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Relative Effects of Furosemide and Ethacrynic Acid on Ion Transport and Energy Metabolism in Slices of Rat Kidney-Cortex

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Summary. The effects of furosemide and ethacrynic acid have been studied using slices of rat kidney cortex incubated in a Ringer medium. At concentrations from 0.2–2.0 mM, furosemide had no significant effect on the tissue ATP content or on the metabolism-dependent net movements of intracellular Na^+, K^+ and Ca^{2+}. It did, however, induce an increase in the net, outward movement of Cl^-; we suggest that this may have arisen from inhibition of a Cl^- accumulating mechanism. In contrast, ethacrynic acid in the same concentration range caused marked reduction of cell respiration and ATP content and virtually total inhibitition of several processes of ion transport (Na^+, Cl^- and Ca^{2+} loss, and K^+ uptake). Concentrations of furosemide greater than 5 mM caused marked inhibition of energy metabolism and transport of ions, and 10 mM furosemide had quantitatively similar effects to 2 mM ethacrynic acid. Electron micrographs of kidney-cortex slices treated with the diuretics at 2 mM show that the ultrastructure was well maintained in the presence of furosemide but that ethacrynic acid caused severe structural disorganisation and necrosis. The mitochondria were generally in the orthodox configuration in the presence of furosemide, but swollen in ethacrynic acid in accord with the marked effects of 2 mM ethacrynate on mitochondrial energy metabolism. Of the effects we have detected, that of low concentrations of furosemide on Cl^- movement appears to be rather specific. Higher concentrations of this agent (5 mM and above), and all concentrations of ethacrynic acid studied (0.1–5.0 mM), have several inhibitory effects which seem to result from primary inhibition of mitochondrial activities and are presumably manifestations of toxicity.

Key words: Furosemide – Ethacrynic acid – Cl^- transport – Cation transport – Energy metabolism – Kidney cortex

Introduction

Furosemide and ethacrynic acid have very similar diuretic effects in vivo and both have been reported to inhibit Cl^- reabsorption in the ascending limb of the loop of Henle (Burg and Green 1973; Burg et al. 1973). They have been used experimentally as inhibitors of specific Na^+ and Cl^- transport processes in a variety of tissues (Cabantchik et al 1978; Candia 1973; Frizzell et al. 1979; Petersen et al 1979; Whittembury and Proverbio 1970). On the other hand, both of these diuretics have been shown to inhibit energy-conserving metabolism of isolated mitochondria (Foucher et al. 1969; Gemba 1974; Manuel and Weiner 1976), an effect which, if it were to occur in intact cells, could contribute to inhibition of transport processes as a result of a reduced availability of ATP.

At rather high concentrations, ethacrynic acid has been found to inhibit the O_2 consumption of renal cortical slices (Jones and Landon 1967; Macknight 1969). Cunarro and Weiner (1978) found that both ethacrynic acid and furosemide reduced the respiration of tubules isolated from renal cortex and outer medulla. The sensitivity of the respiratory inhibition to the ionic composition of the medium, and the close coupling between ion transport and O_2 consumption in kidney cortex (Blond and Whittam 1964), led Cunarro and Weiner (1978) to suggest that the effects of the diuretics on respiration were secondary to an inhibition of Cl^- transport. This interpretation would receive support if it could be shown that tissue ATP levels were not reduced by the diuretics. However, over the range of ethacrynate concentrations (0.2–1.0 mM) studied by Cunarro and Weiner (1978), we have found a close parallel between the inhibition of Na^+ and K^+ transport and declining tissue contents of ATP in kidney-cortex slices (van Rossum and Ernst 1978) so that, at least with this diuretic, the inhibition of respiration and ATP synthesis appeared to be the primary effect.

In view of these findings, we felt it important to carry out a direct comparison of the effects of a range of concentrations of the two diuretics on ion movements and energy metabolism in kidney-cortex slices, and to support these with morphological studies. Furosemide was found to be much less potent than ethacrynic acid as an inhibitor of cell respiration and our results in general support the conclusion of Cunarro and Weiner (1978) that the concentration of furosemide which they used had a primary effect on Cl^- movements. Higher concentrations of furosemide, and a wide range of ethacrynic acid concentrations, caused marked reduction of respiration and ATP contents, presumably representing toxic effects.

