J. Membrane Biol. 85, 1–24 (1985)

The Journal of
Membrane Biology

Morphological and Physiological Studies of Rat Kidney Cortex Slices Undergoing Isosmotic Swelling and its Reversal: A Possible Mechanism for Ouabain-Resistant Control of Cell Volume

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Summary. Slices of rat kidney cortex were induced to swell by preincubation at 1°C in an isotonic Ringer's solution, and their capacity to reverse swelling, by net extrusion of cellular water, was studied during subsequent incubation at 25°C. The recovery from swelling was prevented by the respiratory inhibitor, antimycin A. On the other hand, extrusion of water was little affected by ouabain. The extrusion of water continuing in the presence of ouabain (but not that in its absence) was significantly reduced when furosemide was added or when medium Cl^- was replaced by NO_3^- or I^-. There was substantial variability in the morphological appearance of cells within the cortical slices. Different segments of the nephron showed different structural changes during swelling and its reversal, the proximal tubules being most markedly affected. Proximal tubular cells of swollen slices showed disorganization of brush borders and expansion of their apical surfaces, and contained vesicles in their apical cytoplasm. Upon recovery at 25°C, the apical portions of these cells showed reversal of the expansion, but some apical vesicles remained. These vesicles were much more numerous after recovery in the presence of ouabain, but they were much reduced in numbers, or totally absent, when recovery took place in the presence of furosemide or absence of Cl^-, with or without ouabain. The vesicles seen in the presence of ouabain alone appeared to fuse with each other and with infoldings of the basolateral plasma membrane. Rather similar results were obtained with distal tubular cells in the slices. We suggest that volume regulation in the proximal and distal tubular cells proceeds by way of two mechanisms. The first consists of extrusion of water coupled to the ouabain-sensitive transport of Na^+ and K^+. The other proceeds by way of an ouabain-resistant entry of water into apical cytoplasmic vesicles, following furosemide-sensitive movements of Cl^- and Na^+; the vesicles then expel their contents by exocytosis at the basolateral cell borders.

Key Words volume regulation · kidney cortex slices · ouabain · vesicles in kidney cells · ions and water · morphology of kidney slices

Introduction

There is strong evidence that renal cortical tissue is able to control its cell volume *in vitro* by metabolism-dependent processes which are independent of the ouabain-sensitive transport of Na^+ and K^+ (Macknight, 1968; Whittembury & Proverbio, 1970; Kleinzeller, 1972). The evidence comes mainly from experiments in which slices of the tissue were incubated in isosmotic, saline media at 0 to 1°C, during which they took up water, Na^+ and Cl^-, and lost K^+. Upon restoration of metabolic activity at 25°C, the changes of water, Na^+ and Cl^- were largely reversed, even when the medium contained sufficient ouabain to inhibit reaccumulation of K^+. Mechanisms proposed to account for this reversal of swelling include an ouabain-resistant active transport of Na^+ (Proverbio et al., 1970; Whittembury & Proverbio, 1970) and a contractile system at the cell periphery (Rorive & Kleinzeller, 1972). However, experiments with a different experimental design led Cooke (1978*a*,*b*) to argue against a separate, ouabain-insensitive mechanism for volume regulation in kidney cortex.

The kidney cortex contains many different elements of the nephron, each of which has different transport and permeability properties. Even individual segments, such as the proximal and distal tubules, may be further subdivided according to histological and functional criteria (Valtin, 1977; Giebisch, 1978; Kriz, 1981). The responses of the several tubular elements to swelling at 1°C and subsequent recovery at 25°C may not be identical and initial morphological studies of tissue slices have suggested that this is indeed the case (van Rossum et al., 1981).

Slices of liver undergo similar changes of water and ionic content, with the restoration of volume by

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Table 1. Composition of rat kidney cortex slices before and after preincubation for 90 min at 1°C[a]

	Water content (kg/kg dry wt)			Ions (mmol/kg dry wt)	
	Total	Intracellular	Extracellular	K^+	Ca^{2+}
Fresh tissue	3.44 ± 0.06 (17)	—	—	297 ± 8 (13)	13.6 ± 1.5 (11)
90 min at 1°C	4.63 ± 0.14 (8)	3.17 ± 0.15 (8)	1.46 ± 0.11 (8)	122 ± 9 (8)	30.8 ± 1.2 (8)

[a] "Fresh" tissue was removed from the animal within 15 sec of death. Values are mean ± standard errors of the mean (number of observations). Further details as in Fig. 1 and Materials and Methods.

metabolic activity being partially resistant to ouabain. Morphological studies have been interpreted as suggesting that the ouabain-resistant water extrusion from liver cells is due to accumulation of water in cytoplasmic vesicles which eventually expel their contents into the bile canaliculi by exocytosis (Russo et al., 1977; van Rossum & Russo, 1981, 1984). The ouabain-sensitive fraction of water extrusion from hepatocytes presumably occurs in response to the ion movements driven by the Na^+- and K^+-dependent adenosine triphosphatase (Na,K-ATPase; Leaf, 1956).

This paper describes physiological and ultrastructural studies of renal cortical slices which provide evidence that volume regulation in this tissue also involves at least two different systems, analogous to the Na,K-ATPase-dependent mechanism and the vesicular mechanism proposed for liver.

Materials and Methods

Male, albino rats of a Wistar strain (Zivic Miller, Allison Park, Pa.) were killed by decapitation and their kidneys rapidly removed and placed in ice-cold Ringer's solution. The kidneys were cut sagitally into two, the medullae removed and the cortices cut into slices (0.2 to 0.3 mm thick) with a razor blade guided by a glass slide. All slices were pooled in a beaker containing 30 ml of the basic incubation medium (*see below*) at 1°C and, after 30 min, they were distributed in lots of approximately 100 mg wet wt into a series of either Erlenmeyer flasks (25 ml capacity) or Warburg manometric flasks (15 ml), each containing 3 ml of the medium under study, together with appropriate inhibitors. The manometer flasks contained 5 N NaOH in their center wells. The preincubation at 1°C was then continued for a further 60 min (making 90 min in all), during the last 10 min of which the manometer flasks were attached to manometers and both they and the Erlenmeyer flasks were gassed with O_2. After the 90 min, some of the flasks were removed and their slices collected for analysis. The remaining flasks were stoppered and transferred to a shaking water bath for incubation at 25 or 38°C for the times indicated in Results. Oxygen consumption was determined by standard manometry (Umbreit et al., 1954). The basic incubation medium contained (mM): 136 Na^+, 5.0 K^+, 1.0 Mg^{2+}, 1.2 Ca^{2+}, 143 Cl^-, 1.0 SO_4^{2-}, 2.0 phosphate, 10 Tris-(hydroxymethyl)amino methane (Tris), 10 acetate, 10 glucose and 0.5% (wt/vol) inulin. The pH was 7.4 and the solution was gassed with O_2. In experiments with chloride-free media, the appropriate salts (nitrate, iodide or gluconate) of Na^+ and K^+ were used instead of NaCl and KCl; $CaCl_2$ was replaced with $Ca(NO_3)_2$ in all cases and Tris was neutralized with acetic acid instead of HCl. In view of the chelating properties of gluconate, experiments with the gluconate medium were carried out with both 1.2 and 5.0 mM Ca^{2+}.

Slices were collected for analysis by tipping the flask contents onto a hardened filter paper (Whatman no. 54) held under suction on a sintered glass filter. After gentle blotting, the slices from each flask were divided into two groups and transferred to tared weighing bottles. One group of each pair was extracted with 10% (wt/vol) trichloroacetic acid and analyzed for insulin by a colorimetric method. The other group was dried overnight at 110°C, to determine water and dry wt, and the ions were then extracted with 0.1 N HNO_3. Cations were determined by atomic absorption spectrometry and Cl^- by electrometric titration with Ag^{2+}. The method for Cl^- also responded to I^- and the titration of extracts from slices incubated in media containing I^- (*see below*) therefore gave a measure of residual Cl^- *plus* I^-. Further details of the incubation and analytical methods are described by van Rossum (1972), van Rossum and Ernst (1978) and van Rossum et al. (1981).

For the morphological studies, one slice was removed from each incubation vessel prior to tipping the contents onto the filter. The slices were fixed at 4°C with 2% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) containing $CaCl_2$ (*see also* van Rossum et al., 1981). After post-fixation in 1% OsO_4 in phosphate buffer, the slices were cut into small pieces, dehydrated and embedded in Epon 812. For light microscopy, the embedded samples were sectioned with a glass knife and stained with azure II. For electron microscopy, they were sectioned with a diamond knife and stained with lead hydroxide (Russo et al., 1976). To ensure a thorough study of the samples, at least three tissue blocks from each original tissue slice were sectioned and examined by light and electron microscopy. The areas chosen for presentation in the figures are considered to be representative of the most frequently observed morphological appearance of the tissue in each of the incubation conditions.

Results

Water and Electrolyte Movements

Swelling at 1°C and Recovery at 25°C

During preincubation at 1°C, slices of kidney cortex underwent a considerable increase of water content

beyond that of fresh, unincubated tissue and this was accompanied by a loss of K^+ and gain of Ca^{2+} (Table 1), Na^+ and Cl^- (*not illustrated, but see* for example Mudge, 1951). These changes were unaffected by the presence of the inhibitors ouabain (2 mM), furosemide (1 to 5 mM) and antimycin A (1 to 20 μg/ml). The swelling and ionic movements were substantially reversed when the slices were subsequently incubated at 25°C. The extruded water was derived solely from the intracellular compartment, as estimated from the volume of distribution of inulin, and was completed in 15 min (Fig. 1). During incubation at 25°C, the intracellular K^+ content of the slices increased from 114 ± 3 to 244 ± 10 mmol/kg dry wt, a net change of +130 ± 8 mmol/kg. At the same time, the intracellular Na^+ decreased from 326 ± 16 to 63 ± 18 mmol/kg dry wt, or a change of −263 ± 16 mmol/kg, so that the net change of monovalent cations was −133 mmol/kg dry wt. At the same time, the Cl^- content changed by −171 ± 47 mmol/kg and the Ca^{2+} by −17 ± 2 mmol/kg dry wt. Each of the values quoted is the mean of seven observations.

The net extrusion of intracellular water at 25°C and the associated changes of ionic current were reduced when cellular respiration was inhibited by antimycin A. In Fig. 2*a*, the net extrusion of water is plotted against the rate of respiration persisting in the presence of varying concentrations of antimycin (1 to 20 μg/ml). Little inhibition of water extrusion was seen unless the O_2 consumption was reduced by more than 40%. By contrast, the inhibition of K^+ reaccumulation and the decline of slice ATP content were more closely proportional to the inhibition of respirations (Fig. 2*a*). The net extrusion of Na^+ (Fig. 2*b*) was more strongly inhibited by small degrees of respiratory inhibition than was water extrusion, but was less markedly affected than was K^+ uptake. For example, reduction of O_2 consumption by 40%, to approximately 6 μl/mg protein · hr^{-1} (the lowest rate before the marked inhibition of water extrusion ensued), led to 11% inhibition of water extrusion, 52% inhibition of K^+ uptake and 32% inhibition of Na^+ extrusion. The intermediate position Na^+ in this regard is to be anticipated if it is supposed that Na^+ extrusion results partly in an overall 1 : 1 net exchange for K^+ and partly in a loss of water and Cl^- (*see above*). The net extrusion of Na^+ in excess of the net K^+ accumulation is also shown in Fig. 2*b*; it is nearly equimolar with the Cl^- extruded and shows a relationship to the declining O_2 consumption which is similar to that shown by extrusion of water.

