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Effects of Ethacrynic Acid on Ion Transport and Energy Metabolism in Slices of Avian Salt Gland and of Mammalian Liver and Kidney Cortex

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Summary. Ethacrynic acid greatly inhibited net transport of ions and aerobic, energy-conserving metabolism in slices of avian salt gland, rat liver, and rat and guinea-pig kidney cortex. The effects of increasing concentrations of ethacrynic acid on the transport of Na^+, K^+ and Cl^- ran closely parallel to its effects on tissue ATP levels and respiration. The concentration needed for maximal inhibition of transport reduced ATP levels by 80–90%. Respiration was reduced by 80–90% in salt gland and kidney cortex, and by a maximum of 30% in liver slices. The effects of low concentrations of ethacrynic acid required time to become fully manifest in some tissues, and the development of transport inhibition followed a similar course to decline of respiration and ATP levels. Ca^{2+} extrusion by liver cells was inhibited by ethacrynic acid. The concentration dependence of the inhibition was similar to that shown by the other transport systems inhibited. There was no distinction evident between the sensitivity of Na^+ extrusion and of K^+ accumulation to the diuretic. Lactate production increased as respiration decreased in the presence of increasing concentrations of ethacrynic acid. We conclude that ethacrynic acid acted primarily as an inhibitor of mitochondrial respiration and ATP synthesis in the tissue slices, and that inhibition of ion transport was a nonspecific consequence of the failure of the energy supply.

Ethacrynic acid is believed to exert its diuretic action mainly by inhibiting the reabsorption of NaCl in the ascending limb of the loop of Henle [3], but the mechanism of the inhibition is unresolved [14]. Some workers have provided evidence for a direct inhibition of Na^+ transport processes and ion-sensitive adenosine triphosphatases [17, 25, 28] and have suggested that ethacrynic acid specifically inhibits a ouabain-insensitive, Na^+ transport system which has been detected in kidney and other tissues [12, 23]. However, the diuretic also inhibits glycolysis [10, 11] and aspects of mitochondrial oxidative metabolism [7, 8, 15, 24]. Inhibi-

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tion of the synthesis of ATP provides an alternative means by which ion transport could be blocked, but in this case a more generalized inhibition of cellular activities might be anticipated. A decision as to which of these effects represents the primary mode of action of ethacrynic acid requires a combined study of its effects on ion transport and energy-conserving metabolism. To date, MacKnight's [13] and Epstein's [5] comparisons of the effects of a single, high concentration of the diuretic on ion movements, respiration, and ATP content in slices of rat and rabbit kidney cortex are the only studies which approach this requirement. These authors concluded that the inhibition of energy metabolism could be the main cause of transport inhibition, at least in the slice preparation.

The concentrations of ethacrynic acid required to produce any of the effects described above are higher than may be anticipated to occur during its pharmacological use *in vivo*, and their bearing on the mechanism of diuresis is therefore open to question. However, the suggested specificity of ethacrynic acid as an inhibitor of ouabain-insensitive Na^+ transport [28] makes it important to clarify the mode of action of this agent in experimental preparations *in vitro*.

In preliminary experiments with slices of rat liver and avian salt-gland we found that ethacrynic acid not only inhibited Na^+ extrusion, but also drastically reduced both K^+ accumulation and respiration. In order to distinguish between a primary action on ion transport or energy metabolism, we made a study of the adenine nucleotides in the slices. Further, in an attempt to resolve the opposing conclusions reached in experiments with kidney slices from guinea pig on the one hand [28, 17] and from rat on the other [13], we have also done experiments with slices of kidney cortex from both these species. We find that ethacrynic acid has a marked effect on energy-conserving metabolism in these four tissues and has no specificity as an inhibitor of Na^+ transport as compared to K^+ transport. The primary mode of action in the slices appears to be an inhibition of the mitochondrial synthesis of ATP.

Materials and Methods

Animals and Tissue Preparations

Domestic ducks (*Anas platyrhynchos*) received a few days after hatching were maintained on a commercial chick starting mash, and subjected to osmotic stress by inclusion of 1% NaCl in their drinking water. They were kept for at least 30 days before use, in order

to ensure full development of the salt gland [6]. The ducks were decapitated and their salt glands dissected out with a scalpel. The glands were placed on a hardened (Whatman No. 54) filter paper moistened with Ringer's solution and cooled on a Petri dish containing crushed ice. Surface connective tissue was removed as thoroughly as possible and the glands were then cut into slices (approx. 0.2–0.3 mm thick) in the dorso-ventral plane. Slicing was done with a razor blade guided with a glass slide. Depending on their size, 2 or 3 birds were used for each experiment, the slices from all the glands being randomized by stirring in a beaker of cold Ringer's solution.

Albino rats of a Wistar strain were used at a weight of 200–400 g. They were fed *ad libitum* on a standard diet throughout. They were killed by decapitation and liver slices prepared as described previously [4]. The kidneys were cut throught the hilum, and the papilla and red medulla were carefully dissected out. The remaining outer, brown portion was considered to be the cortex and was sliced as for the salt gland. Two to four kidneys provided enough material for an experiment, the slices being randomized as above. Histological studies show that the slices were composed primarily of cortex with only occasional small areas of outer medulla present.

The guinea pigs used were female albinos (Hartley strain) weighing approximately 500 g. Slices of kidney cortex were prepared as for the rat; the organs of one animal were sufficient for an experiment. Microscopic examination of the slices showed a similar histological composition to the slices of rat kidney cortex.

Incubation

The Tris-buffered Ringer's solution used in most of the experiments contained (in mM): 147 Na^+, 5 K^+, 1 Mg^{2+}, 1.2 Ca^{2+}, 164 Cl^-, 1 SO_4^{2-}, and 10 Tris (pH 7.4). On occasion, a phosphate buffered Ringer's solution was used in which 10 mM phosphate (pH 7.4) replaced the Tris, and the Na^+ and Cl^- concentrations were 165 and 154 mM, respectively; other components were as in the Tris Ringer's. The media were gassed with O_2. No substrates were added unless noted in the text. In experiments with salt gland and liver, the media also contained 0.5% w/v inulin as a marker for the extracellular water compartment. Inulin and ouabain were obtained from Sigma Chemical Co., St. Louis, Mo., and ethacrynic acid was a gift from Merck, Sharp and Dohme.

The slices were pre-incubated for 90–120 min (as indicated in the text) at 1 °C. They were first all placed together in 30 ml of medium which was renewed twice. Approximately 60 min before the end of the pre-incubation, the slices were transferred in lots of 100–200 mg wet wt to Warburg manometer flasks containing 3 ml of the Ringer's solution. The Ringer's now contained inhibitors or substrates at the appropriate final concentrations, and the flasks further held 0.2 ml of 5 N-KOH in the center wells. The flasks were attached to the manometers and gassed while standing in an ice bath. The experimental incubation, at 38 or 25 °C as noted, then permitted recovery of the slices to be studied. Incubations were for 60 min unless otherwise indicated. Readings of O_2 consumption were taken at 10-min intervals, after an inital 10-min equilibration.

At the end of incubation, each flask was rapidly detached from its manometer, the alkali removed with a roll of absorbent paper, and the remaining contents tipped onto a hardened filter paper (Whatman No. 54) supported on a sintered glass funnel under suction. The slices were then treated in one of two ways: (i) they were transferred to weighing bottles for assay of their dry wt, water, and ionic contents [4, 22]; (ii) they were rapidly dropped into 8% $HClO_4$ in 40% ethanol cooled in a salt-ice mixture, and immediately homogenized for 10 sec at 15,000 × rpm with the small head of a Polytron homogenizer (model PT 10, Brinkman Instruments, Westbury, N.Y.). The homogenate

was centrifuged, after which protein was determined in the precipitate and Na^+, inulin, and nucleotides were assayed in the supernatant [22]. It should be noted that tissue K^+ contents could not be reliably assayed in slices subjected to procedure *ii*, because of the insolubility of potassium perchlorate at the low extraction temperatures required for conservation of the adenine nucleotides.

Inulin and lactate in the incubation media were assayed in 1-ml samples of the medium which were withdrawn from the flasks by syringe pipette immediately before tipping the contents into the suction filter. The samples were deproteinized in ice-cold perchloric acid/ethanol.

Assay methods have been described previously [22].

Expression of Results

The results are given as mean ± SEM (number of observations). A single observation refers to the slices incubated in a single vessel. When comparing the effects of different concentrations of an inhibitor, the whole range of concentrations was studied in each experiment, usually with duplicate flasks at each concentration. Similarly, time course experiments involved incubation of groups of slices from the same animal for each of the times studied. Differences were examined for statistical significance by Student's *t* test.

The tissue assay from slices collected by procedure *i* are expressed per unit of slice dry wt; those collected according to procedure *ii* are given per unit of slice protein. In some experiments with salt gland and liver, the intracellular ion contents have been estimated on the basis of assays of the inulin distribution. Where not explicitly called "intracellular contents", the values given refer to total slice contents.