Methods

The methods used for preparation and incubation of slices of rat kidney-cortex and for analysis of the tissue contents were those used by van Rossum and Ernst (1978). The general experimental procedure was to pre-incubate the slices at 1° C for 90 min in order to cause depletion of cell K^+ and to increase the cell contents of Na^+, Ca^{2+} and water, and then to study the metabolism-dependent reversal of these changes during

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Table 1. Effects of incubation conditions on the composition of kidney-cortex slices

Incubation	Protein/ dry wt	Water content (kg/kg dry wt)	K^+ content (mmol/kg dry wt)
90 min at 1°C	0.63 ± 0.05	4.56 ± 0.21	132 ± 5
90 min at 1°C followed by 60 min at 25°C:			
Control	0.56 ± 0.02	3.10 ± 0.14	261 ± 4
Furosemide (10 mM)	0.52 ± 0.02	4.44 ± 0.40	149 ± 18
Ethacrynic acid (5 mM)	0.51 ± 0.04	6.05 ± 0.23	68 ± 5

After the incubation indicated, slices in each incubation vessel were divided into two groups and weighed; one group was analysed for dry wt., water and ion contents and the other for protein. Each value is the mean ± SEM of 8 observations

incubation for 60 min in an oxygenated medium at 25°C. The medium contained (mM): Na^+ 139.0, K^+ 5.0, Ca^{2+} 1.2, Mg^{2+} 1.0, Cl^- 153.4, SO_4^{2-} 1.0, phosphate 2.0, Tris 10.0 and glucose, 10.0. The pH was 7.4 and the medium and reaction vessels were gassed with O_2. Inulin (0.5% w/v) was present as a marker for the extracellular water compartment. The diuretics were added to the media at the 30th minute of pre-incubation at 1°C, so that they were in contact with the tissue for 60 min at 1° and throughout the incubation at 25°C. Readings of O_2 consumption were taken manometrically at 25°C, after an initial 10 min period of equilibration.

Samples of the slices were collected for analysis at the end of the pre-incubation period at 1°C, and the remaining slices after incubation at 25°C. Slices were collected on a filter paper maintained under suction. They were then either weighed, dried and treated with 0.1 N-HNO_3 to extract ions, in which case the composition was expressed relative to the dry wt, or they were homogenised in $HClO_4$(8% v/v) in ethanol (40% v/v) at −20°C to extract ATP and Na^+, when the results were related to the slice protein. For further descriptions of the extraction and assay procedures, see van Rossum (1972) and van Rossum and Ernst (1978).

In order to permit a comparison of results expressed per unit dry wt with those expressed per unit protein, as well as to see whether these bases of reference were markedly affected by the different incubation conditions, we determined the ratio, protein/dry wt, in kidney-cortex slices analysed after incubation as follows: I) 90 min at 1°C; II) 90 min at 1°C followed by 60 min at 25°C; as in II) but in the presence of III) 10 mM furosemide or IV) 5 mM ethacrynic acid. These represented the extremes of the conditions employed in our work and resulted in wide differences of tissue water and K^+ (Table 1). Nevertheless, the ratio, protein/dry wt, was not significantly altered by any of the treatments, although there was a slight tendency for it to decline during incubation at 25°C, especially in the presence of the very high concentrations of diuretics. We conclude that the differences of tissue composition reported in Results at varying diuretic concentrations were not markedly affected by changes in the proportions of protein and dry wt.

The results are expressed as mean ± standard error of the mean (number of observations), where each observation represents the value obtained from the contents of a single incubation vessel. In each experiment, duplicate or triplicate vessels were run for each diuretic concentration tested. Statistical significance of differences was examined by "Student's" *t*-test.

The fixation and treatment of tissue slices for electron microscopy were in general as described by Russo et al. (1977). A comparison was made between fixation in 2.5% glutaraldehyde in the Ringer medium used for experimental incubation of the slices, and 2.5% glutaraldehyde made up in 0.1 M phosphate buffer (pH 7.3); there was no apparent difference between the results. The micrographs presented below are of tissues treated with the latter fixative.

Furosemide was generously provided by Hoechst-Rousell Pharmaceuticals, Inc., Somerville, NJ, USA, and ethacrynic acid by Merck, Sharp & Dohme, West Point, PA, USA.

Results

The effects of increasing concentrations of furosemide on respiration and on the changes of water and ionic content of slices during incubation at 25°C are illustrated in Fig. 1. The diuretic caused a reduction of the rate of O_2 consumption which became statistically significant ($P < 0.01$) at concentrations of 2 mM and above.

Comparison of slices analysed after pre-incubation at 1°C with those analysed after further incubation at 25°C shows a number of changes of intracellular ionic content which can be attributed to metabolism-dependent, ion-transporting activities (Kleinzeller 1972; Macknight 1969; Mudge 1951; van Rossum and Kapoor 1977). Control slices (i. e. incubated in the absence of furosemide) showed a net loss of intracellular Na^+ and Cl^-, a gain of K^+ and loss of total and intracellular water (Fig. 1). At the same time, the Ca^{2+} content decreased (Table 2).