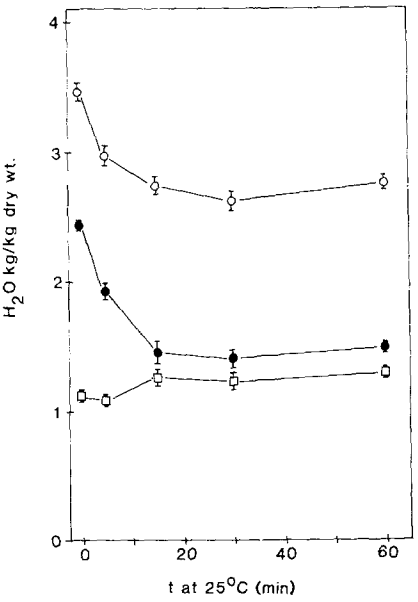


Fig. 1. Time-course of the extrusion of water from kidney cortex slices at 25°C, after preincubation at 1°C for 90 min. For preincubation at 1°C, the slices were initially incubated in two changes of 30 ml of the medium (*see* Materials and Methods, for composition); after 30 min at 1°C they were divided into groups of 100 to 150 mg wet wt, each of which was transferred to 3 ml medium in an Erlenmeyer flask (25 ml capacity). The flasks were gassed with O_2 during the last 10 min at 1°C, after which they were stoppered and transferred to a shaking water bath at 25°C. At the end of preincubation, and again after each of the incubation times indicated, the slices from 2 to 3 flasks were recovered by tipping the flask contents onto a hardened filter paper (Whatman no. 54) which was held under suction. Extracellular water was estimated from the volume of distribution of inulin and intracellular water was determined as the difference between total and extracellular water. Each point represents the mean ± standard error of the mean of seven observations. ○ Total water; ● intracellular water; □ extracellular water

Effect of Transport Inhibitors

In agreement with other work (e.g. Macknight, 1968; Kleinzeller, 1972), the loss of total and intracellular water during the recovery at 25°C in normal, Cl^--containing medium was not significantly reduced by ouabain at any concentration tested between 0.1 and 2.0 mM (e.g. Tables 2 and 3*a*), although K^+ accumulation was completely inhibited by 1 and 2 mM (third column, Table 3*b*). Experiments on the time course of these changes (*not shown*) indicate that a significant water extrusion occurred within 2.5 min at 25°C and that this was little affected by ouabain. However, ouabain did completely inhibit K^+ uptake at this early stage; e.g. control slices took up 40 ± 8 mmol K^+/kg dry wt in 2½ min; while the uptake in the presence of ouabain was 1 ± 11 mmol/kg.

Furosemide, which has been found to inhibit water extrusion from previously swollen slices of guinea pig kidney cortex (Pérez-González de la Manna et al., 1980), had no effect on water extrusion from rat slices in the absence of ouabain (Table 2; *cf.* van Rossum et al., 1981). However, inhibition

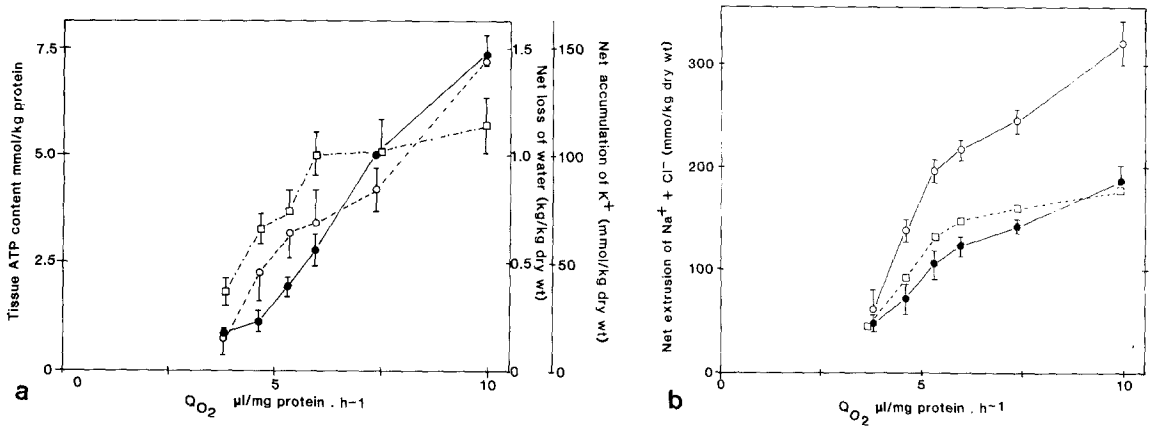


Fig. 2. Relation of (*a*) net extrusion of water, accumulation of K^+ and the content of ATP and (*b*) of Na^+ and Cl^- extrusion, to differing rates of respiration induced by antimycin A. General procedures as for Fig. 1, except that after 30 min at 1°C the slices were transferred to Warburg manometric flasks containing 3 ml of incubation medium with appropriate concentrations of antimycin A (1 to 20 µg/ml), for completion of incubation at 1°C. The medium in all flasks contained 0.5% (vol/vol) ethanol, the solvent for antimycin. The center wells of the flasks contained 0.2 ml 5 N KOH. Readings of O_2 consumption at 25°C were taken at 10-min intervals, after an initial 10-min equilibration period, using standard techniques (Umbreit et al., 1954). The net movements of water and ions, and the ATP contents are grouped according to the rate of respiration of the same sample of slices (*cf.* van Rossum, 1972). To facilitate comparisons, the net extrusions of water and ions are shown as positive values, in this figure. The points are the mean ± standard error of the mean. (*a*) —●— ATP content after 60 min at 25°C (n = 4); ●—□—● net loss of water during 60 min at 25°C (n = 8); --○-- net reaccumulation of K^+ during 60 min at 25°C (n = 8). (*b*) —○— net loss of Na^+ (n = 8); —●— net loss of Cl^- (n = 8); --□-- net loss of Na^+ *minus* net accumulation of K^+

Table 2. Effect of furosemide in the presence and absence of ouabain on the change of water content of kidney cortex slices during incubation at 25°C[a]

	Furosemide (mM)	Total water	Intracellular water
Content after 90 min at 1°C	—[b]	4.60 ± 0.11	2.89 ± 0.14
Then net change after 60 min at 25°C:			
Control	0	−1.30 ± 0.15	−1.27 ± 0.09
	2	−1.19 ± 0.09	−1.32 ± 0.10
	5	−1.21 ± 0.06	−1.34 ± 0.16
Ouabain (2 mM)	0	−1.14 ± 0.13	−1.09 ± 0.07
	2	−0.81 ± 0.11	−0.86 ± 0.12
	5	−0.66 ± 0.14[c]	−0.78 ± 0.10[c]

[a] Slices were incubated for 90 min at 1°C in Tris-buffered Ringer's solution, followed by 60 min at 25°C. The values at 1°C represent the content of water, those at 25°C represent the subsequent net changes of water content during the experimental incubation at 25°C. Ouabain and/or furosemide were added to the preincubation medium after the first 30 min at 1°C. Further details as in Fig. 1. Results are mean ± standard error of the mean of eight observations; water expressed as kg/kg dry wt.

[b] Mean of four values with no inhibitor and four values in the presence of 2 mM ouabain *plus* 5 mM furosemide. The inhibitors had no effect at 1°C.

[c] Significantly different from value without furosemide, P = 0.02 by Student's t-test.

was seen in the presence of ouabain; a concentration of 5 mM furosemide reduced the extrusion of intracellular water by approximately 30% (P = 0.02; Table 2). This concentration of furosemide will have caused a 20% reduction of the rate of respiration of the slices (van Rossum et al., 1981), but the finding that it failed to inhibit K^+ uptake (*not shown*) or water extrusion in the absence of ouabain (Table 2) suggests that a more specific effect was responsible for the inhibition of water loss in the presence of ouabain. The different effects of furosemide in the presence and absence of ouabain suggest that two

Table 3. Effect of medium Cl^- and its replacement by other anions on the net movements of (*a*) Intracellular water and (*b*) intracellular ions[a]

(*a*) Intracellular water (kg/kg dry wt)

Incubation	Principal anion of the medium:			
	Cl^-	NO_3^-	I^-	Gluconate
Content after 90 min at 1°C	2.85 ± 0.09 (16)	3.02 ± 0.11 (14)	3.11 ± 0.15 (8)	1.93 ± 0.06* (10)
Then net change after 60 min at 25°C:				
Control	−1.36 ± 0.11 (21)	−1.01 ± 0.12 (22)	−1.17 ± 0.14 (13)	−0.39 ± 0.14* (13)
Ouabain (2 mM)	−1.37 ± 0.07 (24)	−0.62 ± 0.07* (23)	−0.91 ± 0.19* (14)	−0.47 ± 0.10* (15)

* Significantly different from value in Cl^- medium, $P < 0.01$.

(*b*) Net changes of intracellular ions (mmol/kg dry wt)

Incubation for 60 min at 25°C	Principal anion	Na^+	Cl^-	K^+	(*n*)
Control	Cl^-	−336 ± 20	−194 ± 19	145 ± 17	(21)
	NO_3^-	−317 ± 20	−11 ± 3	105 ± 12	(22)
	I^-	−281 ± 21	−146 ± 24*	90 ± 14	(13)
	Gluconate	−151 ± 15	−8 ± 3	105 ± 10	(13)
Ouabain (2 mM)					
	Cl^-	−199 ± 15	−164 ± 12	4 ± 4	(24)
	NO_3^-	−155 ± 18	−14 ± 4	−17 ± 6	(23)
	I^-	−133 ± 29	−112 ± 21*	14 ± 10	(14)
	Gluconate	−47 ± 16	−12 ± 3	0 ± 5	(15)

* Iodide *plus* residual Cl^- (*see* Materials and Methods).

[a] In (*a*), values shown after preincubation for 90 min at 1°C are the intracellular water contents per kg dry wt, while values shown at 25°C are the subsequent net changes. In (*b*), only the net changes at 25°C are shown. Values represent mean ± standard error of the mean (number of observations).

different processes are involved in the recovery of cellular volume at 25°C, one inhibited by furosemide and one insensitive to furosemide but inhibited by ouabain. When furosemide is the only inhibitor present, the capacity of the latter system is apparently sufficient to compensate for the absence of the furosemide-sensitive activity.

Swelling and Recovery in Cl^--Free Media

The partial inhibition by furosemide suggested that a Cl^- transport system, or a Na^+-Cl^- co-transport, played a role in cellular volume regulation in the presence of ouabain, in much the same way as we have proposed for liver (van Rossum & Russo, 1984). Accordingly, we examined the effect of replacing Cl^- in the incubation medium by other anions.

During preincubation at 1°C, the intracellular water (Table 3*a*), Na^+ and K^+ contents (*not shown*) attained were virtually identical in media containing Cl^-, NO_3^- or I^- as the principal anion. The content of I^- *plus* residual Cl^- in slices incubated in the I^- medium was similar to the Cl^- content of slices in Cl^- medium. On the other hand, the swelling of cells (Table 3*a*) and entry of Na^+ was much reduced in the gluconate medium, presumably because the plasma membranes were impermeable to the gluconate anion. Upon subsequent incubation at 25°C in the absence of ouabain, the extrusion of water (Table 3*a*) and Na^+ (Table 3*b*) was practically unaffected by replacement of Cl^- by NO_3^- or I^-, although K^+ reaccumulation was somewhat less than in Cl^- medium (Table 3*b*). In the presence of ouabain, the extrusion of water was significantly less in the NO_3^- and I^- media than in Cl^- medium. The water extrusion which persisted in the chloride-free media with ouabain was accompanied by a net loss of Na^+ and, in I^- medium, by a loss of I^- *plus* residual Cl^- (Table 3*b*). As in the experiments with furosemide, the different effects of removing Cl^-

Table 4. Effect of ouabain and the replacement of medium Cl^- by NO_3^- on intracellular water and ions at 38°C[a]

Incubation	Principal anion of medium	Intracellular water (kg/kg dry wt)	Intracellular ions (mmol/kg dry wt)	
			Na^+	K^+
Content after 90 min at 1°C	Cl^-	2.95 ± 0.20	503 ± 49	141 ± 10
	NO_3^-	3.09 ± 0.16	557 ± 46	139 ± 8
Then net change after 60 min at 38°C:				
Control	Cl^-	−1.05 ± 0.19	−257 ± 32	90 ± 7
	NO_3^-	−0.50 ± 0.14	−202 ± 37	126 ± 16
Ouabain (2 mM)	Cl^-	−0.76 ± 0.21	−138 ± 36	−37 ± 8
	NO_3^-	−0.15 ± 0.12	−22 ± 27	−33 ± 8

[a] Values shown after preincubation for 90 min at 1°C are the intracellular contents of the swollen slices, while values shown at 38°C represent the net change in contents during 60-min recovery from swelling. The values are the mean ± standard error of the mean of 12 observations at 1°C and 18 values at 38°C.

from media with and without ouabain suggest that at least two mechanisms are involved in volume regulation. An ouabain-insensitive mechanism requires Cl^- for full activity while a second system, not requiring Cl^-, is sufficiently active in the absence of ouabain for water extrusion not to be affected by omission of Cl^-. In addition, it may be necessary to invoke a third mechanism to account for the water extrusion persisting in the presence of ouabain and absence of Cl^-.