Results

Duck Salt Gland

Slices of salt gland that had been pre-incubated for 120 min at 1 °C showed a significant recovery during subsequent incubation at 38 °C for 60 min, with accumulation of K^+ and extrusion of intracellular Na^+, Cl^- and total slice water (Fig. 1*a*, *see also* [16] and [18]. The mean net extrusion of intracellular Na^+ (117 mmol/kg dry wt) somewhat exceeded the reaccumulation of K^+ (70 mmol/kg), the difference being largely accounted for by a net extrusion of Cl^- (35 mmol/kg). Slice Mg^{2+} was not significantly altered during incubation at 38 °C, but the Ca^{2+} content was reduced by 50% (Fig. 1*b*) suggesting that a metabolism-dependent mechanism extruded Ca^{2+} that had entered passively at 1 °C [21].

The effects of a range of concentrations of ethacrynic acid are also shown in Fig. 1, and it is clear that the net movements at 38 °C of all the ions undergoing transport were increasingly inhibited by concentrations above 0.2 mM. The net accumulation of K^+ was totally abolished

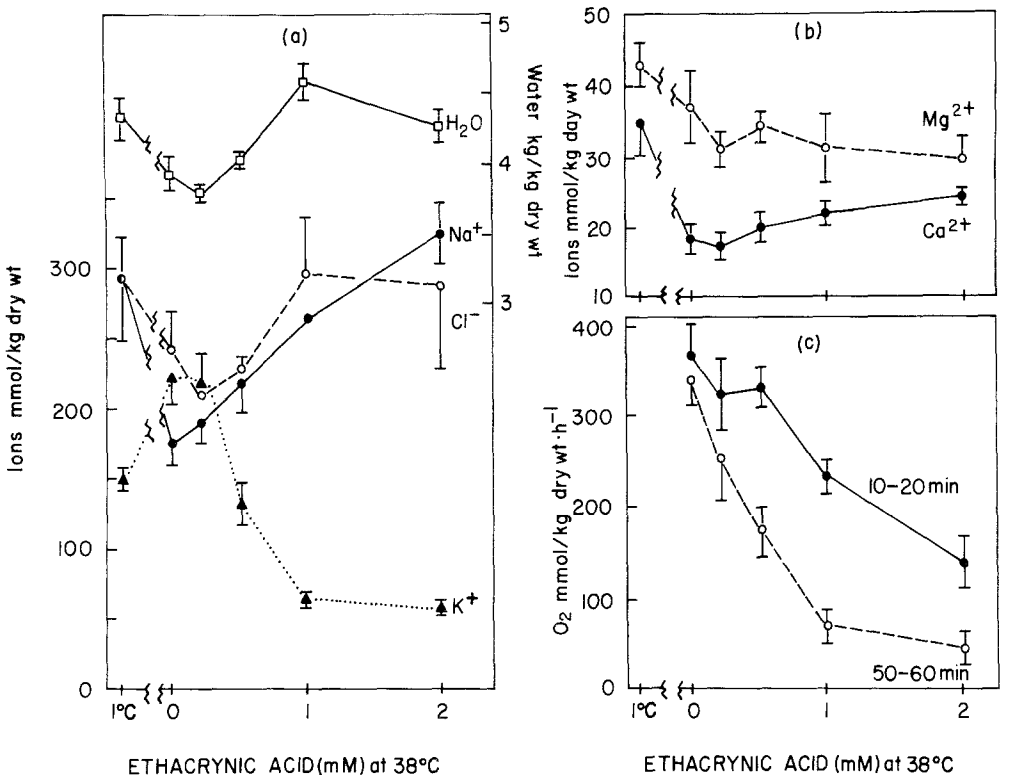

Fig. 1. Effects of ethacrynic acid on ion transport and respiration of salt-gland slices. Slices were incubated for 120 min at 1 °C and 60 min at 38 °C in substrate-free, Tris-buffered Ringer's solution. Samples were analyzed at the completion of incubation at 1 °C, and after incubation at 38 °C in the presence of the indicated concentrations of ethacrynic acid. The diuretic was also present during the last 60 min of the incubation at 1 °C but had no effect on slice composition at this temperature (*cf.* [28]). (*a*): Total water and intracellular monovalent ions: □, Water; ○, Cl^-; ●, Na^+; ▲, K^+. (*b*): Intracellular divalent ions: ○, Mg^{2+}; ●, Ca^{2+}. (*c*): Rate of respiration: ●, during the first 10-min period of observation after 10-min equilibration (i.e., the tenth to the twentieth minute at 38 °C); ○, during the last 10-min period (i.e. 50–60th min). Numbers of observations for each point: 1 °C, 8; 38 °C with ethacrynic acid: nil, 8; 0.2 mM, 9; 0.5 mM, 7; 1.0 mM, 4; 2.0 mM, 8. SEM are indicated

by 0.5 mM ethacrynic acid; higher concentrations caused a net loss of K^+, with a maximal effect at 1 mM. The extrusions of Na^+ and Cl^- showed a sensitivity to the diuretic which was similar to that of the K^+ uptake. This similarity is brought out clearly when, as in Fig. 2, the net extrusion of Na^+ and accumulation of K^+ are plotted according to their absolute magnitude against the inhibitor concentration. Extrusion of Ca^{2+} was substantially less sensitive, 2 mM ethacrynate giving only a 35% inhibition ($p < 0.05$), although the form of the curve (Fig. 1*b*) against diuretic

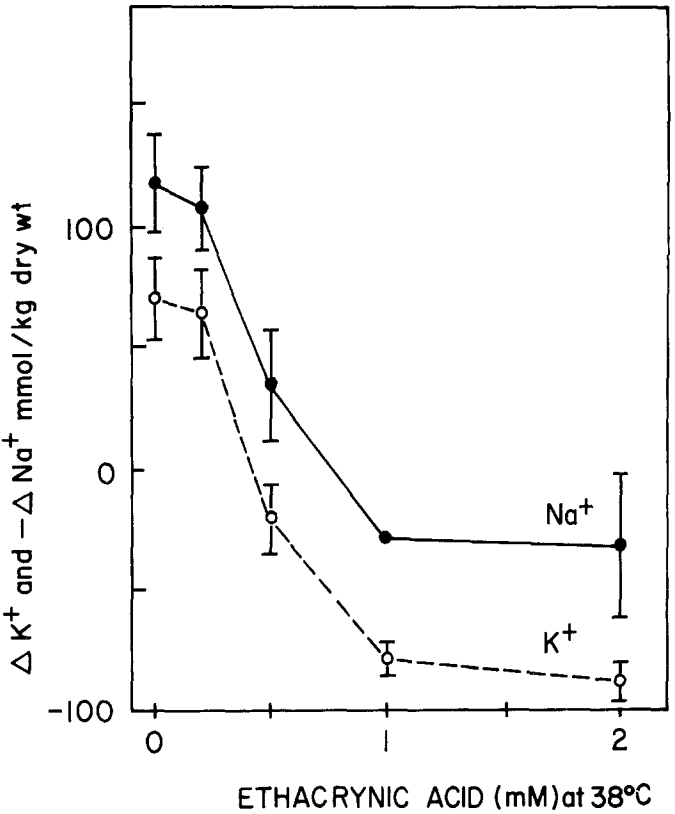

Fig. 2. Comparison of the effects of ethacrynic acid on net gain of K^+ (○) and extrusion of Na^+ (●) by salt-gland slices. The results are from the experiments of Fig. 1; the net ion movements were determined as the differences between the slice contents after incubation at 1 °C and after further incubation at 38 °C

concentration was similar to that of the other ion movements. The Mg^{2+} content was not significantly affected by any concentration.

At 1 and 2 mM, the diuretic also prevented extrusion of water at 38 °C (Fig. 1*a*; *cf.* [28]). This effect will be described in detail elsewhere[1].

Respiration of the salt-gland slices was markedly inhibited by ethacrynic acid and, despite the addition of the agent 60 min before the incubation at 38 °C (*see Materials and Methods*), the effect increased progressively during the course of incubation (*see also* [27]). In Fig. 1*c* the respiratory rates during the first and last 10-min periods of O_2 measurement are plotted against the concentration of diuretic. The effects of ethacrynic acid on K^+ and Na^- transport as determined in these experiments (Figs. 1*a* and 2) refer to the situation existing after 60 min at

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38 °C, and it is therefore noteworthy that the concentration-dependent inhibition of transport followed a pattern similar to that of the respiratory activity in the final observation period (50–60 min) of O_2 uptake (Fig. 1 *c*), and rather different from that in the first 10 min. Furthermore, the concentrations of ethacrynic acid needed for maximal inhibition of Na^+ and K^+ transport reduced respiration by 80% or more.

The above results all suggested that ethacrynic acid was acting largely as an inhibitor of respiratory energy metabolism. To test this further, the adenine nucleotide contents of slices were determined after 60 min at 38 °C. The ATP content was reduced by 95%, and the ADP content halved by the high concentrations of ethacrynic acid, and as a result the energy change [1] fell drastically (Table 1). Moreover, there was a close relationship between the final (50–60 min) rate of respiration, ATP content and the net Na^+ extrusion; this is clearly seen in Fig. 3, where these three factors from the same groups of slices have been plotted, on approximately normalized ordinates, against the ethacrynic acid concentration.