When slices were incubated with 0.5 − 5.0 mM furosemide, the intracellular Cl^- content attained after 60 min at 25°C was significantly lower than that of control slices (Fig. 1) as the result of a greater net loss of this anion; e. g. the net loss of intracellular Cl^- during incubation at 25°C amounted to 195 ± 22(9) mmol/kg dry wt in control slices and to 260 ± 15(9) mmol/kg in presence of 0.5 mM furosemide ($p = 0.01$).

At a concentration of 0.5 mM, furosemide had no significant effect on intracellular cation contents. Increasing the concentration to 5 mM progressively increased the amount of intracellular Na^+ retained (Fig. 1b), and this was accompanied by small increases in the tissue water content (Fig. 1a),

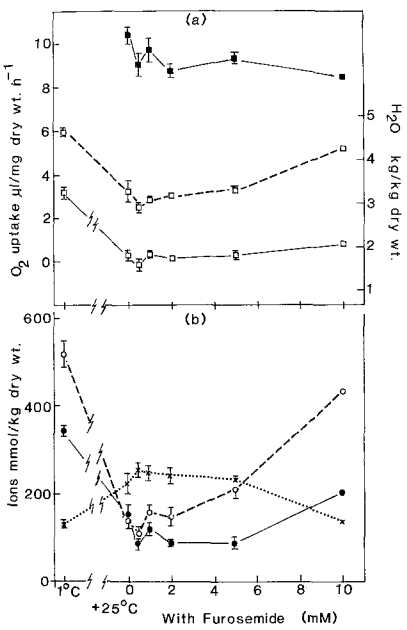

Fig. 1 a and b. Effects of furosemide on the contents of ions and water and on respiration of kidney-cortex slices. The slices were pre-incubated for 90 min at 1°C followed by incubation for 60 min at 25°C in the presence of the concentrations of furosemide indicated. "Intracellular" composition was determined by subtracting the contribution of the inulin-accessible water compartment from the total tissue composition. Each point is the mean ± SEM of 9 observations, except those at 10 mM furosemide which are the mean of 2. **(a)** ■ Rate of respiration; —□— Intracellular water; – –□– – Total water. **(b)** ● intracellular Cl^- content; ○ intracellular Na^+ content; × intracellular K^+ content

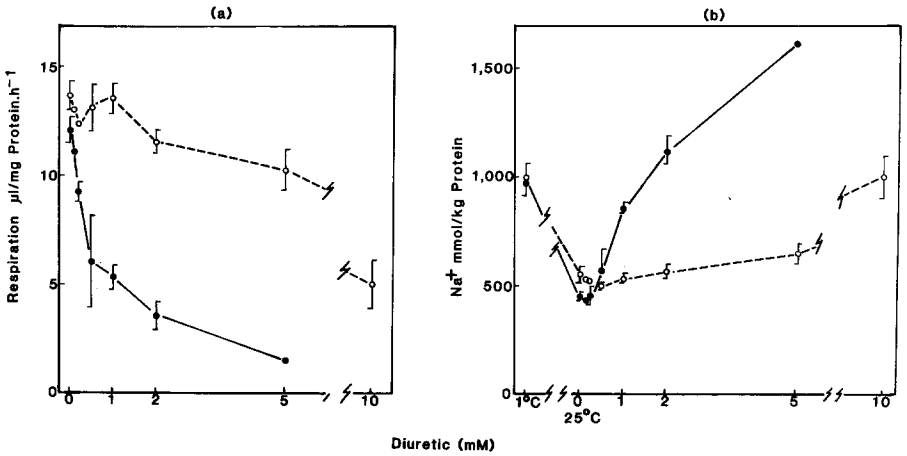

Fig. 2
Effects of ethacrynic acid (●) and furosemide (○) on (**a**) respiration and (**b**) Na^+ content of kidney-cortex slices. Incubation procedure as for Fig. 1. Each point is the mean ± SEM of 4–12 observations for furosemide and of 4 observations for ethacrynic acid

Table 2. Effect of different concentrations of furosemide on the Ca^{2+} content of kidney-cortex slices. Results are from the same experiments as Fig. 1

Incubation	Ca^{2+} content (mmol/kg dry wt)
90 min at 1°C with 0 or 5 mM furosemide[a]	29.8 ± 2.2 (8)
90 min at 1°C followed by 60 min at 25°C with furosemide (mM):	
0	17.0 ± 1.7 (9)
0.5	16.8 ± 1.0 (9)
1.0	16.3 ± 1.5 (9)
2.0	15.7 ± 1.2 (9)
5.0	16.4 ± 1.5 (9)
10.0	23.4, 20.6[b]

