 % (ouabain) and to 7.01 ± 1.90 % (ethacrynic acid).

Effects of ouabain and ethacrynic acid in a medium containing 5 mM $[K^+]_0$

The leached tubules reincubated in a medium containing 5 mM $[K^+]_0$ (Table IV) respond in a similar way to the action of ouabain and ethacrynic acid as the tubules incubated in a nominally K^+-free medium, except for a very small recovery of cellular water amounting to 0.16 ± 0.08 kg of water/kg dry wt which occurs in the presence of ouabain. The extrusion of Na^+ is abolished by both inhibitors. Ethacrynic acid induces, as compared to ouabain, an important supplementary gain of water and Na^+.

Time course of effects of ethacrynic acid on K^+ influx (IMK^+) and ion and water content

Table V shows the inhibitory effect of ethacrynic acid in relation with time on IMK^+ and the water and ions tissular contents.

The tubules, after 20 min incubation with ethacrynic acid, gain 0.32 ± 0.18 kg of water, 174.07 ± 29.59 mequiv of Na^+ and lose 162.54 ± 7.54 mequiv of K^+/kg dry wt. During this lapse of time, IMK^+ decreases by 153.98 ± 6.42 mequiv/kg dry wt which is not significantly different from the K^+ loss that occurred at the same time. Since the change in the K^+ influx is related to net movement of K^+, it is unlikely that the observed uptake of isotope may occur by exchange diffusion, since no net movement is induced by this mechanism. Thus, after 20 min incubation, ethacrynic acid mimicked the inhibitory action of ouabain (see Table III and IV).

TABLE IV

EFFECT OF OUABAIN AND ETHACRYNIC ACID ON ELECTROLYTE AND WATER CONTENT OF SEPARATED RENAL TUBULES REINCUBATED AT 28 °C WITH 5 mM $[K^+]_0$

Tubules treated as in Table I. Each value represents the mean ± S.D. of 6 experiments.

	Conditions		
	0.5 °C ($[K^+]_0 = 0$)	*28 °C* ($[K^+]_0 = 5$ *mequiv/l*) + *ouabain* (*2 mM*)	*28 °C* ($[K^+]_0 = 5$ *mequiv/l*) + *ethacrynic acid* (*1 mM*)
CW (kg water/kg dry wt)	2.60 ± 0.08	2.44 ± 0.07	3.77 ± 0.25
$[Na^+]_T$ (mequiv/kg dry wt)	347.13 ± 22.55	381.24 ± 16.74	595.58 ± 62.38
$[K^+]_T$ (mequiv/kg dry wt)	174.56 ± 6.20	43.43 ± 3.32	56.59 ± 3.45
$[Na^+]_c$ (mequiv/kg cellular water)	132.95 ± 6.50	156.47 ± 4.90	158.64 ± 20.18
$[K^+]_c$ (mequiv/kg cellular water)	66.93 ± 3.06	17.85 ± 1.67	15.08 ± 1.30

TABLE V

TIME COURSE OF ACTION OF ETHACRYNIC ACID ON K^+ INFLUX (IMK^+) AND IONS AND WATER CONTENTS OF SEPARATED RENAL TUBULES

Unleached tubules have been incubated at 28 °C in a medium with 5 mM $[K^+]_0$. Values are mean ± S.D. Figures in parentheses represent numbers of experiments.

Duration (*min*)	*CW* (*kg water/kg dry wt*)	$[Na^+]_T$ (*mequiv/kg dry wt*)	$[K^+]_T$	IMK^+
Ethacrynic acid (*1 mM*)				
20 (5)	2.51 ± 0.19	351.99 ± 29.61	91.33 ± 7.35	56.62 ± 6.42
50 (5)	4.39 ± 0.22	594.10 ± 24.69	70.96 ± 2.38	60.40 ± 7.43
65 (6)	3.99 ± 0.24	603.38 ± 40.92		
Controls				
20 (5)	1.90 ± 0.17	176.94 ± 21.20	253.88 ± 16.26	210.60 ± 17.01
50 (5)	2.18 ± 0.04	158.87 ± 7.67	252.97 ± 11.60	243.70 ± 17.61
65 (6)	1.89 ± 0.21	168.52 ± 13.52		