In the gluconate medium with 1.2 mM Ca^{2+}, the net loss of intracellular water at 25°C was substantially less than in other media (Table 3*a*), although an accumulation of K^+ and extrusion of Na^+ persisted (Table 3*b*). Ouabain inhibited these ionic movements but had no effect on the water loss, indicating that Na,K-ATPase was not responsible for volume control in the gluconate medium. In these experiments, the Ca^{2+} content of the slices at the end of the incubation was slightly reduced in the gluconate medium from 11.0 ± 0.3 mmol/kg dry wt in the Cl^- medium to 7.7 ± 0.6 mmol/kg with gluconate. Slices incubated in the gluconate medium with 5 mM Ca^{2+} showed a final Ca^{2+} content of 14.4 ± 0.7 mmol/kg dry wt, but neither in the presence nor absence of ouabain was the net extrusion of water significantly different from that observed in medium containing only 1.2 mM Ca^{2+}.

Volume Regulation at 38°C

The recovery of water and ionic composition, after prior swelling, was similar at 38°C to that shown above at 25°C (Table 4), despite the lower ATP content of the slices (van Rossum & Ernst, 1978). Replacement of Cl^- by NO_3^- had a greater effect at 38°C, reducing water extrusion by 50% in the absence of ouabain and permitting no significant extrusion in its presence (Table 4). The net movements of Na^+ at 38°C are those anticipated from the effects of the different treatments on the movements of H_2O and K^+ (Table 4).

Morphological Responses

The appearance of cortical slices in response to incubation under any of the conditions described above showed some variability from slice to slice, between different nephronal segments within a single slice and even between adjacent cells within the same section of a tubule. Because of this, the morphological descriptions given below deal mainly with the major changes observed in large fields of cells which appear to be relevant to an understanding of the degree of swelling and mechanism of volume control. Many of the illustrations are therefore of light micrographs or low-power electron micrographs which give overviews of general structural features of large numbers of tubules. Most attention is paid to the proximal tubules, as these are the major component of the cortical slices and so contribute most to the water and ionic contents seen in the tissue assays. But the different morphological responses of other nephronal components in the same slices to the same treatment are also noted, as they illustrate the potential for diversity between adjacent cells. Moreover, the various water-extruding mechanisms which seem, from our physiologi-

cal results (*above*), to contribute to volume regulation in whole slices may possibly involve different types of cells.

Following recovery from swelling, during incubation at 25°C for 60 min, at least 70% of the slice volume consisted of cells that stained well with azure II and exhibited organized structure in the light microscope. Up to 30% consisted of damaged cells which stained poorly with azure II and often appeared to have lost cytoplasmic components. The great majority of the latter (20 to 25% of slice volume) were situated at the slice periphery and had clearly been damaged during preparation and handling of the tissue. The disposition of the remaining damaged cells (5 to 10% of slice volume) lends no support to the frequent assumption that slices of renal cortex are hypoxic in their central regions (Mandel, 1982) for they were scattered randomly among the viable cells of all regions and nephronal segments. Moreover, some morphological features known to be particularly sensitive to mild hypoxia *in vivo* (Mercorella et al., 1983) were seen to be in excellent condition in the slices; these are the foot processes of the glomerular podocytes and glomerular endothelial cells (*not shown*). The descriptions given below refer only to the cells which retained a well-organized appearance. For purposes of comparison we include an electron micrograph of a proximal tubule from a kidney that was fixed by perfusion *in vitro* (Fig. 7), as this provides the most rigorous standard against which to judge the condition of incubated tissues.

Swelling at 1°C

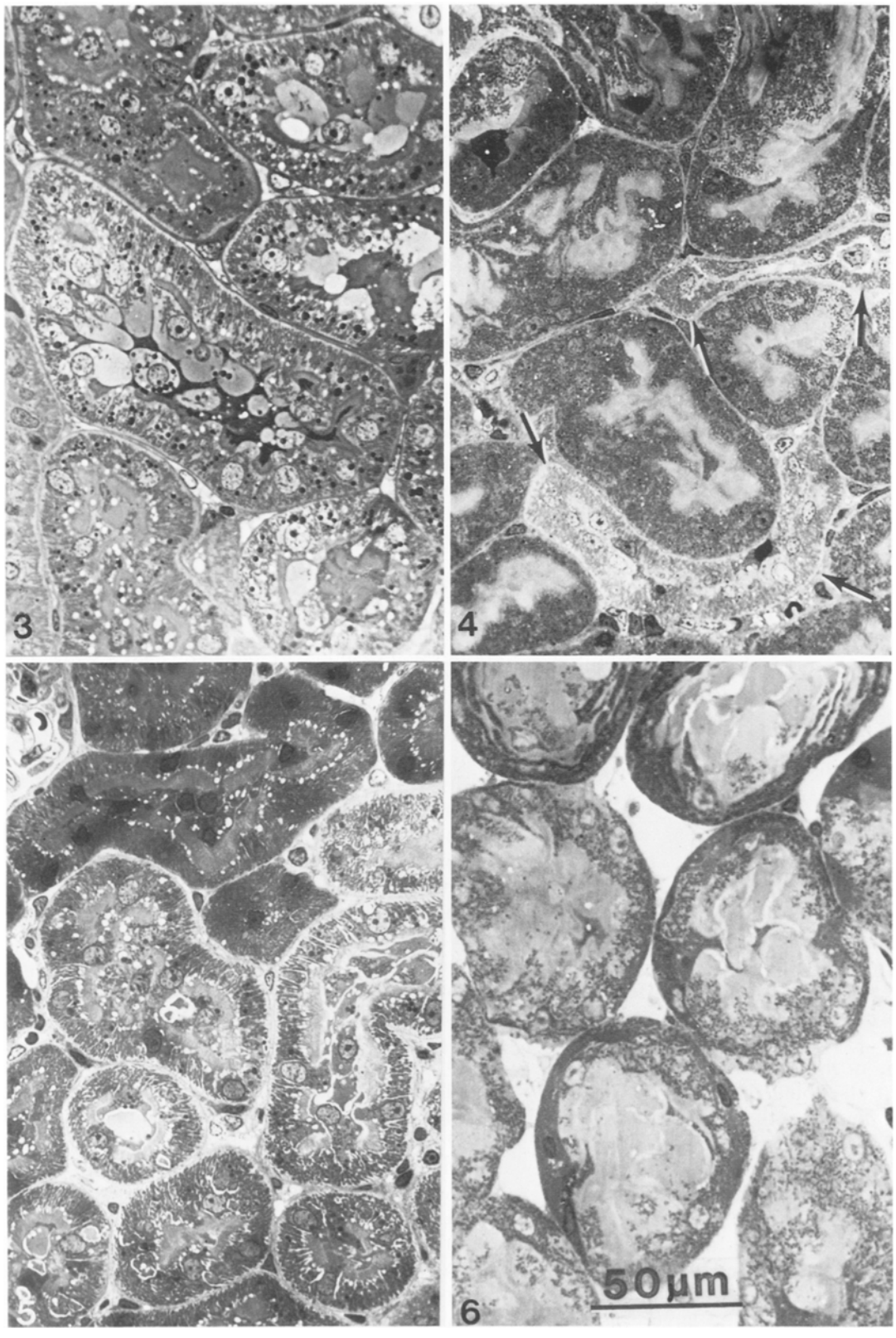
The normal distinction between the segments of the nephron seen in the light microscope was well maintained after 90 min at 1°C. The proximal tubules were the segments most markedly affected by swelling. They were characterized by marked expansion of the apical regions of the cells, accompanied by distortion of the microvillar border and by closure of almost all tubular lumina (Fig. 3). This appearance was reflected at the ultrastructural level by large apical protrusions which contained a highly diluted or vacuous ground substance, as judged by low electron density (Fig. 8). The apical regions below the level of the tight junctions exhibited characteristic, clear, rounded vesicles of various sizes, which were visible with both the light and electron microscopes (Figs. 3 and 8). Although fewer in number, a variety of vesicles and larger vacuoles are seen in freshly mixed, unincubated proximal tubular cells (Fig. 7; *see also* Maunsbach, 1966). The mitochondria varied from orthodox to condensed forms (Figs. 8 and 9) instead of all being in the orthodox configuration, as in the fresh tissue (Fig. 7). In contrast to the high-amplitude swelling of the apical cytoplasm, the basal regions of the same cells generally maintained their normal morphological organizations, although often showing moderate swelling and a variable degree of distortion of basolateral membrane folds (Figs. 8 and 9).

The general structure of the glomeruli was well maintained, with many unswollen elements. However, the glomerular epithelial cells showed a uniform degree of swelling, with light ground substance and condensed mitochondria, despite the maintenance of foot processes (*see above*). The loops of Henle, distal convoluted tubules and collecting tubules (*not shown*) all contained cells in varying degrees of swelling. A few markedly swollen cells protruded into the lumina of some of the distal tubules; nevertheless, the lumina were almost always open. Distal tubular cells contained clear vesicles in their basal regions (Fig. 10).

Recovery at 25°C

The next extrusion of water at 25°C was reflected in a reversal of swelling, as judged by light microscopy (Fig. 4) and by general recovery of normal ultrastructural architecture (Figs. 11–13; *compare* Fig. 7). However, there was some tubular and cellular variability. Proximal tubules showed a high proportion of cells with dense cytoplasm (Figs. 4 and 11) while most mitochondria had reverted to orthodox and intermediate forms (Figs. 12 and 13). The apical expansions characteristic of the swollen cells after preincubation at 1°C (*cf.* Fig. 3 with Fig. 4, and Fig. 8 with Fig. 11) were no longer apparent and microvillar organization was re-established although the tubular lumina remained closed. Some clear, rounded vesicles remained in the apical regions of the proximal epithelial cells, but they were smaller and fewer in number than at 1°C (Figs. 4 and 11).

All types of glomerular endothelial cells, even deep in the slices, had an appearance approximating that in fresh kidney cortex; this was also true of the epithelial cells, which had been markedly swollen at 1°C (*not shown*). Most distal (Fig. 14) and collecting tubules (*not shown*) recovered their normal size and appearance and their mitochondria were in the orthodox configuration. Cells that showed a less complete recovery contained mitochondria in intermediately condensed forms (Fig. 12, right-hand cell). The clear vesicles seen in the basal regions of distal tubules at 1°C were no longer visible after further incubation at 25°C (Fig. 14).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4
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50µm

Effect of Transport Inhibitors

Slices incubated at 1°C with ouabain (2 mM) and furosemide (5 mM), separately or together, appeared qualitatively similar to the swollen slices described above (*cf.* Figs. 3 and 8). After further incubation at 25°C in the presence of ouabain, slices showed a morphological recovery from swelling (Fig. 5) which, in accord with the analytical results (Tables 2 and 3; Fig. 2), was generally similar to that of control slices at 25°C (*cf.* Fig. 4). An important difference was that the slices with ouabain showed many cytoplasmic vesicles that were prominent even by light microscopy (Fig. 5). This was especially so in the proximal tubular cells, the apical regions of which contained an abundance of vesicles of various sizes, which were bounded by membranes and had clear contents (Fig. 15). These were similar to those seen in the apical regions of control cells (without ouabain) but were much more numerous and more widely distributed within the cytoplasm (*compare* Figs. 4 and 5; *see also* Fig. 15). In addition, the basal regions of these cells contained large vesicles as well as large, clear intercellular spaces between the infoldings of the plasma membranes (Figs. 5 and 15); both the vesicles and the spaces were more obvious than in the control slices seen in Figs. 4 and 11. Many images showed instances of the fusion of cytoplasmic vesicles with each other and also with foldings of the basolateral surface, suggesting that their contents opened to the intercellular spaces (Fig. 15, arrows and arrowheads).

Distal and collecting tubules incubated with ouabain also showed a number of vesicles, mainly in the apical regions (Fig. 16). Vesicles were not observed in glomerular cells.