[a] Furosemide had no effect on the Ca^{2+} content at 1°C and the results with and without the diuretic have therefore been pooled
[b] Two single observations

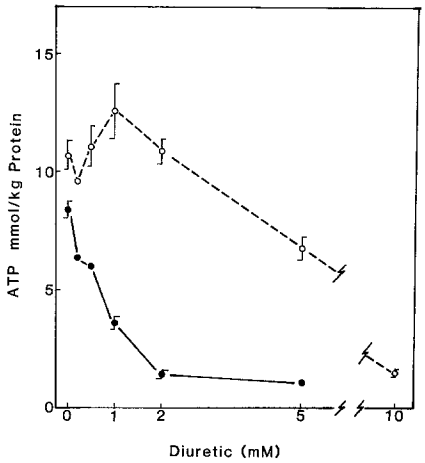

Fig. 3. Effects of ethacrynic acid (●) and furosemide (○) on the ATP content of kidney-cortex slices. Results are from the same experiments as Fig. 2

but neither K^+ accumulation (Fig. 1b) nor the net loss of Ca^{2+} (Table 2) were significantly affected. A further increase of furosemide to 10 mM brought about a substantial inhibition of all the metabolism-dependent transport activities. Thus, it totally prevented K^+ accumulation at 25°C and greatly increased retention of Na^+, as well as inducing a greater retention of Cl^- (Fig. 1b) and Ca^{2+} (Table 2) than that seen in the control slices.

The substantial inhibition by 10 mM furosemide of the metabolism-dependent changes in slice ionic content were reminiscent of the effects of ethacrynic acid, except that with the latter a concentration of 1–2 mM was sufficient to cause an even larger degree of inhibition (van Rossum and Ernst 1978). We therefore next compared the concentration-dependence of the effects of the two diuretics on O_2 consumption, determined during the course of incubation, and slice ATP content, determined at the end of 60 min incubation at 25°C. It should be pointed out that control slices maintain a constant ATP content from the 15th to at least the 60th minute at 25°C (van Rossum and Kapoor, in press).

In this series of experiments, low concentrations of furosemide had effects on respiration similar to those seen in Fig. 1a, with an inhibitory trend becoming statistically significant ($p = 0.01$) at 2 mM (15% inhibition, Fig. 2a). In contrast, furosemide at 0.2–2.0 mM caused no significant alteration of ATP content (Fig. 3). At higher concentrations, furosemide induced marked reductions of ATP content and rate of respiration, changes wich were accompanied by a considerably greater retention of Na^+ (Fig. 2b). The inhibition of respiration given by 10 mM furosemide in this series of experiments was 66%, a value considerably greater than the 20% inhibition seen in the experiments of Fig. 1. The reason for this difference is not clear.

The results of Figs. 2 and 3 also show that energy metabolism was more sensitive to ethacrynic acid than to furosemide at the same concentration. For example, a reduction of ATP by approximately 30% was given by 0.2 mM ethacrynic acid or by 5 mM furosemide, while 85% reduction was given by, respectively, 2 and 10 mM. In each case, the increased retention of Na^+ was closely related to the fall of O_2 consumption and ATP content.

The morphological appearance of slices was studied after incubation in the absence or presence of each diuretic at a concentration of 2 mM. The appearance of control slices after incubation for 90 min at 1°C followed by 60 min at 25°C was similar, in about 80% of the volume of each slice, to that of fresh (unincubated) kidney-cortex tissue. Necrosis was observed in approximately 20% of the slice volume. A large proportion of the necrotic cells was found in the peripheral zone of the slices that had clearly undergone damage during cutting and experimental handling of the tissue; the remaining necrotic cells were scattered throughout the tissue with no apparent localisation to particular regions of the slice or specific segments of the nephrons. In the case of the viable