In the presence of ethacrynic acid, 76 % of the total water gain occurs between the 20th and 50th minute, and during this lapse of time, the net gain of Na^+ is of 242.02 ± 24.74 mequiv and is far more important than the loss of K^+, which is only of 20.36 ± 2.38 mequiv/kg dry wt. The possibility that a toxic effect of ethacrynic acid on kidney tubule cells explains the important gain of water, Na^+ and Cl^-, has been considered, for it has been previously claimed that ethacrynic acid increases the passive influx of Na^+ in rat erythrocytes[30] and uterine norns[31]. In order to test this hypothesis, the loss of soluble proteins by the tubules in the incubation media has been determined. After 50 min incubation, the tubules treated with 1 mM of ethacrynic acid, lose 59.23 ± 6.47 g of proteins/kg dry wt, ($n = 6$), whereas the control tubules lose significantly less proteins, 43.26 ± 7.01 ($n = 6$, $P < 0.05$).

DISCUSSION

The results of this study demonstrate that the same system is responsible for the Na^+–K^+ exchange in physiological situations and for Na^+ extrusion (with Cl^- and water) in a nominally K^+-free medium. Every time that recapture of K^+ lost passively by leached tubules incubated in an initially K^+-free medium is prevented, either by dialysis or ouabain, or ethacrynic acid, the efflux of Na^+ is inhibited. Some of these results are apparently at variance with previously reported ones on kidney slices and deserve comment.

Persistence of Na^+–K^+ exchange into nominally K^+-free medium. Effect of temperature, ouabain and dialysis

The fact that the medium is nominally K^+-free still does not eliminate the possibility that external K^+ can interfere with the extrusion of Na^+. Indeed the passive K^+ loss by the tubules introduces enough K^+ in the medium to make this exchange possible (Tables I and II). In the experiments carried out on leached kidney cortex slices, a net loss of K^+ was observed during their rewarming but no value of external K^+ was given[15,20,22,24]. It is nevertheless obvious that under these conditions K^+ external concentration could not be zero. As is shown on Fig. 2, the suppression of the inhibitory effect of temperature on K^+ influx may indicate that the net extrusion of Na^+ observed at 28 °C needs, as well as the Na^+–K^+ exchange, the simultaneous movement of K^+ towards the inside of cells. When external K^+ is not available to the cells in the presence of ouabain, Na^+–K^+ exchange is inhibited and the extrusion of Cl^- and water is completely blocked (Table III). In dialysis experiments partial removal of medium K^+ induces similar effects to those of ouabain. When dialysis induces an inhibition of K^+ captation (column F, Table II) amounting to 20.71 ± 0.99 % of the ouabain induced one (column F, Table III), the gain of Na^+, Cl^- and water are respectively 24.30 ± 3.66 %, 26.03 ± 26.08 %, 20.90 ± 9.87 % of those induced by ouabain. The fairly total inhibition by ouabain of Na^+ extrusion from hamster and squirrel leached kidney slices has been previously reported by Willis[24]. The extrusion of water in these conditions, however, is not totally inhibited (values not indicated). In Willis' experiments, K^+ tissular content in the presence of ouabain is close to that reported in the present work (column C, Table III). In studies carried out on kidney slices where Na^+ extrusion persists in the presence of ouabain[15,18,20–22], the tissular K^+ contents are twice or three times higher than those observed by Willis[24] and in the present studies, which suggest that K^+ influx was not maximally inhibited.

The efflux of Na^+ and Cl^- in equal amounts, as well as the water loss, observed while rewarming leached slices in a K^+-free medium, has been ascribed by Whittembury and Proverbio[22] to the activity of a Na^+ pump, K^+-independent, insensitive to ouabain, but sensitive to ethacrynic acid: "Pump A". However, these authors observe, while rewarming the slices with ouabain, a net loss of K^+ and a lower inhibition of Cl^- extrusion than that of Na^+. In order to make these results fit with the characteristics of "Pump A", Whittembury and Proverbio[22] propose adding the amount of Na^+ extruded from the slices to the amount which has been exchanged with K^+. The studies reported here also disclose a higher Na^+ than Cl^- net gain, when recapture of K^+ passively lost by tubules is prevented either partially by dialysis (column F,