Furosemide (2 and 5 mM) with and without ouabain caused no gross differences in the general appearance of the slices (*not shown*). Cells of proximal (Fig. 17) and distal tubules (Fig. 17, inset) incubated with ouabain *plus* furosemide had well-organized basal infoldings and apical microvilli, together with orthodox mitochondria. However, a less than full recovery of cytoplasmic density suggests that volume recovery in proximal cells was incomplete when both inhibitors were present together (Fig. 17). A further important difference from both control and, particularly, ouabain-treated slices was that there were only a few clear vesicles in both apical and basal regions when furosemide was included in the medium. The absence of vesi-

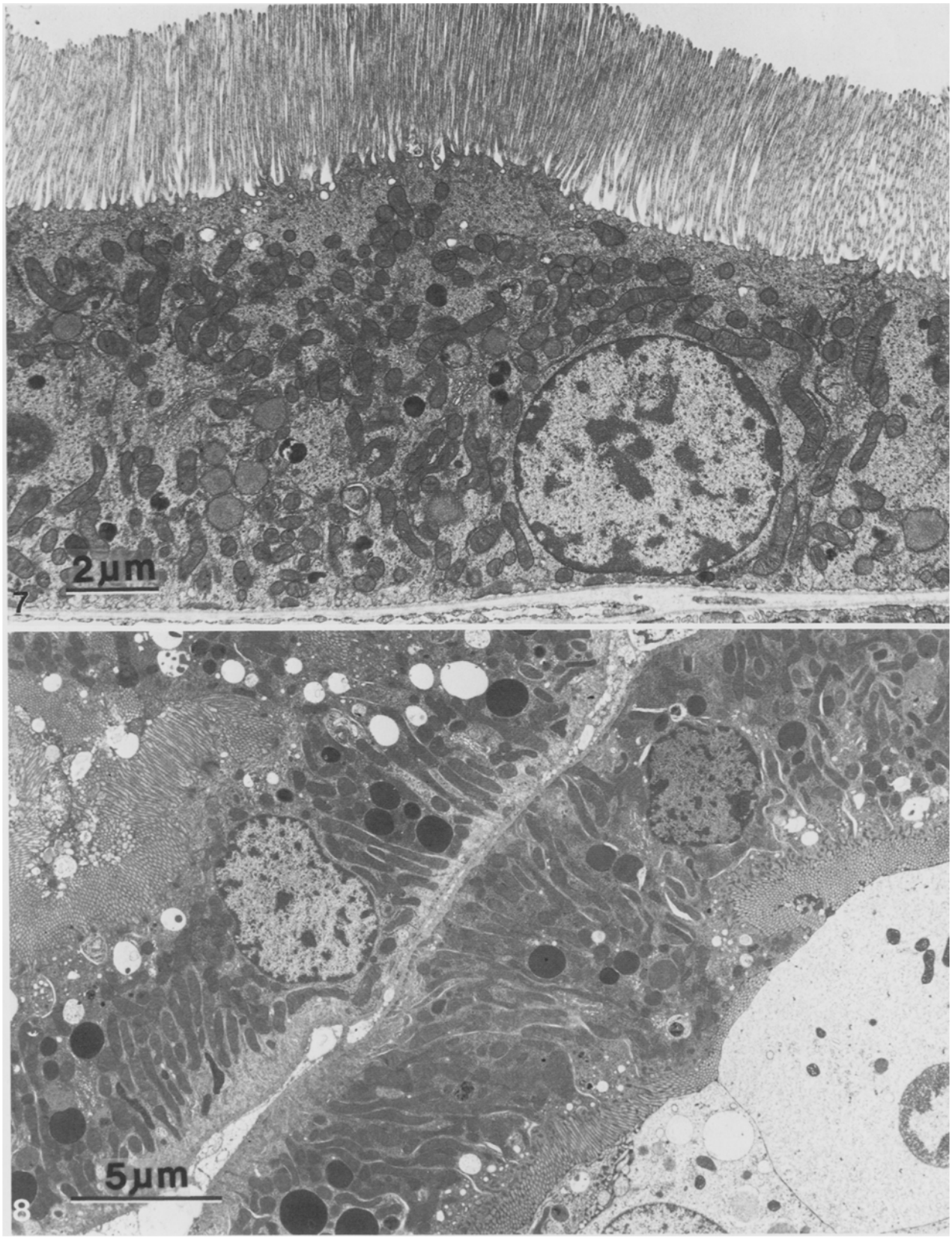
Figs. 3–6 (*facing page*). Light micrographs of plastic sections (1 μm thick) of kidney cortex slices incubated under various conditions. Sections were stained with azure II.

Fig. 3. Slice incubated for 90 min at 1°C. A number of proximal tubules are shown. There is general swelling of these segments, especially shown by large protrusions of the apical parts of the tubular cells. There is an increase in the number of vesicles localized mainly beneath the apical border. While marked swelling of apical regions was a common feature, neighboring proximal tubules (e.g. those at upper and lower left) appeared less affected. A somewhat swollen distal tubule is seen in the extreme lower left-hand corner. An electron micrograph of this tissue is seen in Fig. 8. 480×

Fig. 4. Incubated for 90 min at 1°C followed by recovery from swelling during 60 min at 25°C. This micrograph shows proximal tubules, and two distal tubules (arrows). The cells have largely recovered from swelling, as indicated especially by the density of the cytoplasm and the absence of apical protrusions. Few apical vesicles are present. The lumina of the proximal tubules are occupied by apparently homogeneous areas which are seen in the electron microscope to be well-organized brush borders. A corresponding electron micrograph is seen in Fig. 11. 480×

Fig. 5. Incubated for 90 min at 1°C followed by 60 min at 25°C, in the presence of 2 mM ouabain throughout. Proximal tubules with a small part of a glomerulus (upper left). There is a generally good recovery of cellular volume and appearance from the changes occurring during swelling at 1°C. The most prominent finding is the presence in all tubules of large numbers of small- to medium-sized vesicles, mainly in the apical regions. Homogeneous areas of brush border are present, as described for Fig. 4. In addition, basolateral spaces are visible. The inter-tubular spaces are normal, a further indication of reversal of tissue swelling. A detail of such tubules is shown in Fig. 15. 480×

Fig. 6. Incubated in NO_3^- Ringer's solution for 90 min at 1°C followed by 60 min at 25°C, in the presence of 2 mM ouabain throughout. Some of the cells within a given tubule appeared to be generally recovered from the changes at 1°C (similarly to the recovered cells of Fig. 4) while other cells, particularly in their apical regions, showed a high degree of swelling, often resulting in distortion of the microvillar borders. An electron micrograph of such tubules is shown in Fig. 18. Despite the presence of ouabain, apical vesicles and enlarged basolateral spaces are not present. 480×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5µm
8

cles was most obvious in proximal tubular cells (Fig. 17; *cf.* Fig. 15), but was also noted in distal (Fig. 17, inset) and collecting tubules (*not shown*).

Chloride-Free Media

Slices incubated without ouabain in the Cl^--free media, whether containing NO_3^- or I^- as replacement anion, showed a good morphological recovery from swelling, although there was some expansion of the basolateral vesicles and intercellular spaces in many areas of proximal and distal tubules (*not illustrated*). The vesicles in the apical portions of the cells were, by contrast, reduced in numbers compared to slices in Cl^- medium without ouabain (*not illustrated*).

In the NO_3^- medium, the cells of proximal and distal tubules showed two types of appearance in the presence of ouabain: (a) Swollen cells with light ground substance which almost completely lacked the large, apical vesicles characteristic of ouabain treatment in Cl^- medium (Figs. 6 and 18). The high degree of swelling in the apical region compromised the organization of the microvillar border of proximal cells of this group. In these swollen cells, the mitochondria were in the condensed configuration. (b) Other cells in the same tubules exhibited a dense ground substance and showed reasonably good recovery from swelling. These did contain some of the large, apical cytoplasmic vesicles but the vesicles were very few in number (Fig. 18). Mitochondria were mainly in the orthodox form.

The proximal and distal tubular cells of slices incubated in I^- medium containing ouabain also completely lacked the apical vesicles (Fig. 19). The swelling of the cells was rather less extensive, and the inter-tubular spaces were more expanded in I^- medium than in NO_3^- medium.

When gluconate was the anion used to replace Cl^-, the general structural recovery was similar to that in the I^- medium, with no vesicles either in the apical or basolateral regions of the proximal tubular cells (Fig. 20). However, the basolateral intercellular spaces were dilated and the cells showed a dense ground substance. The latter probably indicates shrinking due to the presence of the nonpenetrating anion, gluconate.

Recovery at 38°C

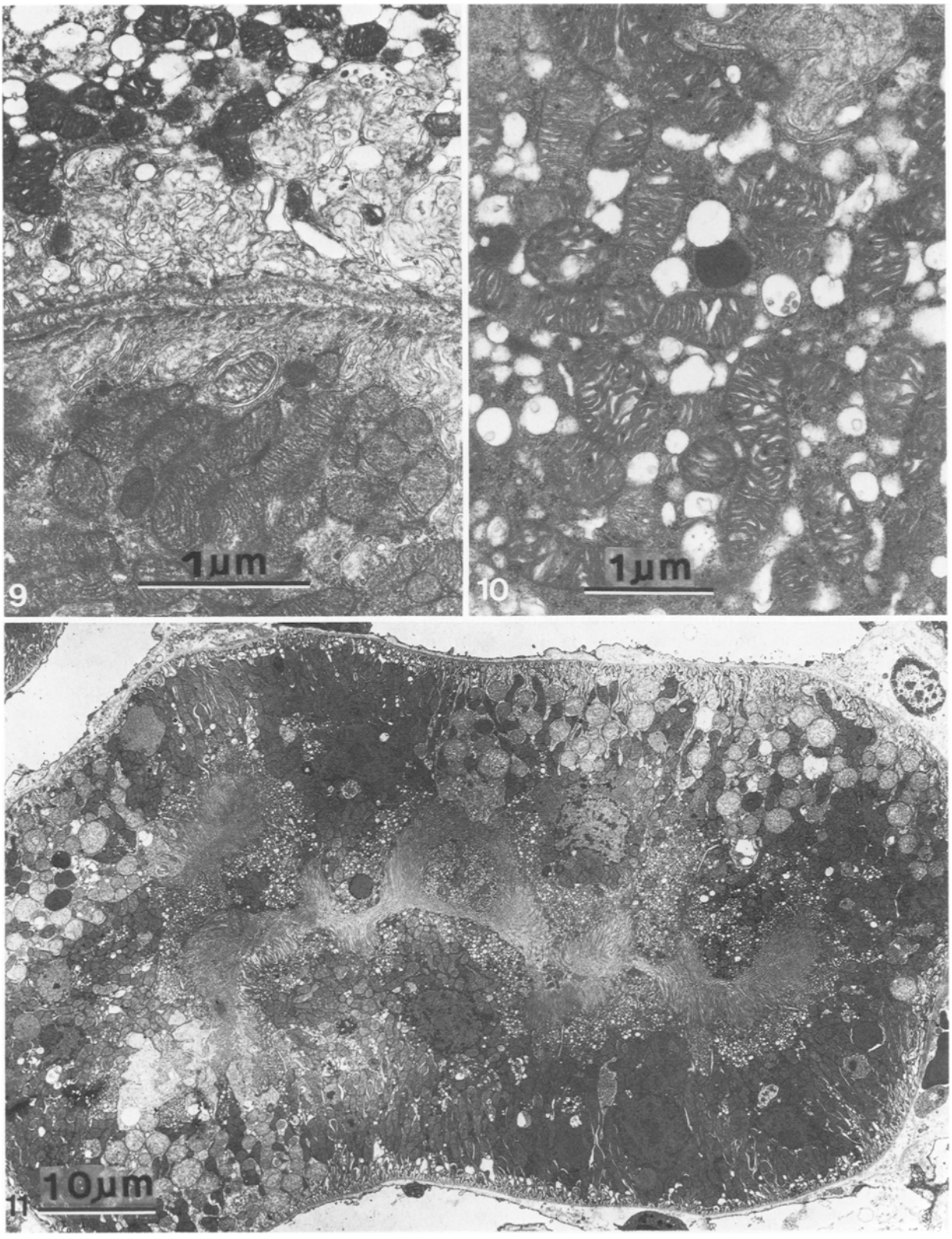
Slices incubated at 38°C without ouabain showed a much more variable recovery of structure than the recovery described above at 25°C, and there was a larger proportion of damaged cells. For these reasons, we did not carry out as thorough a study of morphological changes upon rewarming at 38°C as we did at 25°C. Nevertheless, the recovered cells at the higher temperature were of great interest in displaying large numbers of vesicles in their apical regions, particularly in the proximal tubules, whether or not ouabain was present. These vesicles were similar to the apical vesicles seen at 25°C, but they were more abundant and wider in their distribution at 38°C in the absence of ouabain (Fig. 21), and were more numerous still in the presence of ouabain (Fig. 22). Many of the vesicles appeared to fuse, often forming long chains with an apparently common internal space, while both single vesicles and the terminal vesicles of chains fused with the plasma membrane bordering the intercellular spaces of the basolateral surface (Figs. 21 and 22). The incidence of fusions was much increased in the presence of ouabain and the basolateral intercellular spaces were themselves much dilated (Fig. 22).