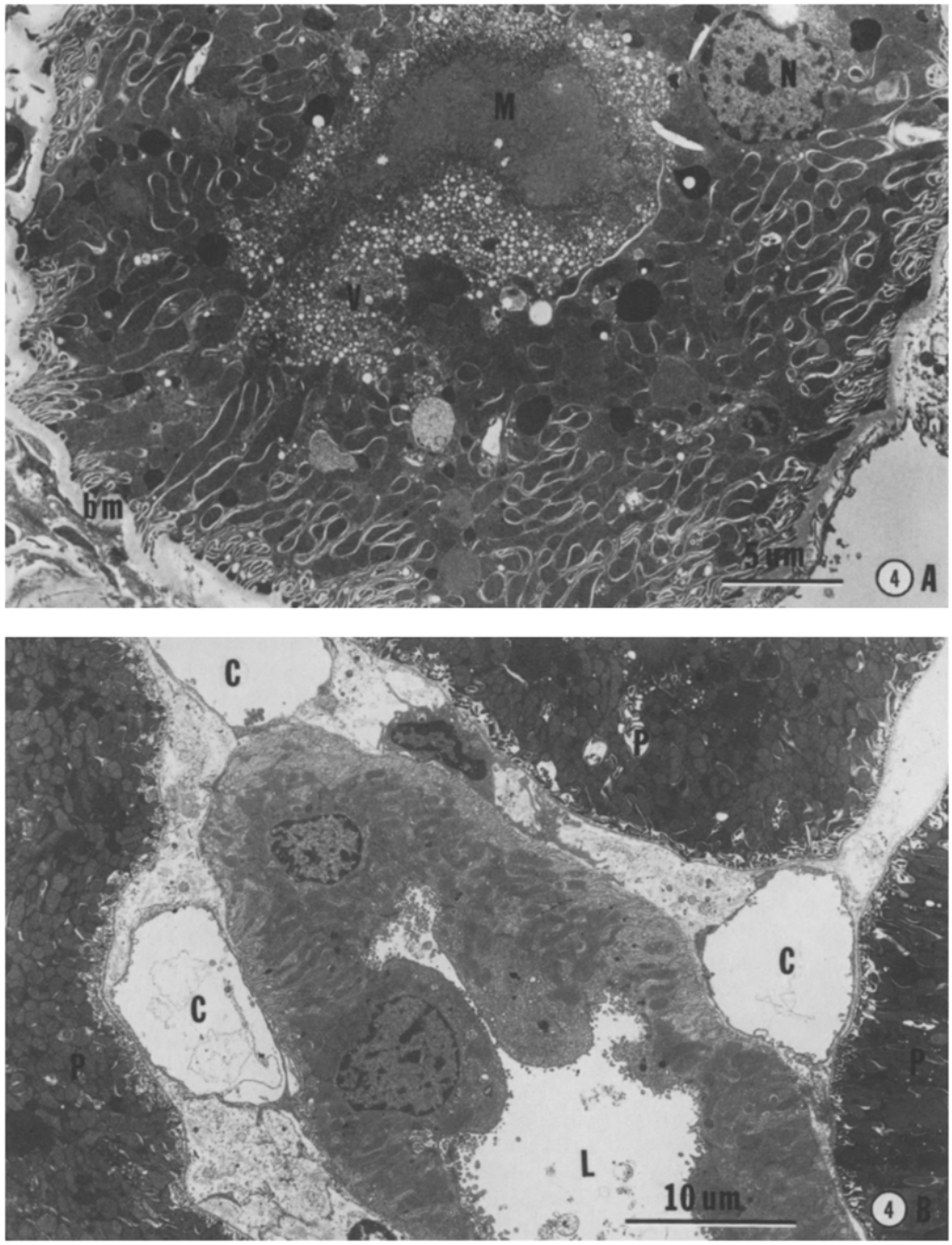

Fig. 4. a Electron micrograph at low magnification of part of proximal tubule in a control slice (i. e. incubated without diuretics) after incubation for 90 min at 1° C and 60 min at 25° C. The majority of the cells are in a very good state of preservation. The apical regions of the cells surrounding the lumen show a number of small vesicles (*V*) and well-preserved microvilli of the brush border (*M*). *BM* basement membrane; *N* nucleus. × 4,600. **b** Low magnification of part of kidney-cortex slice after incubation with 2 mM furosemide showing portions of two proximal tubules (*P*) and one distal tubule. The tissue is well preserved, except that the proximal tubule cells show some degree of swelling and some disorganisation of baso-lateral infoldings; the proximal tubule cells are generally similar to those of the control slice in **a**. In the distal tubule (centre of micrograph) vesicles of various sizes are present. *C* capillaries; *L* lumen of distal tubule. × 3,200

80 % of cells, the most noticeable differences from fresh tissue were that the volume of the cells appeared to be somewhat reduced, the ground substance more electron dense and the extracellular spaces, especially in the basal infoldings, somewhat expanded (Fig. 4a); these differences may be explained by some contraction of cell volume.