Despite the parallel inhibition of energy metabolism and ion transport seen above, the progressive nature of the respiratory inhibition left the possibility that a direct inhibition of a specific transport process occurred in the early stages of incubation at 38 °C. Information on this point was obtained by studying the time course of the effects of an intermediate concentration (0.5 mM) of ethacrynic acid (Fig. 4). In control slices there was an initial 25% fall of ATP during the first 10 min, followed by

Table 1. Effects of ethacrynic acid on adenine nucleotide contents of salt-gland slices[a]

	Ethacrynic acid (mM)				
	0	0.2	0.5	1.0	2.0
ATP	4.74 ±0.55 (12)	4.38 ±0.49 (8)	1.17 ±0.11 (10)	0.33 ±0.05 (10)	0.25 ±0.04 (8)
ADP	0.80 ±0.12	0.78 ±0.11	0.67 ±0.10	0.31 ±0.07	0.29 ±0.08
AMP	0.74 ±0.08	0.64 ±0.07	0.86 ±0.07	0.76 ±0.09	0.66 ±0.12
Energy charge	0.81 ±0.02	0.82 ±0.02	0.56 ±0.02	0.35 ±0.02	0.36 ±0.04

[a] Incubation conditions as in Fig. 1, slices being assayed at the end of the full incubation period. The contents are expressed as mmol/kg protein. Numbers of observations are as indicated for ATP.

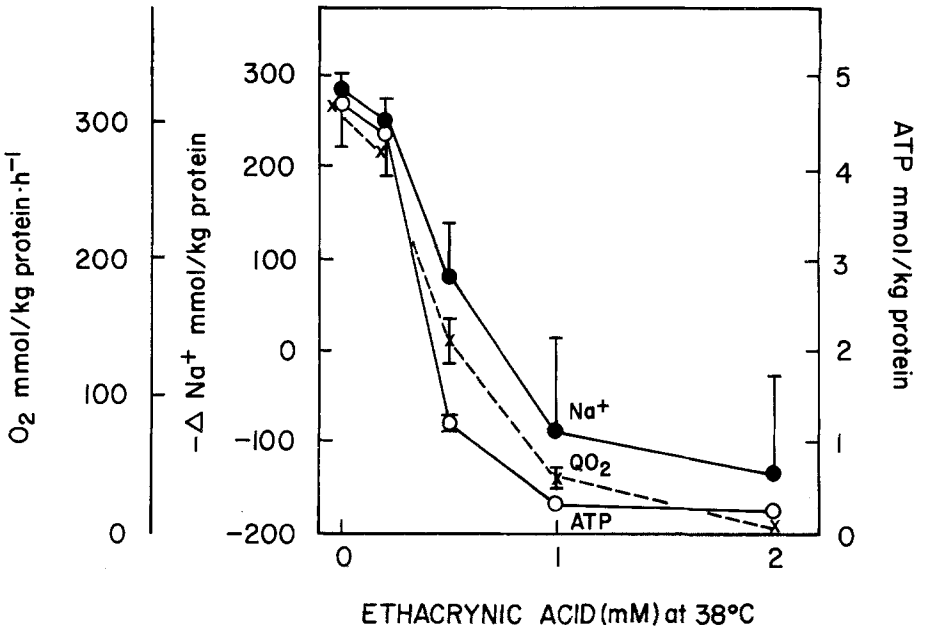

Fig. 3. Comparison of the effects of ethacrynic acid concentrations on Na^+ extrusion, ATP content and respiration in slices of salt gland. Incubation conditions were as in Fig. 1. Numbers of observations at the different ethacrynate concentrations were: nil, $n=12$; 0.2 mM, $n=8$; 0.5 mM, $n=10$; 1.0 mM, $n=10$; 2.0 mM, $n=8$. ●, net Na^+ extrusion, determined as in Fig. 2; ○, slice ATP content determined at end of incubation; ×, rate of respiration during last 10-min period of observation

stabilization or slight recovery (Fig. 4*b*). However, 0.5 mM ethacrynate caused a much more marked fall (65%) of ATP in 10 min, despite the small inhibition of respiration (20%) then prevailing (Fig. 1*c*), with continued loss at longer incubation times. In aggreement with the findings of MacKnight [13] with rat kidney cortex, the effects on Na^+ transport were biphasic. For the first 10 min the extrusion of Na^+ in the presence of ethacrynate was equal to that in the controls (Fig. 4*a*), so that there was no indication of a specific, early inhibition of a sodium-transport system preceding the fall of ATP. Rather, net extrusion of Na^+ only ceased after the fall of ATP exceeded 60%, whereupon a net re-entry ensued. Thus, the indications are that the inhibition of Na^+ transport was a secondary consequence of the falling levels of ATP.

For comparison, measurements were made of the effects of 50 μM ouabain, a concentration which we found to give maximal effects on ion transport and respiration. The latter was reduced by 42% (*cf.* refs. [2] and [19]), an inhibition which was much less than the maximal effect

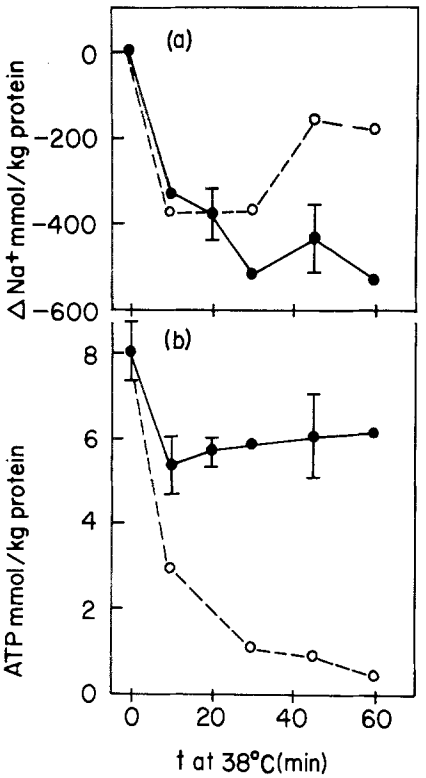

Fig. 4. Time course of (*a*) net Na^+ extrusion and (*b*) ATP content of salt-gland slices during incubation at 38 °C. ●, Controls ($n=2$–3); ○, 0.5 mM ethacrynic acid ($n=2$). Incubation conditions were as in Fig. 1

of ethacrynate. Moreover, instead of causing a fall of ATP levels, ouabain gave a significant ($P=0.05$) increase from 4.9 ± 0.7 mmol/kg protein in control slices (after 120 min at 1 °C *plus* 60 min at 38 °C) to 7.0 ± 0.7 mmol/kg; the corresponding values for the energy charge were 0.81 ± 0.01 in controls and 0.86 ± 0.02 in the presence of ouabain. These results indicate a conservation of ATP resulting from primary inhibition by ouabain of its utilization for ion transport [20, 26]. In contrast, the results obtained with ethacrynic acid are not amenable to this explanation.

Rat Liver

In this tissue, as has been reported previously [4, 22, 23], the net extrusion of Na^+ (267 mmoles/kg dry wt) was more than double the reaccumulation of K^+ (115 mmol/kg) by control slices; an extrusion of Cl^- (193 mmol/kg) approximately accounted for the difference. The effects

of ethacrynic acid on liver slices were broadly similar to the results with salt gland. There was a close parallel between the inhibition of all the transport processes studied, namely K^+ uptake and Na^+ extrusion (Fig. 5), Ca^{2+} extrusion (Table 2), and Cl^- and water extrusion (not

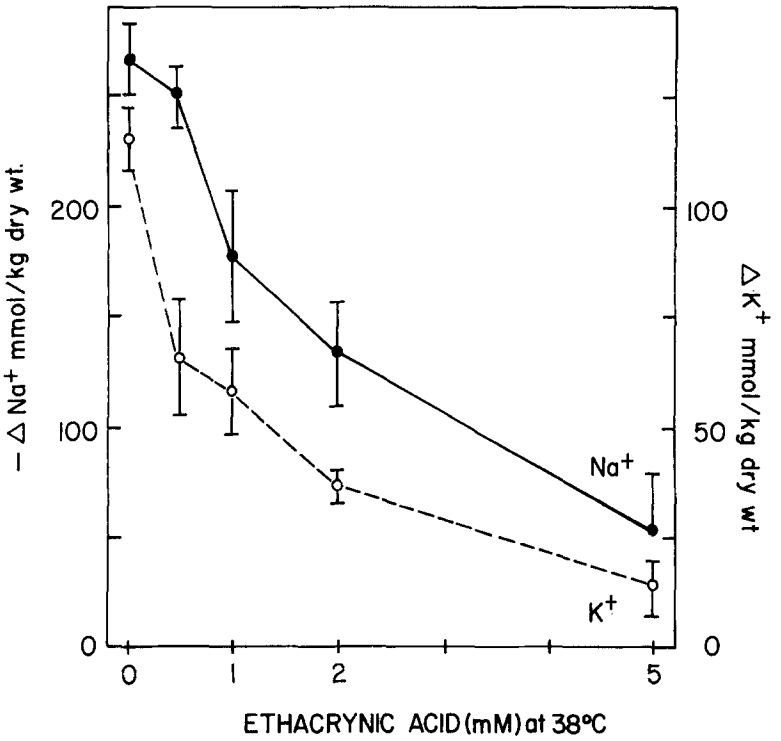

Fig. 5. Comparison of the effects of ethacrynic acid on net gain of K^+ (○) and extrusion of Na^+ (●) by rat-liver slices. Incubation conditions were as in Fig. 1, except that incubation at 1 °C was for 90 min. Net ion movements were determined as in Fig. 2. Numbers of observations at the different ethacrynic acid concentrations: nil, $n=11$; 0.5 mM, $n=9$; 1.0 mM, $n=10$; 2.0 mM, $n=10$; 5.0 mM, $n=8$