Discussion

Our results confirm that the recovery of kidney cortex from a period of isosmotic swelling induced by incubation at 1°C is due to an extrusion of intracellular water, Na^+ and Cl^- which is dependent on respiration and is little affected by ouabain (Mudge, 1951; Macknight, 1968; Whittembury & Proverbio,

Fig. 7 (*facing page, top*). Electron micrograph of proximal tubular cell in fresh, unincubated renal cortex fixed *in situ* by perfusion of fixative. These are the cells most frequently found in cortical slices and are the cells showing the greatest morphological changes during swelling at 1°C and its reversal; this micrograph is therefore included as a standard against which to compare the appearance of the incubated tissue. Note the well-preserved brush border, seen in longitudinal section, orthodox configuration of the mitochondria and the presence of very small and few pinocytotic vesicles. 8,000×

Fig. 8 (*facing page, bottom*). Incubated for 90 min at 1°C. Electron micrograph showing detail of proximal tubules from the slice of Fig. 3. The most prominent finding in most proximal tubules was marked swelling of the apical cytoplasm. As shown by the tubule on the right, intact regions of brush border are often compressed by the apical expansions and a number of vesicles of different sizes are seen, mainly near the luminal borders or within the apical protrusions. Nuclei and a few organelles are generally present within the diluted ground substance of the apical expansions. Mitochondria are in the orthodox or intermediate forms. The portion of the tubule on the left is less swollen, although apical vesicles are apparent (*cf.* Fig. 3). 5,400×


1µm
9
1µm
10
10µm
11

1970; Kleinzeller, 1972). However, our morphological observations show that the picture of ion and water movements in whole slices disguises some differences in the behavior of individual segments of the nephron. Even adjacent cells within the same segment showed contrasting appearances on occasion, possibly due to progressive alterations at different rates during the course of incubation (*cf.* Ginn et al., 1968). This contrasts markedly with the much more uniform appearance of hepatocytes within liver slices undergoing swelling and recovery (Russo et al., 1977).

A number of other experimental protocols have also been used to examine the effects of ouabain on volume regulation by renal cortical slices. According to one (Macknight & Leaf, 1977), an initial "equilibration" period at 25°C permits reversal of the swelling that took place during the preparation and initial hypoxic rinsing of the slices. Subsequent swelling of the slices at 0.5°C and recovery at 25°C showed a marked ouabain-resistance of water extrusion, as in our experiments. Cooke (1978*a*), on the other hand, used only direct incubation at 25°C (corresponding to the "equilibration" period, *above*). He found that the reversal of the swelling which had arisen during preparation was markedly inhibited by ouabain and that an ouabain-resistant portion of the reversal could be accounted for by a loss of cellular K^+ without invoking a separate, ouabain-resistant transport system. Clearly, this latter situation differs from that of our cold preincubated slices, the K^+ content of which was already minimal and showed no further decrease during incubation at 25°C with ouabain (Table 3*b*).

Morphological Damage

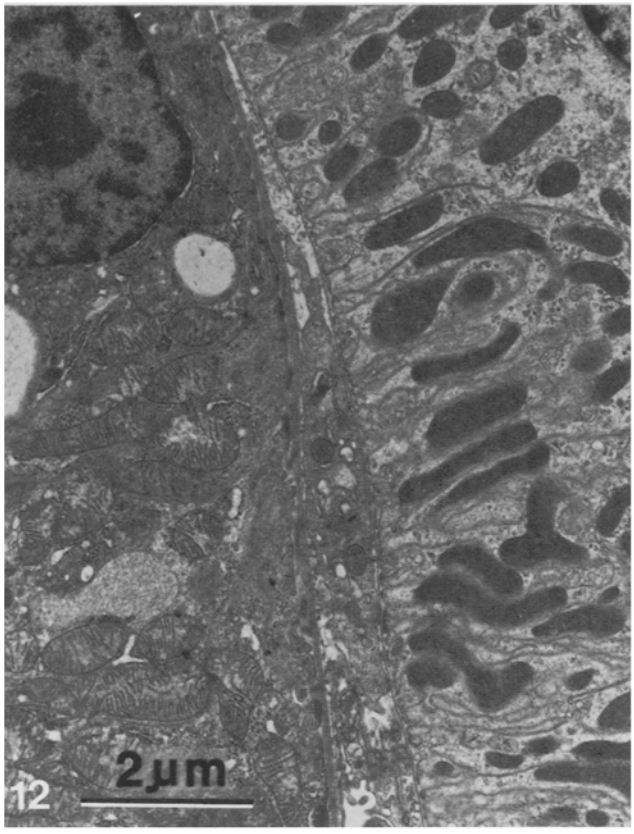
In work with kidney slices, a potentially troublesome factor is cellular damage. Since cells showing histological indications of damage (e.g. failure to be stained by azure II, in our experiment) are presumably unable to maintain normal ionic gradients, a change in the proportion of damaged to viable cells in response to varying incubation conditions can lead to changes in intracellular (i.e. inulin-free) water and ionic content of the whole slices which do not reflect the condition of the viable cells. In our experiments this does not appear to be a major source of error, for the percentage of severely damaged cells after 60 min at 25°C did not differ markedly between the various treatments investigated. Renal cortical slices nevertheless show a larger fraction (approx. 30%) of badly damaged cells at the slice periphery than liver slices do (approx. 10%; Russo et al., 1977). It should be emphasized that the present findings relate exclusively to the experimental protocol in which the slices are preincubated directly at 1°C; the proportion of damaged cells and the nature of the damage may well be different when the slices are treated differently (e.g. after an initial "equilibration" period at 25°C; *see above*).

The cause of the morphological damage to cells scattered in deeper regions of the slices after incubation at 25°C in our experiments is apparently not hypoxia, for the damaged cells are not concentrated at the slice center. Cooke (1979) has shown that renal cortical slices of the same thickness as ours show no reduction of O_2 consumption at 25°C when the pO_2 of the medium is reduced to 40% of the control level, indicating that the cells are adequately oxygenated when the medium is gassed with 100% O_2, as in our experiments. A further indication that hypoxia of the cells is not a serious problem in our studies is the finding that the O_2 consumption of the slices must be reduced by as much as 40%, by an inhibitor of electron transfer, before any inhibition of water extrusion is seen (Fig. 2). Moreover, the glomerular endothelial cells and mesangial foot processes, which are known to be especially susceptible to hypoxic damage (Mercorella et

Fig. 9 (*facing page, top left*). Detail of proximal tubule after 90 min at 1°C. Note partial disorder of the basal infoldings. In the upper cell, mitochondria are in intermediate or condensed forms and are associated with a number of vesicles which are probably derived from endoplasmic reticulum and infoldings of plasma membrane. In lower cells, mitochondria are in the orthodox configuration and the basolateral folds are more organized. 15,000×

Fig. 10 (*facing page, top right*). Detail of distal tubule after 90 min at 1°C. Mitochondria are in intermediate or condensed forms, with well-preserved granules indicative of well-maintained activity. The mitochondria are interspersed with vesicles. 21,000×

Fig. 11 (*facing page, bottom*). Low magnification electron micrograph of a slice incubated for 90 min at 1°C followed by 60 min at 25°C. The tubule shown represents the most frequent picture of recovery of proximal tubules. Many of the cells show very good recovery of all components, including brush border and the apical region. This last contains small vesicles but very few large vesicles. The mitochondria are in orthodox or intermediate configurations. Some of the cells show only partial recovery from swelling, as indicated especially by persistent mitochondrial swelling and dilated basolateral spaces. 2,000×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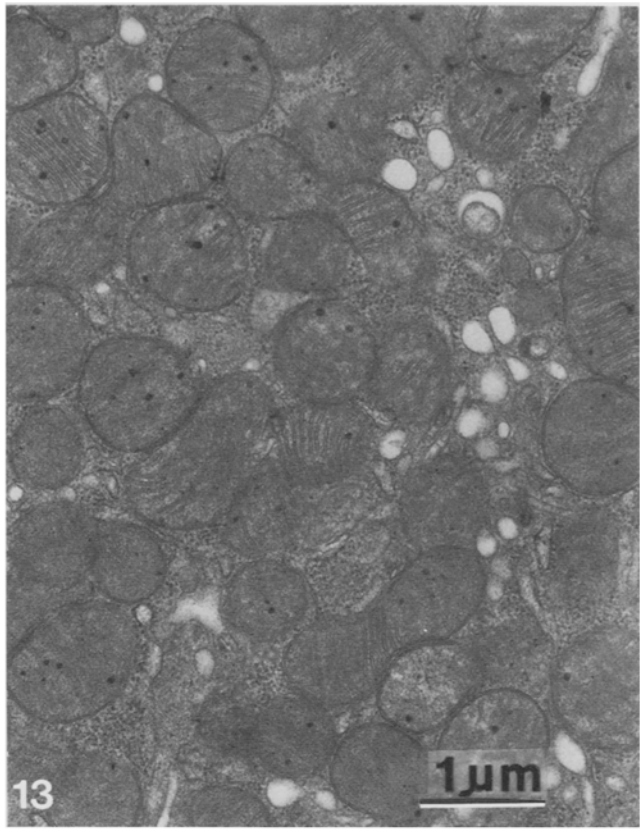
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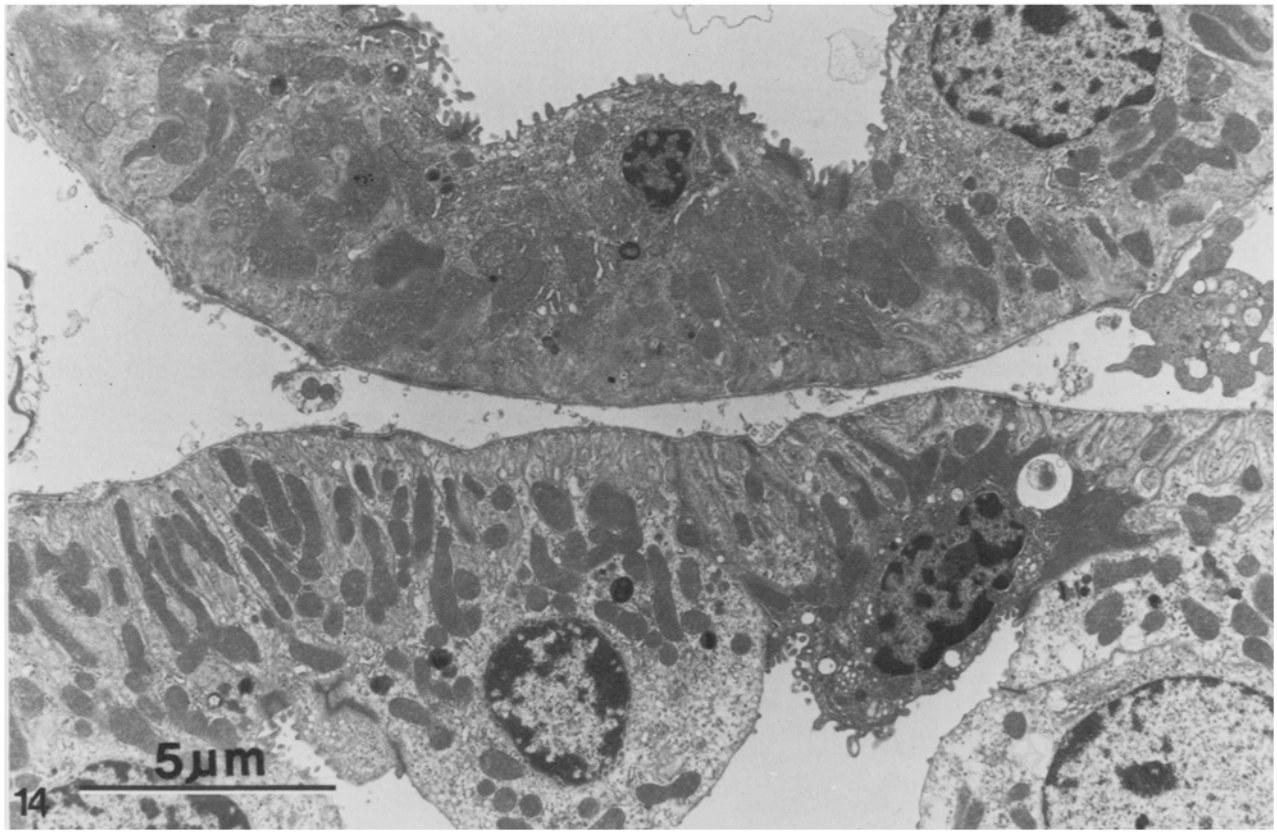
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14

al., 1983), maintain an excellent appearance. The damage thus appears to be due to a more random cause. One likelihood is that it depends on the age of the individual cells.

A greater proportion of cortical cells are subject to damage, and there is a greater morphological variability within slices, after incubation at 38°C than at 25°C and the much lower ATP levels at the higher temperature (van Rossum & Ernst, 1978) suggest that hypoxia may be a more important factor in this case (Balaban et al., 1980; Mandel, 1982). These findings indicate that caution should be exercised in interpretation of physiological data obtained from kidney slices at 38°C.