The overall appearance of slices after incubation with 2 mM furosemide was very similar to that of the control slices (Fig. 4b), a finding in general accord with most of the analytical data discussed above. In contrast, slices incubated with 2 mM ethacrynic acid had a marked appearance of general necrosis and disorganisation (Fig. 5). This was particularly true of proximal tubular cells, in which the cytoplasmic ground substance exhibited varying degrees of condensation, clumping and vesiculation, often accompanied by disruption or loss of basolateral plasmalemmal infoldings;

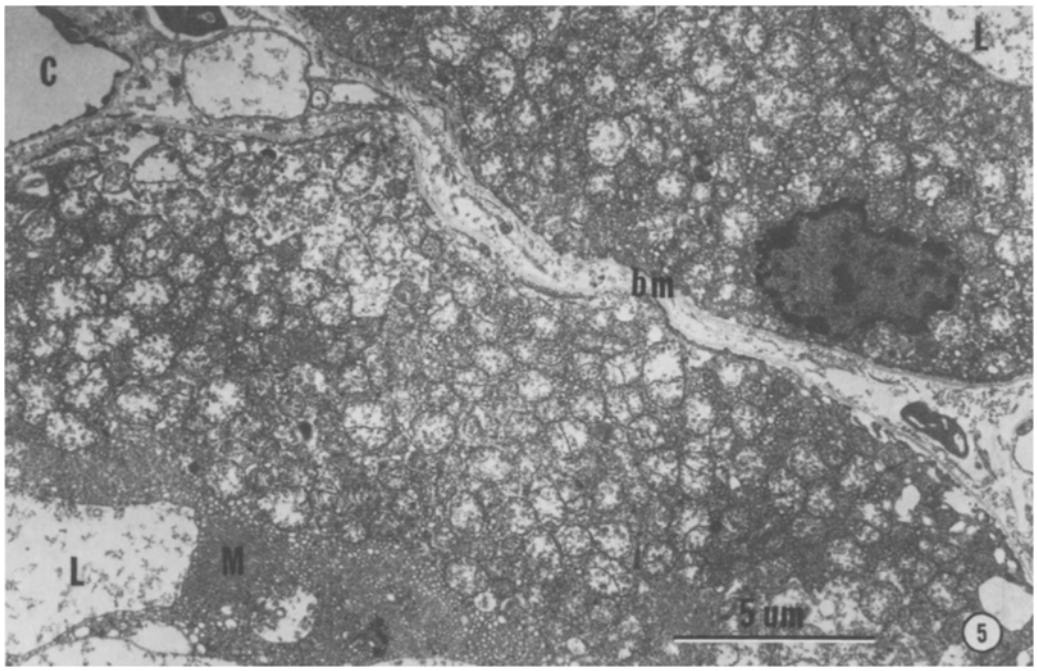

Fig. 5. Kidney-cortex slice after incubation with 2 mM ethacrynic acid. The figure illustrates parts of two proximal tubules which show the general appearance of damage caused by ethacrynic acid, as discussed in the text. In addition, note the nuclear changes, particularly the alterations of the nuclear membranes and the clumping of chromatin, which are similar to those seen in liver (Russo and van Rossum, in preparation). *C* Capillary; *BM* basal membrane; *L* tubular lumen. ×6,900

these changes are similar to those reported by Petersen et al. (1979) with Guinea pig gall bladder. Generally, distal tubules were less affected by ethacrynic acid, although some showed moderate disruption of fine structure.

The morphology of mitochondria in the slices was of especial interest, in view of the effects of the two diuretics on respiration. Mitochondria in all nephron segments of the control slices were similar to those of fresh tissue; they were almost all in the classical orthodox configuration, with a matrix of medium density, parallel cristae and some dense particles (Fig. 6a). Mitochondria in the furosemide-treated slices (Fig. 6b) were also in a well-preserved, orthodox form, but they were largely without dense granules and their matrix frequently appeared less electron dense than in control slices. These latler findings are often indicative of a mild disruption of energy metabolism in mitochondria and appear to be consistent with our finding that 2 mM furosemide is the lowest concentration at which we observed a significant decline of respiratory rate in the slices. In the slices incubated with ethacrynic acid, all mitochondria were swollen to a degree which varied from a slight swelling, in which they were still rod-shaped but had a less dense matrix and completely lacked dense granules, to high-amplitude swelling in which they were spherical, the matrix light and inhomogeneous and the cristae fragmented and vesiculated (Figs. 5 and 6c).

The only other region of the cell showing some affect of incubation with furosemide was the apical region. This was usually well preserved, but there were occasional areas in wich the microvilli showed fragmentation (Fig. 6d). In contrast, the microvillar borders of proximal tubules in slices treated with ethacrynate were consistently in various states of disorganisation and apparent dissolution, while the tubular lumina were often filled with cellular debris and microvillar remnants (Fig. 6e).

Discussion

The readjustments of ionic composition that take place when kidney-cortex slices which have been pre-incubated at 1°C are incubated at 25°C are brought about by the metabolism-dependent transport of ions (Mudge 1951). A number of different transport processes is involved. They include an oubain-sensitive uptake of K^+ coupled to an extrusion of intracellular Na^+, as well as a second component of Na^+ loss, insensitive to ouabain, which occurs together with a net loss of intracellular Cl^- and water (Kleinzeller 1972; Macknight1969; Whittembury and Proverbio 1970). A net reduction of Ca^{2+} content also occurs, probably as a result of extrusion by yet another transport system (Gmaj et al. 1979; van Rossum and Kapoor 1977).