Table 2. Effect of ethacrynic acid on the Ca^{2+} and Mg^{2+} contents of liver slices

Incubation:	90 min	90 min at 1 °C followed by 60 min at 38 °C				
Ethacrynic acid (mM)	at 1 °C 0	0	0.5	1.0	2.0	5.0
Ca^{2+}	18.8 ±2.3 (12)	10.3 ±0.5 (11)	12.1 ±1.0 (9)	16.1 ±1.5 (10)	14.7 ±1.4 (10)	19.2 ±1.2 (8)
Mg^{2+}	29.5 ±1.3	26.0 ±0.8	25.8 ±1.4	26.7 ±1.0	26.2 ±0.7	25.3 ±1.0

[a] The results are from the same experiments as Fig. 5. Values are expressed as mmol/kg dry wt; numbers of observations for Mg^{2+} are as those given for Ca^{2+}.

shown). Again, Mg^{2+} contents were not significantly affected (Table 2). Four quantitative differences from the salt gland were noted: (i) respiration was only inhibited by 30% (Fig. 6), and the effect did not increase with time; (ii) net Ca^{2+} extrusion was totally inhibited (Table 2); (iii) the concentration of ethacrynate required for maximal effects (5 mM) was greater than with salt gland; (iv) at any given concentration of ethacrynic acid, the inhibition in mmol Na^+ extruded per kg dry wt was approximately double the decrease in the mmol K^+ reaccumulated. The last point may, at first sight, suggest that ethacrynic acid was a more effective inhibitor of Na^+ transport than of K^+ transport. However, the extrusion of Na^+ from control slices incubated without ethacrynic acid was double the reaccumulation of K^+ (*see above*) and the relative effectiveness of the diuretic as an inhibitor of transport of the two cations is therefore obtained by comparison of its proportionate effect on each. In Fig. 5, the net transports of Na^+ and K^+ are plotted on approximately normalized scales; it is seen that the effect of ethacrynic acid concentrations on each was very similar, 1.0–1.5 mM giving approximately 50%

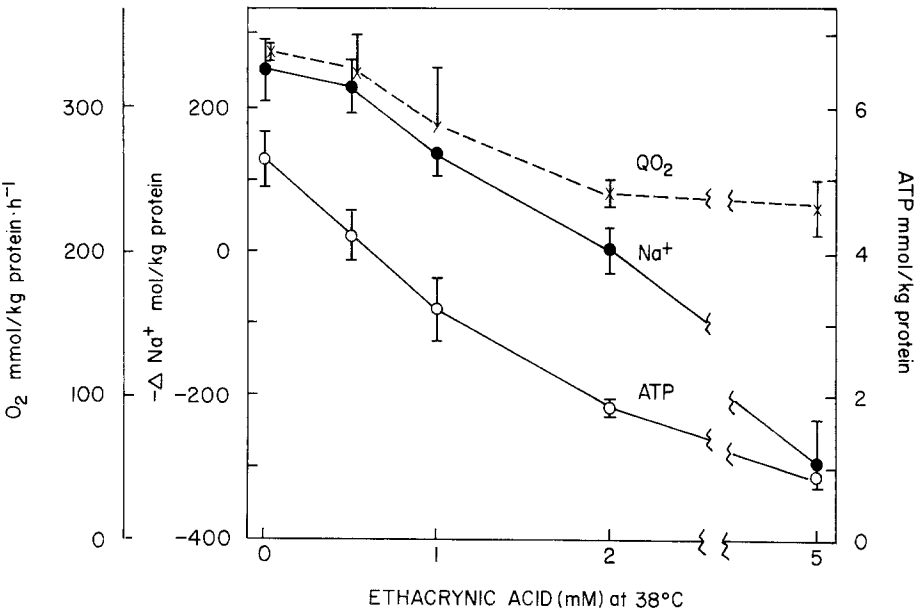

Fig. 6. Comparison of the effects of ethacrynic acid concentrations on Na^+ extrusion, ATP content and respiration in slices of rat liver. Incubation conditions as in Fig. 5. Observations are the means ± SEM of 6 observations, except at 0 and 2 mM ethacrynate, where $n=8$. ●, net Na^+ extrusion, determined as in Fig. 2; ○, slice ATP content determined at end of incubation; ×, mean rate of respiration throughout observation period of 50 min, (after 10-min equilibration)

Table 3. Comparison of the effects of ouabain and ethacrynic acid on liver slices[a]

	90 min at 1 °C	90 min at 1 °C followed by 60 min at 38 °C			
		Control	Ouabain (2 mM)	Ethacrynate (3 mM)	Ouabain (2 mM) + ethacrynate (3 mM)
Water	3.49 ± 0.10	2.59 ± 0.14	3.07 ± 0.22	3.81 ± 0.08	3.85 ± 0.10
Na^+	461 ± 36	155 ± 22	362 ± 48	430 ± 32	376 ± 32
Cl	552 ± 57	318 ± 29	496 ± 64	370 ± 31	433 ± 39
K^+	76 ± 7	270 ± 21	106 ± 9[b]	100 ± 6[b]	77 ± 8[c]
Ca^{2+}	20.5 ± 1.9	11.0 ± 1.0	12.9 ± 1.1	20.0 ± 1.6	20.3 ± 1.8
Respiration	—	334 ± 20	273 ± 8	220 ± 11	216 ± 14

[a] Incubation conditions as for Fig. 5. Values for ions represent the intracellular contents and are expressed as mmol/kg dry wt. Water contents are of total slice water, as kg/kg dry wt. Respiration is expressed as mmol O_2/kg dry wt · hr^{-1}. Each value is the mean ± SEM of 10 observations for ions and water, and of 6 observations for O_2 consumption.

[b] Significantly different from values at 1 °C, $P = 0.02$.

[c] Significantly different from value with ethacrynate alone, $P = 0.02$.

inhibition of each, while 5 mM gave 90% inhibition. Table 3 shows a comparison of the effects of 3 mM ethacrynic acid and 2 mM ouabain. Ethacrynic acid was less specific since it not only inhibited K^+, Na^+ and Cl^- transport, but also totally prevented water extrusion (which is only partially sensitive to ouabain—*see also* [23]) and Ca^+ extrusion (insensitive to ouabain—*see also* [21]). Although 2 mM ouabain is usually sufficient totally to inhibit K^+ reaccumulation by liver slices [23], in Table 3 a small uptake, significant at the 2% level, was observed. In this series of experiments, therefore, 2 mM ouabain was a slightly submaximal concentration and thus offered a good comparison to the 3 mM ethacrynic acid, which is also somewhat less than maximal (Fig. 5). It will be noted that the two inhibitors each inhibited K^+ uptake to a similar extent. Addition of ouabain in the presence of ethacrynate gave no significant inhibition beyond the effects of the diuretic alone on respiration or transport, except for a small further inhibition of K^+ accumulation (Table 3).

Table 4 shows that 3 mM ethacrynic acid prevented statistically significant net movements of intracellular Na^+, K^+, and Cl^- at all incubation times at 38 °C. This contrasts with the initial extrusion of Na^+ from salt-gland slices (Fig. 4), but is consonant with the finding that respiratory inhibition in liver slices did not require time to develop fully.

Table 4. Time-course of net ion movements in liver slices incubated with and without 3 mM ethacrynic acid[a]

	Ethacrynate (mM)	Incubated 90 min at 1 °C, then at 38 °C for (min)				
		0	5	15	30	60
Na^+	0	376±20	352±19	188±38	131±13	106±20
	3		349±40	326±28	348±37	374±64
Cl^-	0	355±62	317±18	173±36	225±23	220±23
	3		395±94	307±31	418±95	372±69
K^+	0	63± 6	80± 6	92± 3	127± 8	155±17
	3		66± 4	73± 4	68±22	58±15

[a] The results are for intracellular ion contents expressed as mmol/kg dry wt. Numbers of observations were 4 at each time, except at 15 min where there were 6 observations. Incubation conditions as for Fig. 5.