In general, it is clear that renal cortical slices are a less satisfactory model for correlating physiological and morphological indications of volume regulation than are liver slices, both because of the greater histological and functional heterogeneity of the former and their greater susceptibility to damage. Nevertheless, the fact that cortical slices have been widely used for the study of cellular volume regulation makes it important to examine the relationship between their structure and function during volume-regulating activity.

Swelling at 1°C

Preincubation of the renal cortical slices in the cold led to swelling of most types of cells, although the various portions of the nephron differed somewhat in their response. Proximal tubules were the most, and glomeruli the least affected by swelling. Within each segment, individual cells showed markedly different degrees of swelling, but in no case did the swelling go beyond stage 3 of the classification by Ginn et al. (1968). Kidney cells are thus more resistant to swelling than liver cells, since the latter become uniformly swollen and show characteristics of stage 4 and stage 5 swelling (Russo et al., 1977).

It has been shown that the tubular lumina of kidney cortex rapidly collapse after cessation of blood flow and that the lumina in cortical slices are all in the occluded state (e.g. Bojesen & Leyssac, 1965). The micrographs of the present paper support this. As a consequence, the apical borders of the cells probably become inaccessible to the medium even before the onset of the preincubation at 1°C so that any swelling due to water entering across the apical border would have occurred from fluid present in the lumina prior to incubation. The bulk of the water and ion exchanges taking place during the cold incubation must then have occurred across the basolateral borders of the cells. It is possible that the specifically apical distribution of the large vesicles in the swollen slices (e.g. Fig. 3) may indicate that they arise from early swelling during the collapse of the lumina; however, we have made no observations of the time-course of the morphological changes during swelling which would provide direct evidence on this point.

Recovery at 25°C

The restoration of conditions favorable to metabolic activity at 25°C resulted in a net extrusion of intracellular water accompanied by reduction in cellular size and a recovery of ultrastructure which shows that the great majority of cells were viable. The ground substance became denser and mitochondria returned to orthodox conformations. The size and number of the apical vesicles was substantially reduced, implying that some of the lost water was derived from their contents. However, many apical vesicles remained (Figs. 4 and 11), especially those of smaller size, and the numbers persisting were markedly greater when recovery was allowed to proceed at the higher temperature of 38°C (Fig. 21).

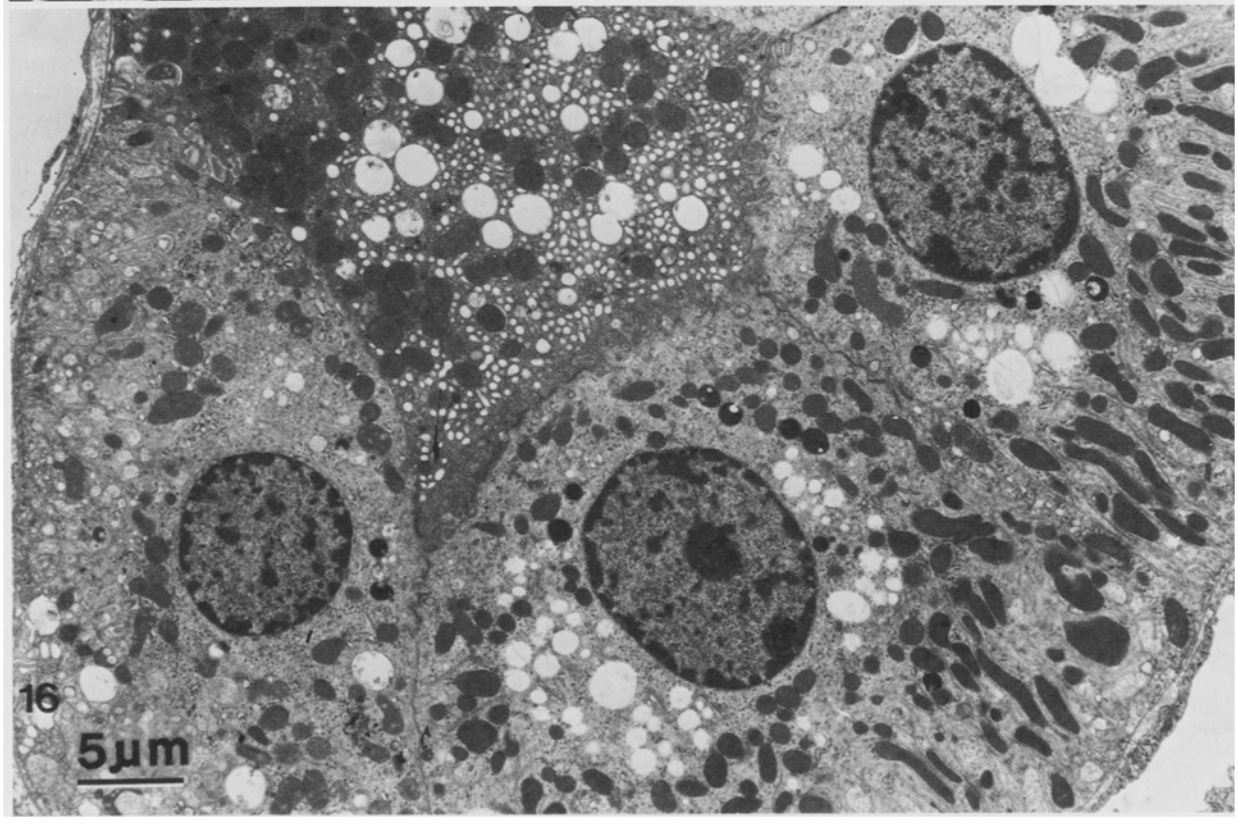
The proposal of a volume-regulating mechanism which is independent of the Na^+- and K^+-transporting system is based largely on the considerable resistance of the water extrusion to ouabain

Fig. 12 (*facing page, top left*). Detail of a slice incubated for 90 min at 1°C followed by 60 min at 25°C. Well-recovered cells from a proximal (*left*) and distal (*right*) tubule are shown. The basolateral infoldings are well recovered. The mitochondria are mainly in the orthodox form in the proximal tubule and intermediate in the distal tubule cells, although different forms are also seen in each tubule. 12,000×

Fig. 13 (*facing page, top right*). Detail of mitochondria from proximal tubule after incubation for 90 min at 1°C and 60 min at 25°C. The mitochondria have recovered well from swelling and the interspersed vesicles seen at 1°C (Figs. 9 and 10) are reduced in numbers. 16,000×

Fig. 14 (*facing page, bottom*). Low magnification electron micrograph of distal segments of nephron from a slice incubated for 90 min at 1°C and 60 min at 25°C. These tubules have recovered well from swelling at 1°C and are similar in appearance to fresh, unincubated tissue. 6,800×


15
2µm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5µm

(e.g. Macknight & Leaf, 1977). It has been shown that ouabain does not bind to or inhibit the Na,K-ATPase in the cold, although a fairly rapid binding occurs at 25°C (Schwartz et al., 1975; Hootman & Ernst, 1981). The failure of binding at 1°C seems unlikely to account for the ouabain resistance of water extrusion in our experiments, for even after only $2\frac{1}{2}$ min at 25°C no K^+ uptake was seen, while water extrusion was well advanced. Furthermore, there was no tendency for the intracellular water content to start to increase again at longer incubation times, as would be expected if ouabain had a delayed action on the mechanism of volume regulation. Our work also provides an extra indication of the considerable independence of the volume regulation mechanism from the Na,K-ATPase in that K^+ accumulation, presumably a measure of the activity of the ATPase, is much more sensitive to the limitation of cellular energy supply, by the action of antimycin A, than is water extrusion.

Relationship of Vesicle Formation to Water Extrusion

The apical vesicles seen in many of the types of cells were markedly affected when incubation at 25°C was carried out under conditions which were expected to alter ion-transporting activities, although there was no immediately obvious correlation with the ability of the cells to extrude water. Thus, in the presence of ouabain, the net extrusion of water was little affected (Tables 2 and 3) and apical vesicles increased in number and average size (Figs. 5 and 15). Incubation with furosemide or in Cl^--free medium also had little effect on water extrusion at 25°C in the absence of ouabain yet the number of vesicles was drastically reduced. In contrast, incubation in these same conditions, but with ouabain, caused an inhibition of water extrusion (Tables 2 and 3) while still reducing the number of vesicles (Figs. 17–20).

We suggest that these contrasting relationships between vesicle formation and water extrusion can be explained if at least two different mechanisms are responsible for water extrusion, one of which involves the vesicles (indicated schematically in Fig. 23). First, our observations on apical vesicles, especially in the proximal tubules, suggest that the vesicles may form a vehicle for water extrusion which is similar in mechanism, although opposite in cellular orientation, to the vesicular mechanism we have proposed for ouabain-resistant volume regulation in liver slices (Russo et al., 1977; van Rossum & Russo, 1981, 1984). Secondly, following the proposal of Leaf (1956) and our earlier conclusions with liver slices, we propose that part of the water extrusion is driven by the Na^+- and K^+-dependent adenosine triphosphatase at the basolateral membrane, the water probably traversing the membranes by way of an aqueous channel.

The crucial observations on which we base the proposed vesicular mechanism are: i) The extrusion of water at 25°C in the presence of ouabain is significantly inhibited by furosemide or by substitution of NO_3^- or I^- for Cl^- (Tables 2 and 3), and these same conditions drastically reduce the number of apical vesicles (*compare* Figs. 5 and 6 and Figs. 15–19). This suggests that the vesicles may be formed by entry of water from the cytosol in response to a furosemide-sensitive movement of Cl^- and Na^+ across the vesicular membrane. ii) Vesicles are seen to fuse with the basolateral plasma membranes at 25°C and at 38°C, especially in the presence of ouabain (Figs. 15, 21 and 22), suggesting that expulsion of the vesicular contents to the intercellular fluid may be the final step of water extrusion. iii) The number of vesicles seen in the cytoplasm is greatly increased when ouabain is present (*compare* Figs. 5 and 6, and Figs. 11 and 15). This suggests an increase of vesicular activity when water extrusion coupled to the Na,K-ATPase is inhibited; however, this can only form a link in our argument if it can be shown that Na,K-ATPase does, in fact, act as the driving force for one mechanism of water extrusion (*see below*). iv) The increased number of vesicles in control slices at 38°C is associated with an in-

Fig. 15 (*facing page, top*). Proximal tubule after incubation for 90 min at 1°C and 60 min at 25°C in the presence of 2 mM ouabain. Despite partial recovery from swelling and ultrastructural changes, two obvious feature are: i) a large number of apical vesicles; ii) the basolateral spaces are dilated, partially distorting the infoldings of the plasma membrane. Vesicles are frequently seen to fuse with each other (arrowheads) and with the lateral spaces (arrows). Mitochondria are in orthodox configurations. 8,500×

Fig. 16 (*facing page, bottom*). Distal tubule of slice incubated for 90 min at 1°C and 60 min at 25°C in the presence of 2 mM ouabain. Many vesicles are present, although fewer than in the proximal tubules (*cf.* Fig. 15). The vesicles are found throughout the cytoplasm, including the apical region. The basolateral parts of the cells are well recovered from swelling and contain few vesicles. 2,800×