Table II) or completely by ouabain (column F, Table III). This probably is due to the fact that, besides its specific inhibition of water, Na^+ and Cl^- efflux, ouabain also dissipated the concentration gradients of ions between the tubules and the medium. Obviously this latter action of ouabain will not be effective on H_2O nor on Cl^- transport since $[Cl^-]_c$ is already identical at 0.5 °C with $[Cl^-]_0$ in the absence of the drug. In keeping with this view is the observation that net losses of water and Cl^- in a medium devoid of ouabain (column F, Table III), are equal to the gain of these elements in the presence of the drug (column F). The net gain of Na^+ in the presence of ouabain (column F, Table III) exceeds by 127.92 ± 26.27 mequiv/kg dry wt the amount of Na^+ extruded in the absence of the drug (column F). This additional gain of Na^+ is not significantly different from that induced by the suppression of the concentration gradient: in the presence of ouabain (Table III), the cellular water content (kg/kg dry wt) is 2.68 and $[Na^+]_c$ increases about 43.03 mequiv/kg cellular water which gives a net gain of $2.68 \times 43.03 = 115.40 \pm 25.70$ mequiv/kg dry wt.

The "cell contraction" hypothesis proposed by Kleinzeller and Knotova[15] to explain the extrusion of electrolytes and water in a K^+-free medium cannot meet the conditions of extrusion of Na^+, Cl^- and water observed here. The results reported here demonstrate that the tubules do lose an isotonic NaCl solution but that they keep most of their K^+. It is therefore difficult to understand how a non specific cellular concentration would be able to induce the extrusion of Na^+, Cl^- and water, but not that of K^+.

Inhibition by ouabain of Na^+ extrusion in a medium with K^+

Willis[24] observed that while the extrusion of Na^+ is nearly blocked by ouabain in a K^+-free medium, the inhibition is diminished by one half in a K^+ medium. No such a protective action of K^+ towards ouabain was observed in present studies (Table IV). This suggests again that the extrusion of Na^+ which occurs in a nominally K^+-free medium, or in a medium with K^+, is dependent on the activity of the coupled Na^+–K^+ pump sensitive to ouabain. No alternative pathway could be observed in the present studies. However, it has been reported that Li^+, Rb^+ and Cs^+ can substitute for K^+ in stimulating ATPase[32,33] and that in the Li^+ medium renal cells are able to maintain volume as in the Na^+ one[34].

Biphasic action of ethacrynic acid

It has been observed, in this study, that ethacrynic acid has an early action comparable to that of ouabain, and later inhibits Na^+ and water transport more intensely than ouabain. It is doubtful whether ethacrynic acid has a selective action on a Na^+-pump regulating the cellular volume. Indeed the amount of Na^+, water and Cl^- gained by the tubules in the presence of this drug by far exceeds the amount of these elements excluded in its absence. On the other hand, the inhibitory action of ethacrynic acid on the Na^+–K^+ pump, comparable to that of ouabain, is sufficient to explain the inhibition of Na^+, Cl^- and water extrusion. As Whittembury and Proverbio[22] do not observe any inhibitory effect of ethacrynic acid on the coupled pump but only the effects of this drug on Na^+, Cl^- and water transport, they logically draw the conclusion that ethacrynic acid inhibits specifically the "pump A", which is precisely involved in the extrusion of these elements. As in our experimental con-

ditions "pump A" does not exist, it is obvious that any effects of ethacrynic acid on this hypothetical "pump A" will be added to its inhibitory effects on the Na^+–K^+ pump. Such a biphasic action of ethacrynic acid was previously reported by Daniel *et al.*[31] on rat uterine horns and by Macknight[35] on rat kidney cortex slices.

In these studies, changes in tissue composition mainly represent changes in composition of proximal tubules, which constitute approximately 80 % of tubular suspensions[6]. K^+ changes must be due mainly to modifications of the slow K^+ compartment, located chiefly in proximal tubules[36] and representing about 95 % of total tissular K^+[37].

In conclusion the present results show that the extrusion of Na^+, Cl^- and water in a nominally K^+-free medium and the Na^+–K^+ exchange are induced by the same transport system which activity is regulated in some manner by the cellular electrolyte composition. Whatever the experimental conditions may be there is always a rough reciprocal relationship between the intracellular concentration of Na^+, Cl^- and that of K^+. The characteristics of the extrusion of Na^+ (with Cl^- and water) in a nominally K^+-free medium, or in a medium with K^+, can be considered as identical, if one presumes that the keeping of this relation by the tubules regulates the transport of the ions and water. Holding to that view, it is obvious that, in nominally K^+-free solutions the solitary movement of K^+ on the carrier towards the inside of cell without net gain cannot restore anything more that the normal cell volume through a loss of isotonic NaCl solution from the cell. On the other hand, in the presence of a sufficient amount of K^+ in the medium, the uptake of K^+ by the tubules yields a net gain and the net extrusion of Na^+ and Cl^- that follows exactly balances the increase in $[K^+]_c$.