The rather small degree of respiratory inhibition caused by ethacrynic acid in liver (Fig. 6) was similar to that given by ouabain [4, 20]; however, it was accompanied by drastic falls of the ATP content (Fig. 6) and energy charge (Table 5) which contrast with the unchanged levels in the presence of ouabain [23]. The inhibition of Na^+ extrusion closely followed the fall of ATP (Fig. 6). Ethacrynic acid inhibits glycolysis in some cells [10], but this could not account for the fall of liver-slice ATP since the lactate production increased somewhat (Table 5). Rather, this repre-

Table 5. Effects of ethacrynic acid on energy metabolism in liver slices[a]

Ethacrynate (mM)	0	0.5	1.0	2.0	5.0
Respiration (mmol O_2/kg protein hr^{-1})	339 ±7	324 ±25	285 ±42	241 ±10	231 ±20
Lactate formed mmol/kg protein hr^{-1}	45 ±8	57 ± 8	70 ±16	67 ±11	71 ±11
Lactate/ pyruvate	17 ±3	20 ± 3	30 ± 6	65 ±15	65 ±13
Energy charge	0.76±0.02	0.70± 0.03	0.65± 0.4	0.53± 0.02	0.41± 0.05
(*n*)	(8)	(6)	(6)	(8)	(6)

[a] Incubation conditions as for Fig. 5. Incubation was for 90 min at 1 °C followed by 60 min at 38 °C in the presence of the concentrations of ethacrynate shown.

sents a Pasteur effect, which indicates that the primary action of ethacrynic acid on these slices was to interfere with mitochondrial oxidative phosphorylation. Also indicative of this mode of action is the finding of an increase in the ratio, lactate/pyruvate, which was related to the diuretic concentration (Table 5).

Rat Kidney Cortex

MacKnight [13] and Epstein [5] have previously obtained results which are also consistent with ethacrynic acid inhibiting ion transport by virtue of an inhibition of energy metabolism. We have attempted to corroborate their findings, studying effects of a wider range of ethacrynic acid concentrations on the adenine nucleotides.

In a first series of experiments, we duplicated the conditions used for salt gland and liver, conducting the experimental incubation at 38 °C.

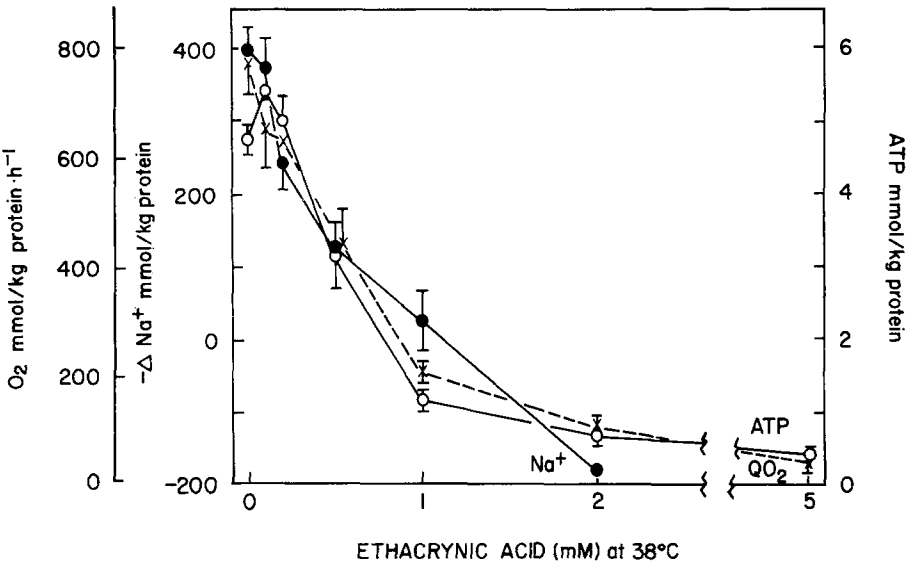

Fig. 7. Comparison of the effects of ethacrynic acid concentrations on Na^+ extrusion, ATP content, and respiration in slices of rat kidney cortex. ●, net Na^+ extrusion, determined as in Fig. 2; ○, slice ATP content determined at end of incubation; ×, rate of respiration during last 10-min period of observation (50–60th min of incubation). (*a*): Experimental incubation period at 38 °C; conditions as in Fig. 5. Numbers of observation at the different ethacrynic acid concentrations: nil, $n=10$; 0.1 and 0.2 mM, $n=5$; 0.5 to 2.0 mM, $n=8$; 5.0 mM, $n=4$. (*b*): Experimental incubation at 25 °C; incubation was for 90 min at 1 °C followed by 60 min at 25 °C in Tris-buffered Ringer's solution containing 10 mM acetate. Numbers of observations at ethacrynic acid concentrations: for nil, 1.0 and 2.0 mM, $n=4$; at 0.2, 0.5, and 5.0 mM, $n=2$

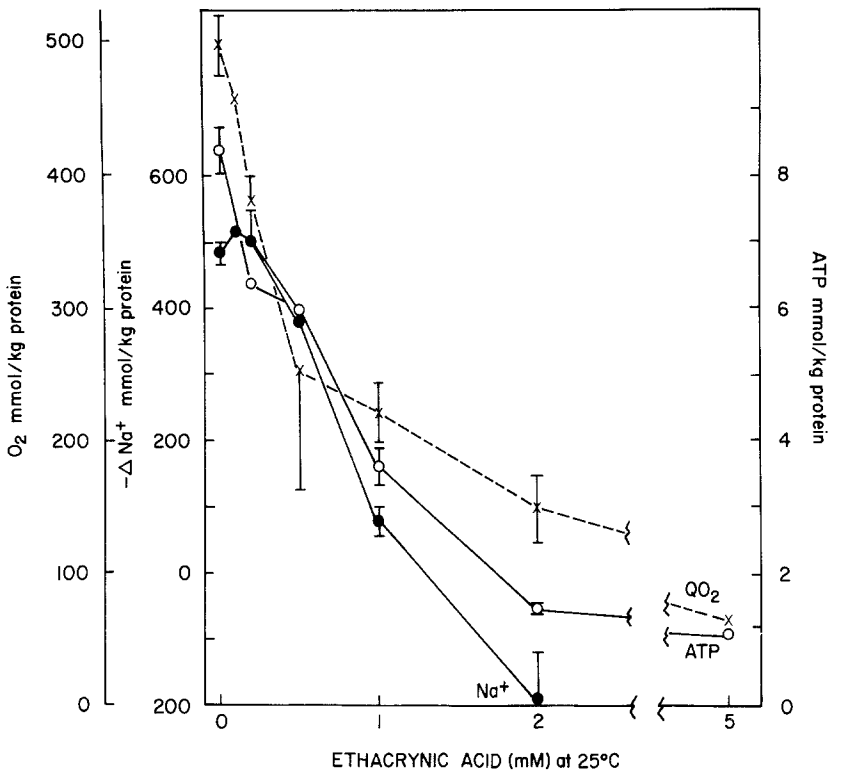

Fig. 7b

The results are very similar to those obtained above. The inhibition of respiration was progressive and extensive; e.g., after 60 min in the presence of 5 mM ethacrynic acid at 38 °C, O_2 uptake was inhibited by 90% (Fig. 7*a*). The fall of Na^+ extrusion at increasing ethacrynic acid concentrations was closely related to the decline of respiration and ATP content (Fig. 7*a*). Lactate production was nil in control slices but rose to 1.0 ± 0.4 mmol/kg protein/hr at 0.5 mM ethacrynic acid, and attained a maximum of 2.7 ± 0.5 mmol/kg/hr at 1.0 mM (no exogenous glucose was present). These results again indicate a primary inhibition of respiratory-coupled production of ATP by the diuretic. However, electron microscopic examination of the slices showed that even the controls exhibited marked alteration in normal fine structure at 38 °C. In further work we therefore conducted the experimental incubation at 25 °C and added 10 mM acetate as substrate [13]. The ultrastructure of the control slices was much better maintained under these conditions.

The ATP content of the control slices was significantly higher at 25 °C (8.4 ± 0.4 mmol/kg protein) than at 38 °C (4.7 ± 0.2 mmol/kg), de-