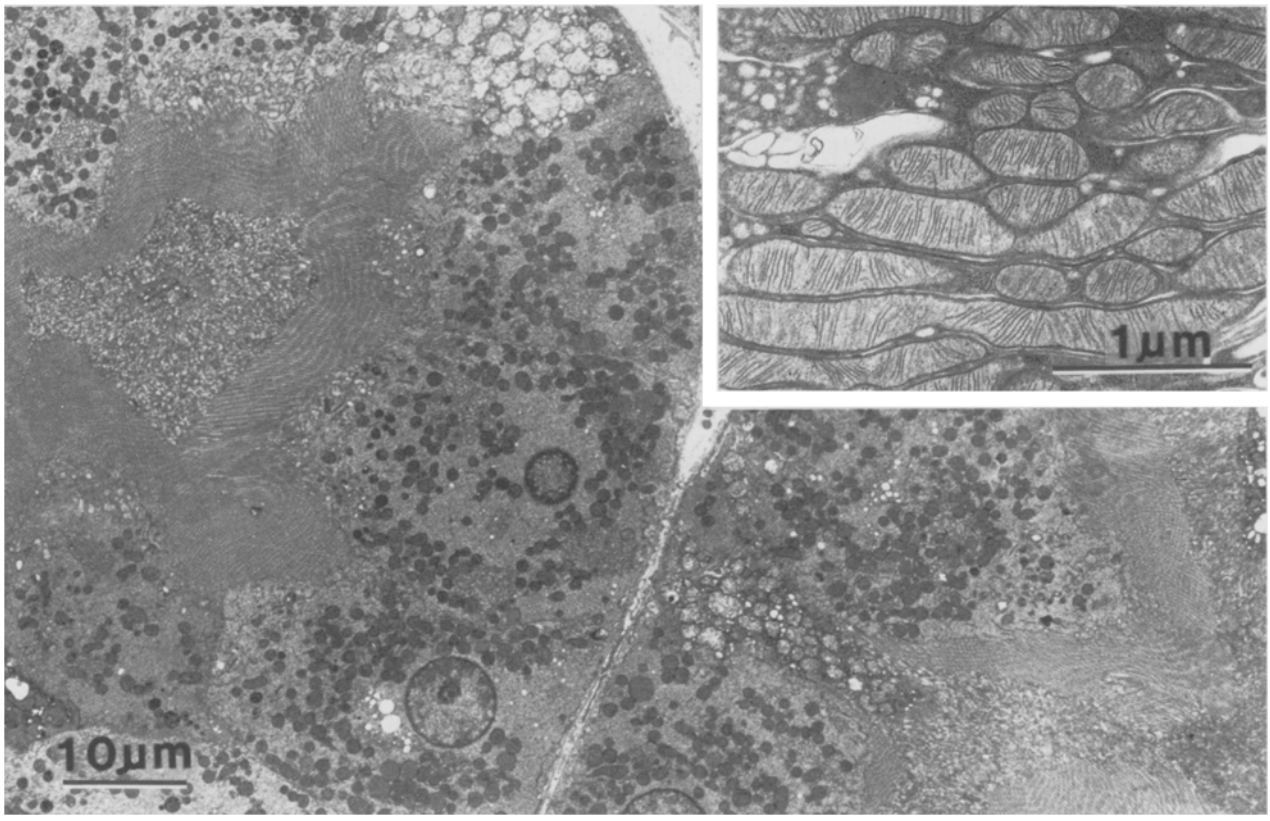

Fig. 17. Proximal tubule of slice incubated for 90 min at 1°C and 60 min at 25°C in the presence of 2 mM ouabain *plus* 2 mM furosemide. Apart from the region of structural damage (*top right*), the cells show a recovery of organization from the incubation at 1°C and are devoid of vesicles. Indications of swelling remain in that the ground substance is electronlucent and some mitochondria are in swollen forms. The *Inset* shows a detail of well-recovered mitochondria from a distal tubule of the same slice of cortex. These mitochondria are in the orthodox configuration; between them are seen a few, very small vesicles. Main figure 1,600×; Inset 26,500×

creased inhibition of water extrusion in chloride-free medium (Table 4), i.e. with an increased dependence of the cell on the chloride-dependent system of volume control. These four lines of evidence show that the apparently variable relationship between water extrusion and vesicle formation can be accounted for by the proposed vesicular mechanism. It is of interest that similar vesicles have been observed in isolated tubules of renal cortex, especially in the presence of ouabain (Rabinowitz, Garcia-Cañero, Russo & van Rossum, *in preparation*).

Cooke (1978*a*,*b*) has provided evidence that the Na,K-ATPase plays an important role in the extrusion, from kidney cells, of the water that enters them during slicing and the first few minutes of anaerobic incubation, but the evidence that it plays a role in extrusion of the water after the longer period of swelling imposed in our experiments is rather indirect. Indeed, a role for it appears, at first sight, to be excluded by the failure of ouabain to cause a significant inhibition of water extrusion in the control medium (Tables 2, 3; *see also* Macknight, 1969; Kleinzeller, 1972) and by the greater sensitivity of K^+ uptake than of water extrusion to the partial inhibition of respiration (Fig. 4). However, these observations are not conclusive, for it is possible that inhibition of the Na,K-ATPase-dependent system is compensated for by increased activity of an alternative system, such as the vesicular mechanism proposed above. It is clear from our results that there must be a volume-regulating mechanism in addition to the furosemide- and chloride-sensitive system, for inhibition of the latter in the absence of ouabain prevents vesicle formation without affecting net extrusion of water. The most likely candidate for this role would be one driven by the ouabain-sensitive Na,K-ATPase. Finally, it may be noted that in some of our experiments (e.g. Table 4) there was a tendency for ouabain to reduce the extrusion of water, even though the effect was not statistically significant. In sum, we feel justified in proposing the existence of the two mechanisms for volume regulation that are depicted in Fig. 23.

The large, electron lucent intercellular spaces between infoldings of the basal membranes must contain water and their occurrence varied with the incubation conditions in a way which suggested that

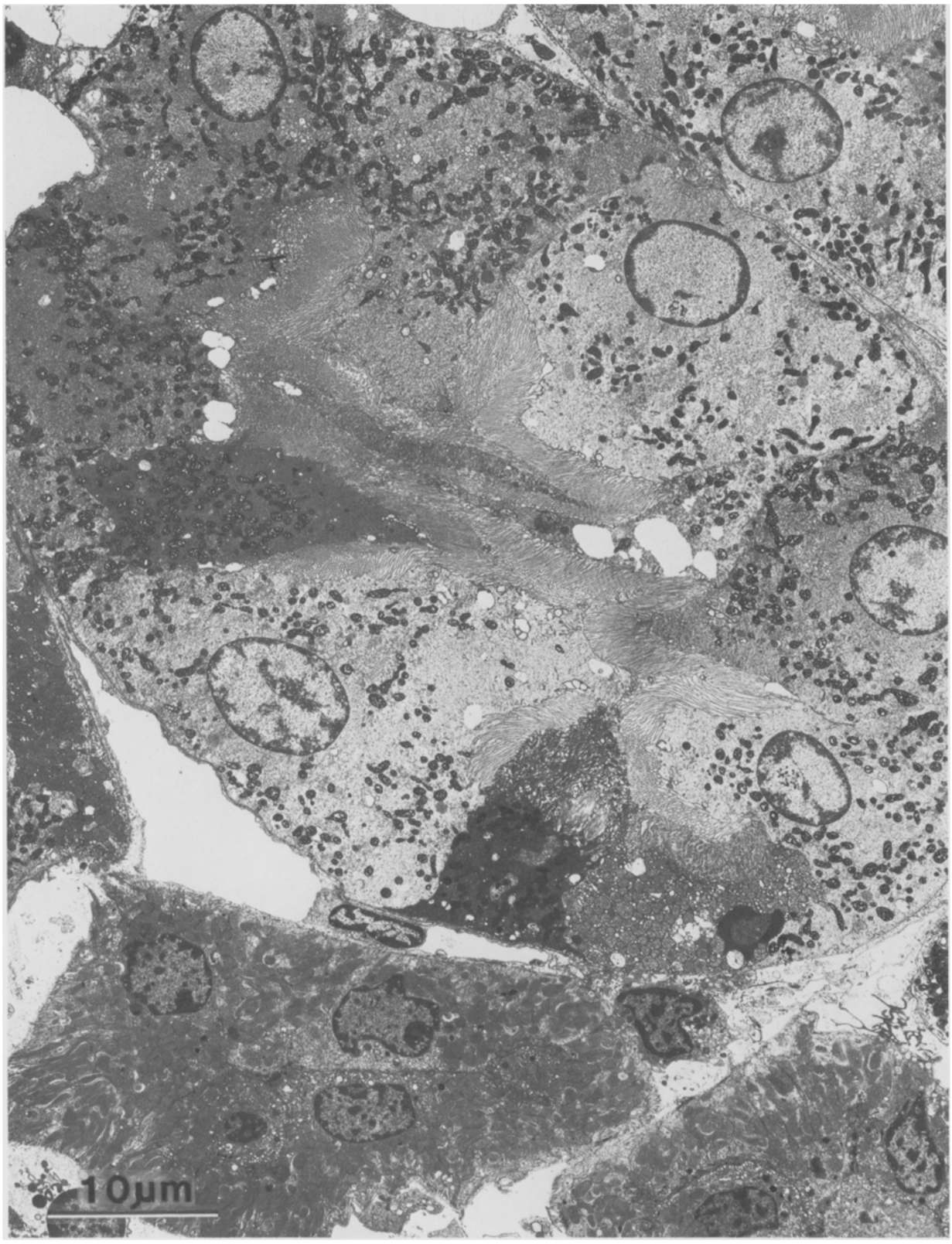

Fig. 18. Low-power electron micrograph of cortical slice after incubation for 90 min at 1°C followed by 60 min at 25°C in NO_3^- medium containing 2 mM ouabain. The central portion of this micrograph illustrates a proximal tubule and a portion of another is seen at top right. Ultrastructure shows some partial recovery but many of the proximal cells remain quite swollen (e.g. light ground substance *cf.* Fig. 6). By contrast with the situation in the presence of ouabain and Cl^- at 25°C, very few cytoplasmic vesicles are to be seen (*cf.* Figs. 15 and 16). The brush border is generally better organized than in the comparable tubules of Fig. 6, although in some areas it is displaced by the expanded apical cytosol. Mitochondria are in condensed or intermediate forms, except in the structurally disorganized cells where they are swollen. In the lower part of the micrograph are parts of two distal tubules in which structural recovery is well advanced but no vesicles of the type normally seen with ouabain are present. Lumina of the blood vessels between tubules are dilated. 2,900×

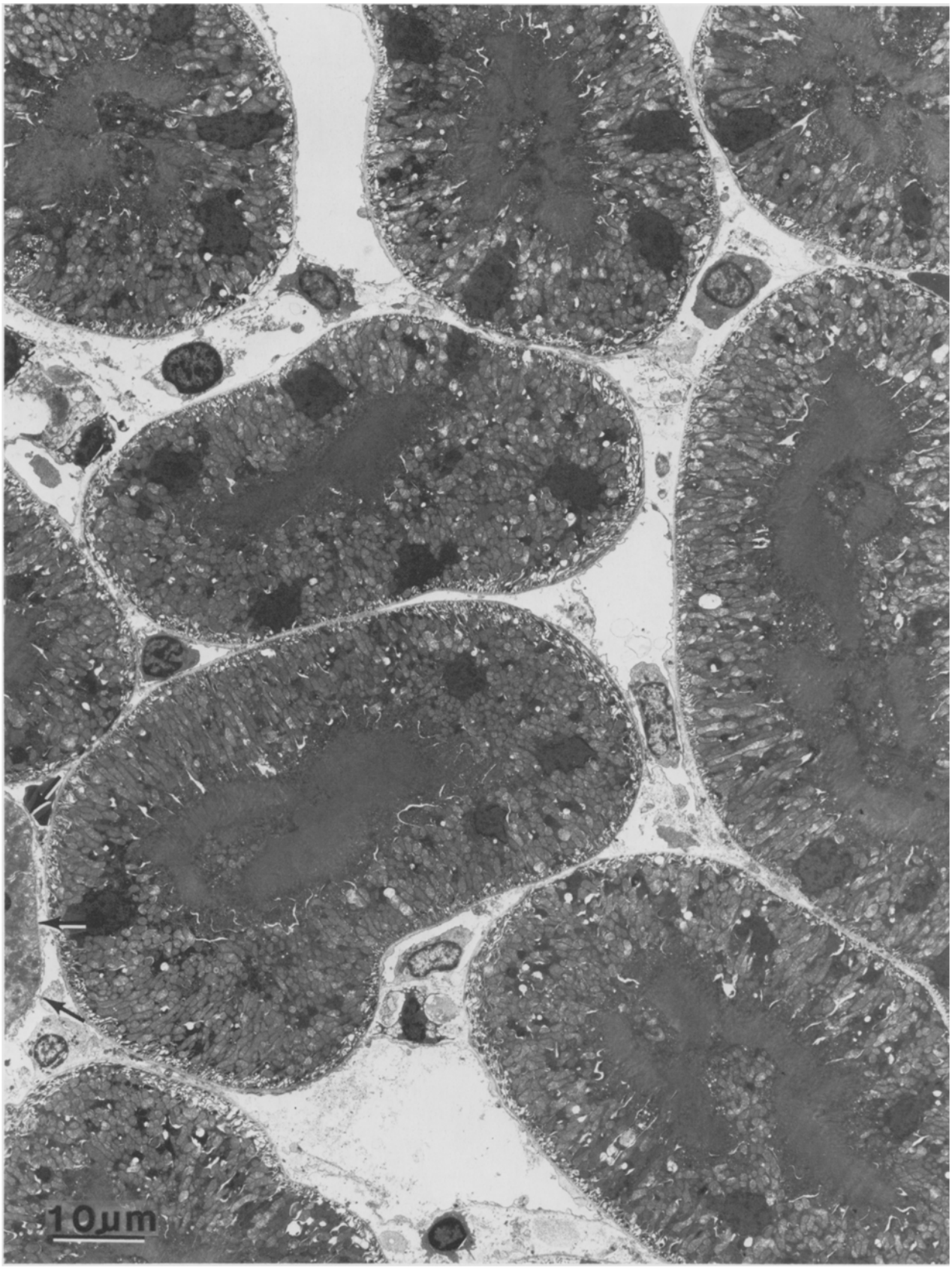

Fig. 19. Tissue incubated for 90 min at 1°C and 60 min at 25°C in I^- medium containing 2 mM ouabain. The micrograph illustrates proximal tubules and a small part of a distal tubule (arrows). The cells show a very good, general recovery and are devoid of the vesicles normally associated with incubation in the presence of ouabain and Cl^- (*cf.* Figs. 15 and 16). There is a general increase in the electron density of all structures, compared to that seen in Cl^- or NO_3^- media; this could arise either because the cells are somewhat shrunken or, more likely, from the intrinsic electron density of I^-. 1,600×