As anticipated from the above, intracellular Cl^- in our experiments showed a net, outward movement from the kidney-cortex slices at 25°C but, unexpectedly, this movement was increased by about 30% in the presence of furosemide concentrations between 0.5 and 5.0 mM. The changes in tissue composition of other ions were not significantly affected by furosemide, unless its concentration was at least 5 mM. To account for this affect on Cl^-, furosemide must either have stimulated a mechanism which, directly or indirectly, extruded Cl^- from the cells, or it must have inhibited a mechanism which normally accumulated Cl^-. Since the only known effects of furosemide on Cl^- movements are inhibitory (e. g. Burg et al. 1973; Frizzell et al 1979), we favour the latter explanation. The inhibition of a transport system required by this explanation is consistent with the results of Cunarro and Weiner (1978), who found a chloride ion-dependent inhibition of respiration in tubules of kidney cortex by 1 mM furosemide (the only concentration they studied). These workers suggested that furosemide acted by

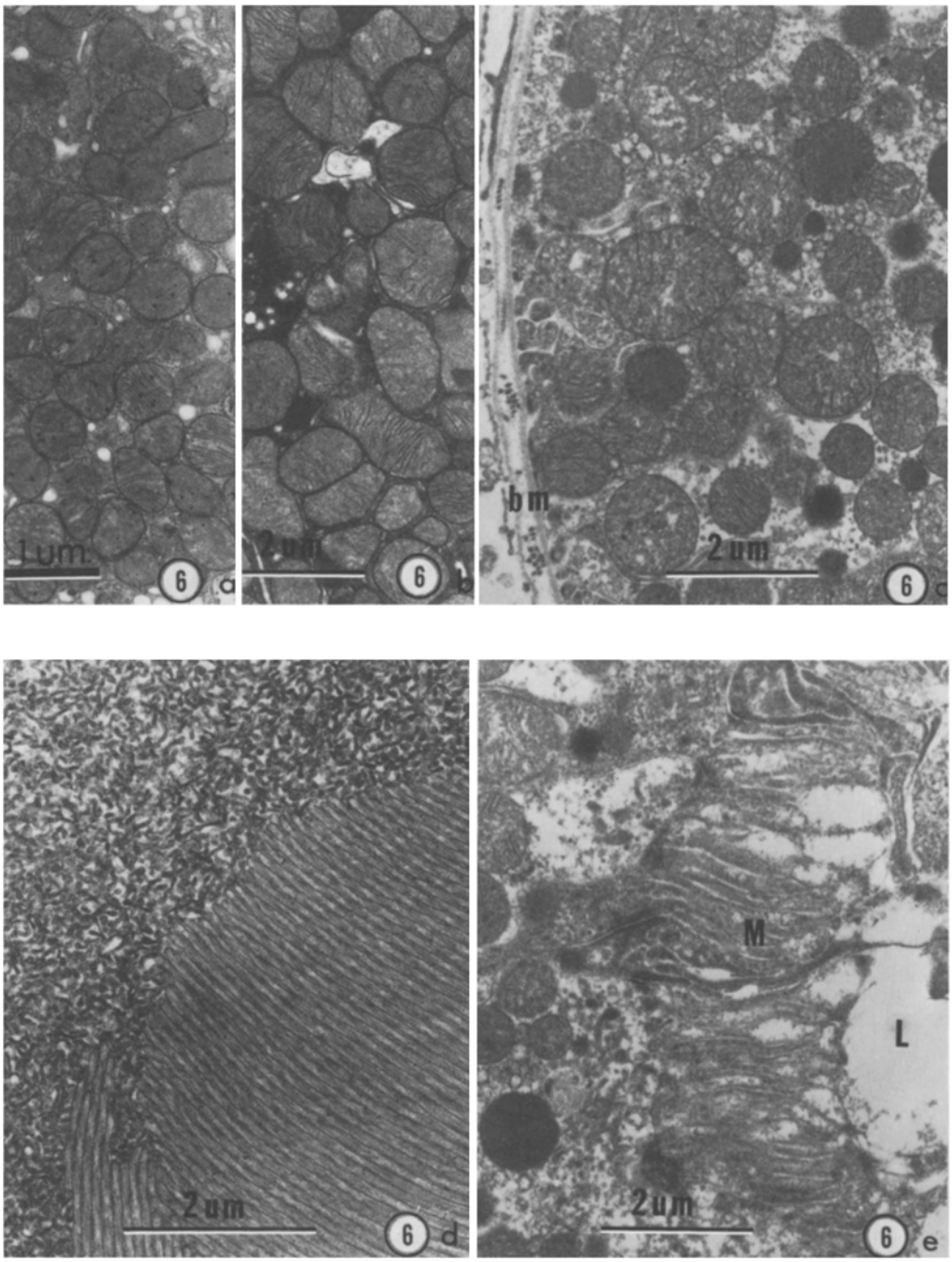

Fig. 6. Details of mitochondria (**a—c**) and apical regions (**d, e**) of kidney cortex slices after incubation for 90 min at 1°C and 60 min at 25°C in the absence or presence of diuretics. **a** Mitochondria from proximal tubule of a slice incubated without diuretics. The mitochondria are in the orthodox conformation and show dense granules. Small vesicles of the Golgi apparatus and smooth endoplasmic reticulum can also be seen. Mitochondria from distal tubules were very similar (not illustrated). ×17,000. **b** Mitochondria from proximal tubule of slice incubated with 2 mM furosemide; they are in the orthodox form but lack dense granules and have a less dense matrix than the control mitochondria in **a**. ×12,000. **c** Mitochondria from slice incubated with 2 mM ethacrynic acid. These mitochondria are in the best preserved state noted with ethacrynic acid, the more usual appearance being that of the mitochondria seen in Fig. 5. *BM* basement membrane. ×13,500. **d** Detail of brush border of kidney cortex slice incubated with furosemide. Well preserved microvilli are seen in transverse section, together with a region (upper left) in which the microvilli have become fragmented and disorganised. ×17,000. **e** Detail of apical portion of proximal tubule in a kidney cortex slice incubated with 2 mM ethacrynic acid. For description, see text. *M* microvilli; *L* lumen of proximal tubule. ×13,500

directly inhibiting a Cl^- transport system, with a consequent, secondary inhibition of respiration, rather than by directly inhibiting mitochondrial respiration. Our finding that the ATP content of slices was not reduced over most of the range of concentrations (0.2 – 2.0 mM) at which furosemide reduced slice Cl^- content, supports their interpretation.