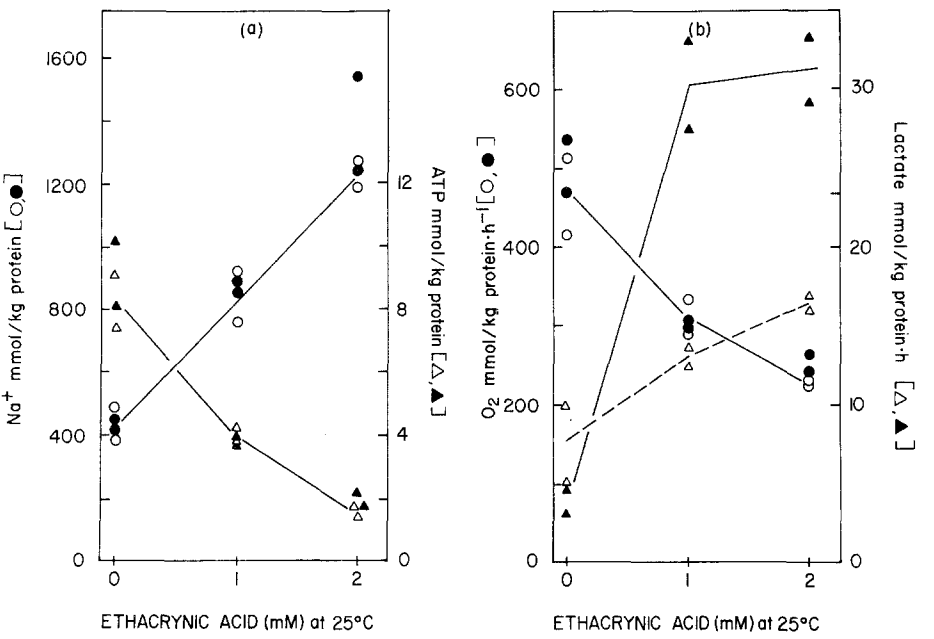

Fig. 8. Effects of ethacrynic acid on slices of rat kidney cortex at 25 °C in the presence and absence of glucose. Conditions as for Fig. 7*b*, except for the presence (filled symbols) or absence (open symbols) of 10 mM glucose, in addition to acetate, as substrate. (*a*): ○, ●, Na^+ content; △, ▲, ATP content. (*b*): ○, ●, rate of O_2 consumption; △, ▲, rate of lactate production. $n=2$, for all points

spite the control O_2 consumption (486 ± 26 mmol/kg protein/hr) being substantially lower than at 38 °C (780 ± 35 mmol/kg hr). The progressive nature of the respiratory inhibition by ethacrynate was less marked at 25 °C. But despite these quantitative differences, the close relationship of the inhibition of Na^+ extrusion to the fall of respiratory activity and ATP content at increasing ethacrynic acid concentration was seen at both temperatures (Fig. 7*a–b*).

In a single experiment, glucose (10 mM) as well as acetate was added to the medium at 25 °C, in order to duplicate the substrate mixture used by Whittembury and Proverbio [28] for guinea-pig kidney. Glucose gave no protection against the inhibitory effects of 1 and 2 mM ethacrynic acid on transport and ATP, despite the presence of a Pasteur effect which ensured a substantial increase in the rate of lactate production as respiration declined (Fig. 8).

Guinea-Pig Kidney Cortex

In view of the concordance of results with the three tissues studied thus far, it became important to reinvestigate the situation in guinea-pig kidney

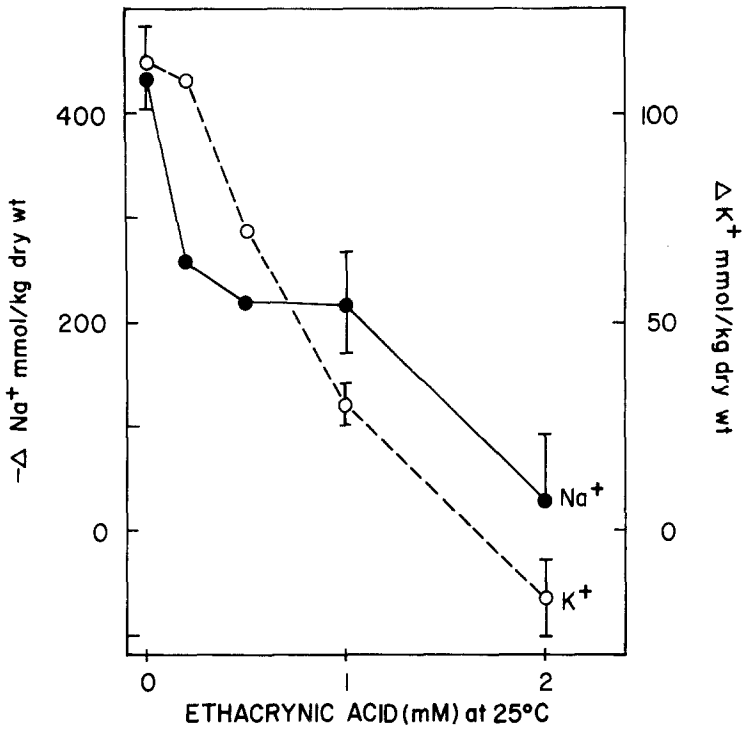

Fig. 9. comparison of the effects of ethacrynic acid on net gain of K^+ (○) and extrusion of Na^+ (●) by guinea-pig kidney-cortex slices at 25 °C. Incubation was for 90 min at 1 °C followed by 60 min at 25 °C, in phosphate-buffered Ringer's solution containing 10 mM acetate and 10 mM glucose. Net ion movements were determined as in Fig. 2. Numbers of observations: at nil, 1.0 and 2.0 mM ethacrynate, $n=10$; at 0.2 and 0.5 mM, $n=2$

cortex slices [17, 28]. Slices were incubated for 90 min at 1 °C and for 60 min (unless noted) at 25 °C. We first used a phosphate-buffered Ringer's solution containing 10 mM acetate; these conditions are similar to those of Whittembury and Proverbio [28] except for the absence of HCO_3^-. Figure 9 shows that 2 mM ethacrynic acid not only prevented the net extrusion of Na^+, but also totally inhibited K^+ reaccumulation, with no obvious difference in sensitivity. Extrusion of water and Cl^- showed a similar pattern. Also, addition of 0.5 mM ouabain in the presence of 2 mM ethacrynate resulted in a small, further increase of Na^+ content, from 955 to 1180 mmol/kg dry wt, as well as a small loss of K^+, from 120 to 71 mmol kg. As in rat kidney cortex, ethacrynic acid induced a Pasteur effect, increasing lactate production from 17.8 to 38.5 mmol/kg dry wt/hr.

The time-course of the effects of 2 mM ethacrynic acid was studied under the same conditions (Fig. 10). Respiration was progressively inhibited and, in accordance with MacKnight's result from rat kidney [13], there was a partial initial recovery of composition at 25 °C while the rate of respiration was still relatively high. After 30 min, however, O_2 consumption was reduced by more than 60% and there ensued large increases of tissue Na^+ and Cl^- contents beyond the values found in cold-incubated slices.

Substitution of phosphate by 10 mM Tris-HCl as the buffer caused a 20% reduction in the control rates of respiration and lactate production, but gave no significant difference in the net transport of water and ions. The sensitivity of these activities to 1 and 2 mM ethacrynic acid

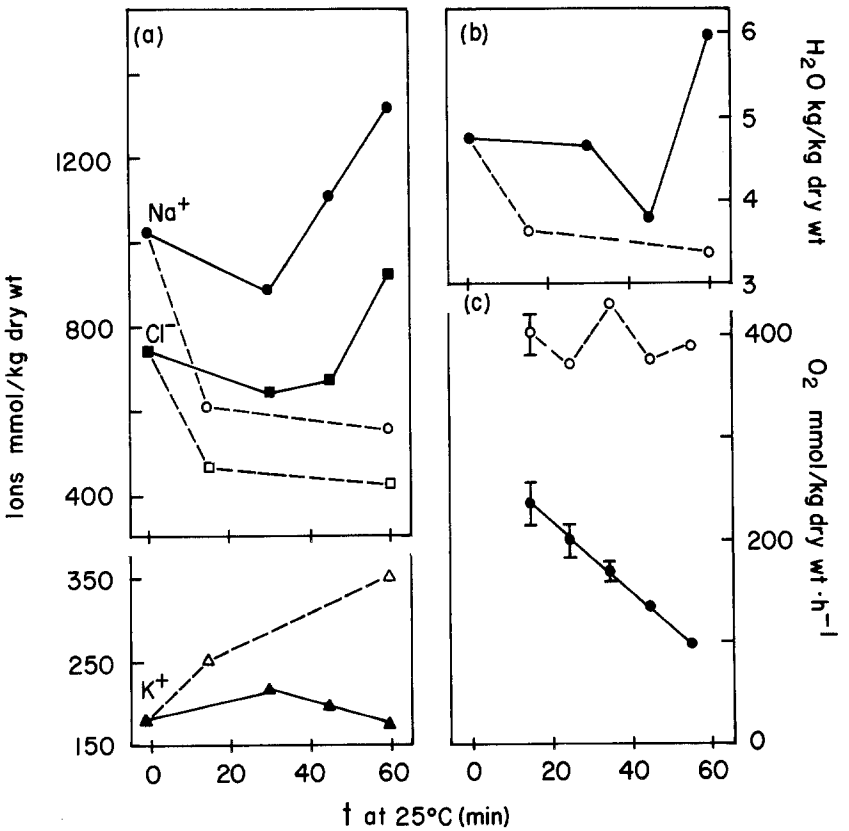

Fig. 10. Time-course of net ion movements in slices of guinea-pig kidney cortex. Incubation conditions were as in Fig. 9. Open symbols are control slices, closed symbols are for slices incubated with 2 mM ethacrynic acid. (*a*): ●, ○, Na^+ content; ■, □, Cl^-; ▲, △, K^+. (*b*): Water content. (*c*): Rate of respiration

Table 6. Effects of ethacrynic acid, in the presence and absence of glucose, on Na^+ transport and energy metabolism in slices of guinea-pig kidney cortex[a]

	Medium glucose (mM)	90 min at 1 °C	Then 60 min at 25 °C with ethacrynic acid		
			0	1 mM	2 mM
Na^+	0	819 ±40	409 ±17	699 ±27	890 ±84
	10		489 ±52	668 ±23	924 ±58
ATP	0	8.8 ± 0.6	10.8 ± 0.3	5.6 ± 0.1	1.9 ± 0.2
	10		12.5 ± 1.0	5.2 ± 0.5	2.2 0.2
Energy charge	0	0.74± 0.02	0.85± 0.02	0.71± 0.02	0.46± 0.05
	10		0.86± 0.01	0.68± 0.05	0.48± 0.04
O_2 uptake (50–60 min)	0	–	384 ±28	139 ±20	82 ±14
	10		408 ±21	154 ±17	108 ±14
Lactate production	0	–	5.9 ± 3.0	14.9 ± 1.9	22.7 ± 2.6
	10		15.2 ± 2.5	33.3 ± 2.2	41.3 ± 5.1

[a] Slices were incubated in Tris-buffered Ringer's solution containing 10 mM acetate for 90 min at 1 °C followed by 60 min at 25 °C. Neither glucose nor ethacrynic acid had any effect during incubation at 1 °C. Results for Na^+ and ATP contents are expressed as mmol/kg protein, and for O_2 consumption and lactate production as mmol/kg protein/hr. Values are mean ± SEM of 4 observations.

were also similar in the two media, and there was no evidence of a smaller sensitivity of K^+ transport than of volume control to the diuretic (results not illustrated).