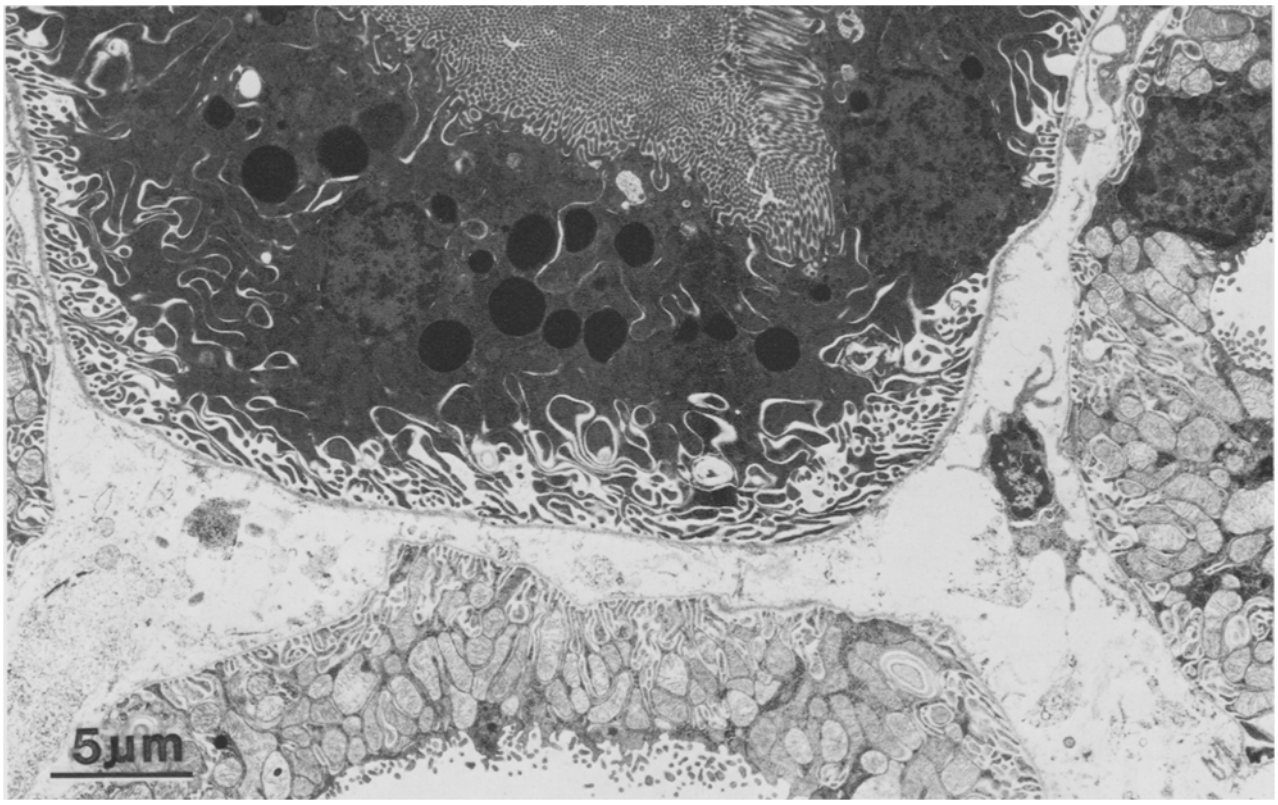

Fig. 20. Tissue incubated for 90 min at 1°C and 60 min at 25°C in gluconate medium containing 2 mM ouabain. The upper portion of the micrograph shows a proximal tubule, while the lower and right-hand segments are parts of two distal tubules (*pars recta*). The proximal tubule shows a fairly good recovery but appears to be shrunken, probably as a result of the osmotic effect of the nonpenetrating anion, gluconate. No vesicles are present, but the basolateral spaces are dilated and the infoldings partially disorganized. The distal tubules appear to be less electron dense and generally less affected than the proximal tubule. 3,800×

they may be a consequence of the activity of the proposed vesicular mechanism of transport. Thus, at 25°C they were most in evidence in the presence of ouabain (Figs. 5 and 15), but were greatly diminished in size and numbers in the further presence of furosemide (Fig. 17) and when Cl^- was replaced by NO_3^- (Fig. 18). At 38°C they were abundant in the absence, and increased further in the presence of ouabain (Figs. 21 and 22), and the results with chloride substitution (Table 4) indicate that substantial activity of the vesicular mechanism occurs at 38°C even in the absence of ouabain. One possibility is that the intercellular spaces arose from an accumulation of water that had been extruded by exocytosis into the basolateral infoldings of the cells. However, the situation is by no means clearcut, for basal intercellular spaces were observed when the Cl^- was replaced by I^- or gluconate (Figs. 19 and 20) rather than by NO_3^-.

Our results also show clearly that some water extrusion is able to continue when both of the above systems should be inhibited, i.e. upon incubation in the presence of ouabain either together with furosemide or in the absence of Cl^- (Tables 2 and 3). We have no indication of the mechanism of such a third component of water extrusion, at present. It seems at least possible that it may be present mainly in those nephronal segments, principally the glomeruli, loops of Henle and collecting tubules, which show little vesicle formation and yet recover their structure well at 25°C. Further experiments suggest that this third mechanism is not inhibited by cytochalasins, whereas the vesicular mechanism is blocked (van Rossum, Russo & Ernst, *in preparation*).

These results with kidney may be compared with our previous work with liver slices (Russo et al., 1977; van Rossum & Russo, 1981, 1984). In the latter, the existence of an ouabain-sensitive mechanism of volume regulation is much clearer, and together with the ouabain-resistant vesicular system it can account for practically all the water-extruding capacity. An apparent contrast between the tissues is that the exocytotic expulsion of vesicular water from liver cells appears to take place at the canalicular (i.e. apical) cell membrane, whereas in the kidney cells it appears to occur at the basolateral membrane. Nevertheless, there is a striking similarity

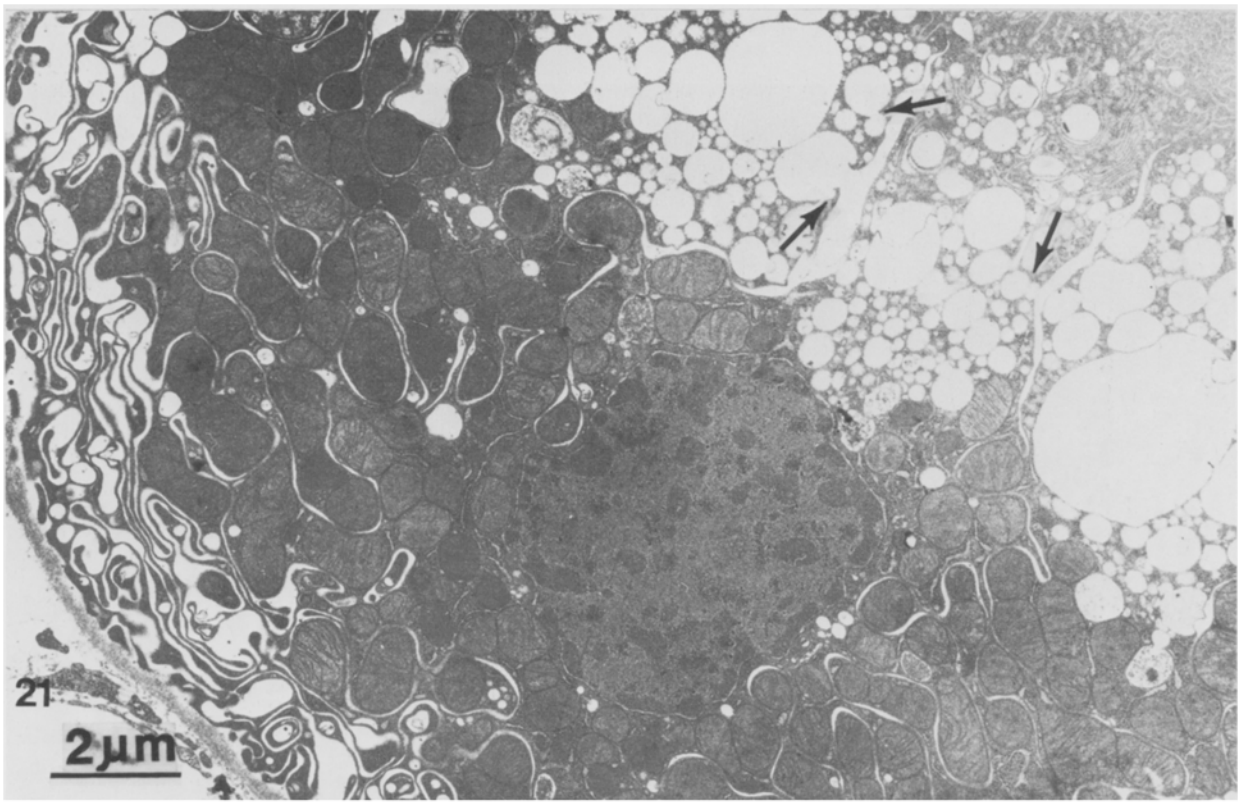

Fig. 21. Part of proximal tubule after incubation for 90 min at 1°C followed by 60 min at 38°C. A large number of vesicles of many sizes are present, mainly localized at the apical region. The appearance is similar to that of slices incubated with ouabain at 25°C (*cf.* Fig. 15) except for the larger number of vesicles at 38°C. Many vesicular profiles show fusion with neighboring vesicles and with the basolateral plasma membrane bordering focal expansions of the intercellular spaces (arrows). 8,000×

Fig. 22. Detail of proximal tubule after incubation for 90 min at 1°C and 60 min at 38°C in the presence of 2 mM ouabain. Vesicles pack the apical cytoplasm and in many places fuse with one another to form chains (arrows). 3,800×

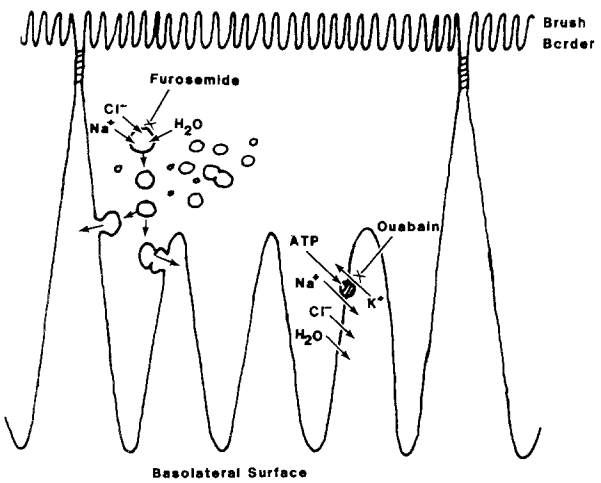

Fig. 23. Schematic diagram of the two mechanisms proposed for the extrusion of water, Na^+ and Cl^- from cells of proximal and distal tubules. In the left-hand apical portion of the epithelial cell is shown the ouabain-resistant entry of ions and water into apical vesicles, followed by fusion of vesicles with the basolateral plasma membrane. In the right-hand, basal portion is indicated the ouabain-sensitive extrusion of water. For further details, *see* text

between the results we have obtained with these two tissues which tends to reinforce the suggested importance of a vesicular mechanism for water extrusion as being one way in which cells are able to regulate their volume.

This work was supported by grant no. R806616 from the U.S. Environmental Protection Agency and grant no. AM 27559 from the U.S. Public Health Service. M.A.R. received support from the Fondazione "Assicurazioni Generale," Trieste, Italy. We are most grateful to Mrs. O.O. Holowecky, Miss B. DeFeo and Messrs. C. Franchi, J. Palacios and J. Schreiber for technical, and Mrs. G. King for secretarial assistance.

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Received 5 June 1984; revised 13 November 1984