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REFERENCES

1 I. M. Glynn, *J. Physiol. London*, 134 (1956) 278.
2 R. L. Post and P. C. Jolly, *Biochim. Biophys. Acta*, 25 (1957) 118.
3 V. Koefoed-Johnson and H. H. Ussing, *Acta Physiol. Scand.*, 42 (1958) 298.
4 J. Orloff and M. B. Burg, *Am. J. Physiol.*, 199 (1960) 49.
5 D. C. Tosteson and J. F. Hoffman, *J. Gen. Physiol.*, 44 (1960) 169.
6 M. B. Burg and J. Orloff, *Am. J. Physiol.*, 211 (1966) 1005.
7 R. L. Post, C. R. Merritt, C. R. Kinsolving and C. D. Albright, *J. Biol. Chem.*, 235 (1960) 1796.
8 E. T. Dunham and I. M. Glynn, *J. Physiol. London*, 156 (1961) 274.
9 R. Whittam and K. P. Wheeler, *Biochim. Biophys. Acta*, 51 (1961) 622.
10 J. S. Charnock and R. L. Post, *Aust. J. Exp. Biol. Med. Sci.*, 41 (1963) 547.
11 I. M. Glynn, *Pharmacol. Rev.*, 16 (1964) 381.
12 J. C. Skou, *Physiol. Rev.*, 45 (1965) 596.
13 A. I. Katz and F. H. Epstein, *Israel J. Med. Sci.*, 3 (1967) 155.
14 A. Kleinzeller, in A. Kleinzeller and A. Kotyk, *Membrane transport and Metabolism*, Academic Press Inc., New York, 1961, p. 527.
15 A. Kleinzeller and A. Knotova, *J. Physiol. London*, 175 (1964) 172.
16 G. Whittembury, *J. Gen. Physiol.*, 48 (1965) 699.

17 G. Whittembury, *Acta Cient. Venez.*, 16 (1965) 140.
18 G. Whittembury, *Abstr. 2nd Int. Biophys. Congr., Vienna, 1966*, No. 309.
19 J. S. Willis, *J. Gen. Physiol.*, 49 (1966) 1221.
20 A. D. C. Macknight, *Biochim. Biophys. Acta*, 150 (1968) 263.
21 G. Whittembury, *J. Gen. Physiol.*, 51 (1968) 303 S.
22 G. Whittembury and F. Proverbio, *Pflügers Arch.*, 316 (1970) 1.
23 F. Proverbio, J. W. L. Robinson and G. Whittembury, *Biochim. Biophys. Acta*, 211 (1970) 327.
24 J. S. Willis, *Biochim. Biophys. Acta*, 163 (1968) 516.
25 M. B. Burg and J. Orloff, *Am. J. Physiol.*, 205 (1963) 139.
26 M. B. Burg and J. Orloff, *Am. J. Physiol.*, 203 (1962) 327.
27 O. Schales, in M. Reiner, *Standard Methods of Clinical Chemistry*, Vol. 1, Acad. Press, New York, 1953, p. 37.
28 O.H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
29 D. C. Tosteson and J. S. Robertson, *J. Cell Comp. Physiol.*, 47 (1956) 147.
30 J. F. Hoffman and F. M. Kregenow, *Ann. N.Y. Acad. Sci.*, 137 (1966) 566.
31 E. E. Daniel, A. M. Kidway, K. Robinson, D. Freeman and S. Fair, *J. Pharmacol. Exp. Ther.*, 176 (1971) 563.
32 R. Whittam and M. E. Ager, *Biochem. J.*, 93 (1964) 337.
33 J. S. Willis and L. S. T. Fang, *Biochim. Biophys. Acta*, 219 (1970) 486.
34 A. Kleinzeller, D. A. Ausiello, J. A. Almendares and A. H. Davis, *Biochim. Biophys. Acta*, 211 (1970) 293.
35 A. D. C. Macknight, *Biochim. Biophys. Acta*, 173 (1969) 223.
36 M. B. Burg and M. Abramow, *Am. J. Physiol.*, 211 (1966) 1011.
37 M. B. Burg, E. F. Grollman and J. Orloff, *Am. J. Physiol.*, 206 (1964) 483.