The effects of ethacrynic acid on the adenine nucleotides of these slices were studied in Tris-buffered Ringer's containing 10 mM acetate as substrate. Table 6 shows that 1 and 2 mM ethacrynic acid reduced ATP levels by 50 and 85%, respectively, and gave commensurate decreases of total nucleotides, energy charge, and the final rate of respiration. Na^+ extrusion was totally blocked by 2 mM ethacrynate, and the concentration-dependence of the inhibition closely followed the fall of respiratory rate and ATP content (Fig. 11). Addition of glucose had no significant effect on the results, except to increase the rate of lactate production both in the presence and absence of the diuretic (Table 6). It will be noted that the only concentration of ethacrynic acid tested on guinea-pig kidney-cortex slices by Whittembury and Proverbio [28], namely 2 mM, was found in our experiments to block active movements of both Na^+ and K^+ totally and, in addition, to reduce respiration by 75–80% and to lower ATP contents by 85%.

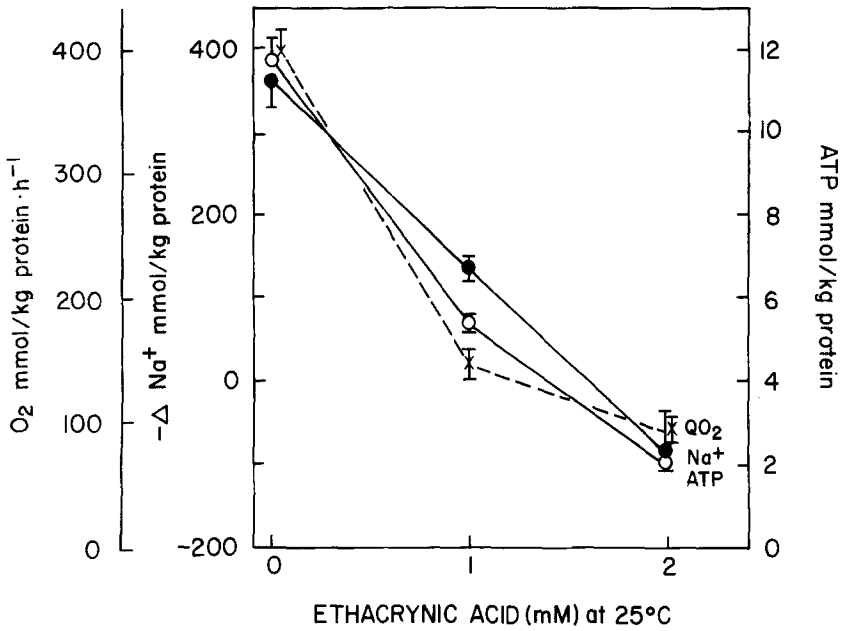

Fig. 11. Comparison of the effects of ethacrynic acid concentrations on Na^+ extrusion, ATP contents and respiration in slices of guinea-pig kidney-cortex. The results are from the experiments of Table 6, the values with and without glucose present having been combined in view of the absence of a significant difference between them. At all points, $n=8$. ●, net Na^+ extrusion, determined as in Fig. 2; ○, slice ATP contents at end of incubation; ×, rate of respiration during last 10-min observation period

Morphological Effects of Ethacrynic Acid

The inhibitory effects of ethacrynic acid on salt gland and kidney slice respiration and on electrolyte and nucleotide content were paralleled by marked alteration in cellular fine structure in these tissues. In particular, 2 mM ethacrynic acid caused pronounced disorganization of plasmalemmal specializations (microvilli in renal proximal tubules, basolateral folds in proximal tubular and salt gland epithelial cells), disruption of mitochondrial structure and vesiculation of cytoplasm. The cytotoxic effects of ethacrynic acid on these tissues will be described in detail in a subsequent publication.

Discussion

The results show that ethacrynic acid inhibits aspects of energy-conserving metabolism and several transport activities in slices of the four

tissues studied. In addition to its relation to the question as to whether the action of ethacrynic acid as a diuretic is due to primary inhibition of ion transport or of energy provision, our work elucidates two other unresolved questions. These are: whether in whole cells ethacrynic acid inhibits mainly glycolysis or mitochondrial oxidative metabolism; and whether the diuretic has a specific inhibitory effect on a Na^+ transport system which is insensitive to K^+ and ouabain. These latter two points will be discussed first.

Effects on Energy Metabolism

In slices of kidney [11] and in Ehrlich ascites tumor cells [9, 10], ethacrynic acid has been found to inhibit both glycolysis and respiration. That these are two independent effects is shown by the fact that glycolysis is inhibited in mammalian red cells [10] and that respiration and phosphorylation are inhibited in isolated mitochondria [7, 15, 24]. In considering which effect occurs in intact cells, Gordon and de Hartog [10] noted that respiratory inhibition by ethacrynic acid in ascites cells differed from the effects of respiratory chain inhibitors, such as antimycin A and cyanide, in that no Pasteur effect was produced. They concluded that both glycolysis and respiration were inhibited in their cells. The tissue preparations in which we have measured lactate production (liver and kidney cortex) did show a Pasteur effect, the increased lactate production being inversely related to the rate of respiration. It follows that, in these tissues, respiratory metabolism was at least more markedly inhibited than glycolysis. Thus, the main cause of the decline in tissue energy levels should be the fall in respiratory activity, and this is consistent with the close parallel we have observed in salt gland and kidney cortex between the effect of the diuretic on the final rate of respiration and on tissue ATP contents. Our present results are not sufficient to decide whether ethacrynic acid acted as an inhibitor of the mitochondrial electron-transfer chain, or of phosphorylating reactions.

In the case of the liver, there was a marked lowering of slice ATP at maximal ethacrynate concentrations despite the persistence of 70% of the maximal O_2 consumption. This may be due to the uncoupling properties which ethacrynic acid has been reported to show at low (0.1 mM) concentrations [11], or to a microsomal origin of the ethacrynate-resistant respiration. The latter seems the more likely explanation in view of the greater respiratory inhibition given in tissues with less mixed-function oxidase activity (salt gland and kidney).

Specificity of Effects on Ion Transport

As a result of their work on ion and water movements in slices of guinea-pig kidney cortex, and on Na^+-K^+-dependent adenosine triphosphatases, Whittembury and co-workers [17, 28] concluded that ethacrynic acid had a marked specificity towards the K^+- and ouabain-insensitive, "second" Na^+ pump which has been implicated in volume-controlling activities in various tissues [12, 14, 23]. In particular, they observed that K^+ accumulation was only inhibited 30% by 2 mM ethacrynate (the only concentration used), while Na^+ extrusion was more strongly blocked. By contrast, we have found both Na^+ and K^+ transport to be totally inhibited by 2 mM ethacrynic acid in kidney and salt-gland slices, and by 5 mM in liver. Moreover, in each of the tissues we have studied, the effects of increasing concentrations of ethacrynic acid on K^+ accumulation ran closely parallel to the effects on Na^+ extrusion (Figs. 2, 5, and 9). MacKnight [13] using rat kidney cortex also failed to find a difference between the action of this diuretic on Na^+ and K^+ movements. None of the changes of incubation conditions that we introduced (temperature, substrate, buffer) altered the relative effects of ethacrynic acid towards these two ions. There remain a number of differences between the experimental conditions used for kidney-cortex slices by ourselves and by Whittembury and Proverbio [28]. Thus, the latter authors used only the outermost slice of the cortex, conducted the cold pre-incubation in a K^+-free medium and continued it for 150 min, and used a medium for all incubations which had a smaller osmotic activity by about 20 mosM. They also used 2 mM or 16 mM K^+ for incubation at 25 °C, instead of the 5 mM which we have used; however, Whittembury and Proverbio reported a comparable insensitivity of K^+ reaccumulation to ethacrynic acid at both concentrations of medium K^+ [28].