The precise nature of the furosemide-sensitive mechanism which, we speculate, accumulates Cl^- in the cortical cells and, in particular, whether it is coupled to Na^+ movements (Frizzell et al 1979), cannot be determined from our experiments. We can, however, assert that it is probably not related to the Cl^- transporting mechanism of the ascending thick

limb of the loop of Henle (Burg et al. 1973), as our morphological studies show these regions of the nephron to be practically absent from our cortical slices.

At higher concentrations (5–10 mM), furosemide induced closely parallel effects on tissue ATP contents and several aspects of cellular ionic composition. The same was true for ethacrynic acid, except that the effects were observed over the whole range of concentrations tested, from 0.1 mM upwards (see van Rossum and Ernt 1978, for the effects of ethacrynate on ions other than Na^+). We conclude that these effects were all due to primary, inhibitory actions of the two diuretics on mitochondria, which are related to their known inhibition of respiration in isolated mitochondia (Foucher et al. 1969; Gemba 1974; Manuel and Weiner 1976). The importance of the mitochondria as the site of action of the higher concentrations of furosemide in the slices is also indicated by the finding that, at 2 mM, the diuretic induced minor morphological modifications in the slice mitochondria of a type presaging alteration of oxidative phosphorylation (i. e. loss of dense granules and matrix density), while few other changes were noted in the cells.

Our results agree with the work of Manuel and Weiner (1976) and of Cunarro and Weiner (1978) in showing that ethacrynic acid is a more potent inhibitor of mitochondrial activity than furosemide, and that this difference is seen in whole cells. Over the concentration range studied, we have been unable to distinguish an effect of ethacrynate on ionic movements which could be dissocated from a fall of tissue ATP content. At first sight this appears remarkable since Burg and Green (1973) had to perfuse the lumina of isolated nephrons with at least 0.5 mM ethacrynic acid order to obtain a significant inhibition of Cl^- transport. But our results may be at least partly due to the long incubation times we have employed (i. e. 60 min at 25° C) since some preliminary results suggest that the ATP content of cortical slices treated with 2 mM ethacrynic acids falls by less than 30% in the first 15 min (Russo and van Rossum, unpublished observations). In general, may of the effects of the two diuretics that we have observed would appear to be of a toxic, rather than a specific, pharmacological nature. This is indicated by the decline of ATP, non-specific inhibition of several ion-transport processes and, in the case of ethacrynic acid, marked morphological alterations. However, the effects of furosemide at concentrations up to 1–2 mM are of a more specific nature.

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