The net movements of Cl^- and water were also inhibited by ethacrynic acid according to a similar pattern as the cation movements, with total inhibition at 2–5 mM, depending on the tissue (Fig. 1*a*, Table 3). Thus, the diuretic clearly inhibited both of the two, independent processes which transport Na^+ (i.e., the ouabain-sensitive and ouabain-insensitive mechanisms) and together control cell volume [12, 23, 28]. A third transport process inhibited by ethacrynic acid in liver slices is the extrusion of Ca^{2+} (Table 2), a mechanism which is independent of the Na^+ transport systems in this tissue [21].

Mechanism of Transport Inhibition

The finding that so many transport activities have a similar sensitivity to ethacrynic acid suggests that the latter acts by inhibiting a single process common to all of them. Since the three transport processes appear to be independent of each other, the most likely common factor is the energy supply which our work shows to be very sensitive to the diuretic.

Concentrations of ethacrynic acid necessary to give complete inhibition of Na^+ and K^+ transport in each tissue also lower ATP levels by 80–90%. The close relationship between the effects of the diruetic on energy levels and on Na^+ and K^+ transport is seen in the time-course studies (especially Fig. 4) as well as in the concentration dependence of the inhibitions. The latter relationship is clearly seen in Figs. 3, 6, 7, and 11. Inhibition of Na^+ transport, inhibition of respiration, and reduction of ATP levels all followed a similar pattern in each tissue and, in the case of rat kidney, at both incubation temperatures. The values in Table 7 are the concentrations of ethacrynic acid giving half-maximal inhibition of the parameters we have measured. The values for respiration and tissue ATP were very similar. Estimates of the half-maximal inhibition of Na^+ extrusion are complicated by the fact that,

Table 7. Concentrations of ethacrynic acid giving half-maximal inhibition of Na^+ transport and energy metabolism in tissue slices[a]

Tissue	Concentration of ethacrynic acid (mM) giving half-maximal inhibition of			
	Respiration	ATP content	Na^+ extrusion	
			(*a*)	(*b*)
Duck salt gland	0.46	0.37	0.42	0.74
Rat liver	0.95	1.08	0.83	3.15
Rat kidney cortex				
38 °C	0.58	0.58	0.38	2.59
25 °C	0.43	0.75	0.83	1.93
Guinea-pig kidney cortex	0.61	0.75	0.79	1.98

[a] As described in the text, the half-maximal inhibition of Na^+ transport was estimated from two ways of assessing the maximal inhibition: (*a*) from zero net extrusion of Na^+ at 25 °C; (*b*) from extrapolation of double reciprocal plots to infinite inhibitor concentration. The values for O_2 uptake refer to the rate during the last 10 min of observations (i.e., the fiftieth to sixtieth minutes).

especially in kidney and liver, high concentrations of the inhibitor not only prevent net extrusion, but induce a large, further entry of Na^+ which did not attain a maximum over the concentration range tested. In view of the consequent uncertainty as to how to estimate maximal inhibition, we have adopted two estimates: first, maximal entry of Na^+ was estimated by extrapolation of double reciprocal plots to infinite inhibitor concentration; second, maximal inhibition was equated to zero extrusion of Na^+. In the latter case, estimates of the concentrations of ethacrynic acid needed for half-maximal effects agree well with those for respiration and ATP contents. For the estimates from double reciprocal plots, much higher estimates of ethacrynate are obtained. However, in either case it is clear that transport was not more sensitive to ethacrynic acid than was aerobic energy metabolism, and we conclude that the inhibition of enery-conserving reactions was the primary effect of the diuretic in the slices and is the cause of the transport inhibition. Whether this is also the basis for the diuretic action *in vivo* is open to question in view of the high concentrations of ethacrynic acid (between 0.2 and 0.5 mM) needed to produce significant effects in the tissue slices.

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References

1. Atkinson, D.E. 1968. Citrate and the citrate cycle in the regulation of energy metabolism. *In:* Metabolic Roles of Citrate. T.W. Goodwin, editor. pp. 23–40. Academic Press, New York
2. Borut, A., Schmidt-Nielsen, K. 1963. Respiration of avian salt-gland in tissue slice experiments. *Am. J. Physiol.* **204:**573
3. Burg, M., Green, N. 1973. Effect of ethacrynic acid on the thick ascending limb of Henle's loop. *Kidney Int.* **4:**301
4. Elshove, A., Rossum, G.D.V. van. 1963. Net movements of sodium and potassium, and their relation to respiration, in slices of rat liver incubated *in vitro*. *J. Physiol. (London).* **168:**531
5. Epstein, R.W. 1972. The effects of ethacrynic acid on active transport of sugars and ions and on other metabolic processes in rabbit kidney cortex. *Biochim. Biophys. Acta* **274:**128
6. Ernst, S.A., Ellis, R.A. 1969. The development of surface specialization in the secretory epithelium of the avian salt gland in response to osmotic stress. *J. Cell Biol.* **40:**305
7. Foucher, B., Geyssant, A., Goldschmidt, D., Gaudemer, Y. 1969. Mise en évidence du mécanisme d'action de l'éthacrynate de sodium sur des mitochondries de foie de rat. *Eur. J. Biochem.* **9:**63

8. Gemba, M. 1974. Effect of diuretics on ion transport of kidney cortex mitochondria. I. Inhibition of ion accumulation by ethacrynic acid and furosemide. *Jpn. J. Pharmacol.* **24**:213
9. Gordon, E.E. 1968. Site of ethacrynic acid action on Ehrlich ascites tumor cells. *Biochem. Pharmacol.* **17**:1237
10. Gordon, E.E., Hartog, M. de. 1969. The relationship between cell membrane potassium ion transport and glycolysis. The effect of ethacrynic acid. *J. Gen. Physiol.* **54**:650
11. Jones, V.D., Landon, E.J. 1967. The effect of ouabain, Meralluride and ethacrynic acid on respiration and glycolysis in kidney slices. *Biochem. Pharmacol.* **16**:2163
12. Kleinzeller, A. 1972. Cellular transport of water. *In:* Metabolic Pathways. (3rd Ed.) L.E. Hokin, editor. Vol. VI., pp. 91–131. Academic Press, New York
13. MacKnight, A.D.C. 1969. The effects of ethacrynic acid on the electrolyte and water contents of rat renal cortical slices. *Biochim. Biophys. Acta* **173**:223
14. MacKnight, A.D.C., Leaf, A. 1977. Regulation of cellular volume. *Physiol. Rev.* **57**:510
15. Manuel, M.A., Weiner, M.W. 1976. Effects of ethacrynic acid and furosemide on isolated rat kidney mitochondria: inhibition of electron transport in the region of phosphorylation site II. *J. Pharmacol. Exp. Ther.* **198**:209
16. Peaker, M. 1971. Avian salt glands. *Proc. R. Soc. London B* **262**:289
17. Proverbio, F., Robinson, J.W.L., Whittembury, G. 1970. Sensitivities of (Na^+-K^+)-ATPase and Na^+ extrusion mechanisms to ouabain and ethacrynic acid in the cortex of the guinea-pig kidney. *Biochim. Biophys. Acta* **211**:327
18. Rossum, G.D.V. van 1966. Movements of Na^+ and K^+ in slices of herring-gull salt gland. *Biochim. Biophys. Acta* **126**:338
19. Rossum, G.D.V. van 1968. Relation of the oxidoreduction level of electron carriers to ion transport in slices of avian salt gland. *Biochim. Biophys. Acta* **153**:124
20. Rossum, G.D.V. van 1970. On the coupling of respiration to cation transport in slices of rat liver. *Biochim. Biophys. Acta* **205**:7
21. Rossum, G.D.V. van 1970. Net movements of calcium and magnesium in slices of rat liver. *J. Gen. Physiol.* **55**:18
22. Rossum, G.D.V. van. 1972. The relation of sodium and potassium ion transport to the respiration and adenine nucleotide content of liver slices treated with inhibitors of respiration. *Biochem. J.* **129**:427
23. Russo, M.A., Rossum, G.D.V. van, Galeotti, T. 1977. Observations on the regulation of cell volume and metabolic control *in vitro*; changes in the composition and ultrastructure of liver slices under conditions of varying metabolic and transporting activity. *J. Membrane Biol.* **31**:267
24. Sawa, H., Weinman, E.J., Hyde, S.E., Eknoyan, G. 1976. Renal and hepatic mitochondrial effects of diuretics in the rat. *Biochem. Pharmacol.* **25**:2649
25. Wald, H., Gutman, Y., Czaczkes, W. 1977. Differences in Na and K transport in kidney cortex and medulla indicated by ouabain, ethacrynic acid and other inhibitors. *Biochem. Pharmacol.* **26**:711
26. Whittam, R. 1961. Active cation transport as a pacemaker of respiration. *Nature (London)* **191**:603
27. Whittembury, G. 1968. Sodium and water transport in kidney proximal tubular cells. *J. Gen. Physiol.* **51**:303*s*
28. Whittembury, G., Proverbio, F. 1970. Two modes of Na extrusion in cells from guinea-pig kidney cortex slices. *Pfluegers Arch.* **361**:1