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ETHACRYNIC ACID AND KIDNEY CELL METABOLISM

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Abstract—Cellular effects of ethacrynic acid were examined in the rabbit and rat kidney. Emphasis was placed on levels of the compound *in vitro* lower than those employed in earlier studies. In the present study *in vitro*, ethacrynic acid is without effect on sodium–potassium-dependent adenosine triphosphatase (ATPase) activity. When 0·1 mM ethacrynic acid is incubated with rabbit kidney cortex slices, it inhibits the maintenance of intracellular potassium but has no effect on cellular respiration or cell volume. Ethacrynic acid (0·05 mM) inhibits the respiration of isolated mitochondria and suppresses membrane ATPase stimulation of mitochondrial respiration. Ethacrynic acid administered *in vivo* at a dose sufficient to elicit a diuretic response does not affect kidney mitochondrial respiration or inhibit the sodium–potassium-dependent ATPase activity. Ethacrynic acid labeled with carbon-14 localizes in part in the kidney plasma membrane. About 90 per cent of the plasma membrane-bound ethacrynic acid is found in association with proteins comprising the low molecular weight fraction. The compound is not bound significantly to cell membrane macromolecules associated with the ATPase system.

ETHACRYNIC acid and furosemide are potent diuretic compounds with high ceilings of activity that have contributed new therapeutic efficacy to medical problems such as refractory edema and partial renal failure. It is not known whether ethacrynic acid and furosemide, which are chemically dissimilar compounds, have some common mechanism of action which brings about a diuretic response. Ethacrynic acid effects have been studied for several years in kidney, red cells and Ehrlich ascites cells in the hope of gaining insight into the diuretic mechanism and cell membrane function.

Most observations *in vitro* with ethacrynic acid have been made with approximately 1 mM levels of ethacrynic acid. Proverbio *et al.*[1] and Macknight[2] examined the effects of ethacrynic acid on guinea pig and rat renal cortex slices. With 1 mM levels of ethacrynic acid, they observed an apparent inhibition of cell volume regulation. Macknight[2] attributes this effect on cell volume to an associated inhibition of cellular respiration. Regulation of cell volume in tissue slices appears in general to be coupled to cellular metabolism and is insensitive to ouabain.[3,4]

Levin and Cortes[5] report that 1 mM ethacrynic acid appears to depress the glycolytic pathway in incubated rabbit kidney slices. The medulla is more sensitive than the cortex. The site of inhibition appears to be at the triose phosphate level.

Examination of kidney cell fractions shows that particulate fractions with membrane sodium–potassium-dependent adenosine triphosphatase (ATPase) activity are inhibited by incubation with 1 mM or 0·5 mM levels of ethacrynic acid.[6–9] Klahr *et al.*[10] found that a 1 mM level of ethacrynic acid *in vitro* greatly depressed the formation of lactate from glucose 6-phosphate or fructose-1,6-diphosphate in cell-free fractions of rat and rabbit renal cortex and medulla.

Similar observations *in vitro* have been made with Ehrlich ascites cells and with red

cells. Ethacrynic acid at a 1 mM level is a potent inhibitor of glycolysis and respiration in the Ehrlich ascites cell[11,12] and a potent inhibitor of glycolysis in the red cell.[11] Hoffman and Kregenow[13] have observed that 1 mM ethacrynic acid inhibits a non-ouabain-sensitive extrusion of sodium from the red cells. They postulate a sodium pump additional to that associated with ouabain-sensitive sodium–potassium-dependent ATPase activity. Dunn[14] re-examining this concept, argues that ethacrynic acid is blocking cation-exchange diffusion rather than a sodium pump in the red cell.

In the present study, a variety of observations *in vitro* have been made with rabbit and rat kidney at levels of ethacrynic acid lower than those employed in the studies cited above. These observations *in vitro* have been supplemented with observations on cellular effects of ethacrynic acid administered *in vivo*. The observations tend to support the concept that ethacrynic acid, at dose levels eliciting a diuretic response, does not act primarily on metabolic pathways or on sodium–potassium-dependent ATPase activity of the cell membranes in the species examined. Ethacrynic acid administered *in vivo* also does not appear to reach a cellular level approaching the levels employed in earlier studies *in vitro* cited above. Clinically, some diuretics are used at considerably higher levels, e.g. for management of refractory edema. In these instances their earlier observations *in vitro* are potentially relevant to interpretation of the clinical response.

METHODS

Incubations. The procedure for incubation of kidney slices and measurement of tissue respiration, potassium and water have been described previously.[15] The procedure for incubation of mitochondria is as follows. Mitochondria (about 2·5 mg protein) are incubated in Warburg flasks containing 3 ml medium and 0·1 ml KOH in the center well. The flasks are shaken at 37° and O_2 uptake is measured manometrically. The medium contains 77 mM KCl, 17 mM $NaPO_4$ (pH 7·4), 1 mM ADP (unless otherwise designated), 3 mM $MgCl_2$, 75 mM sucrose and 10 mM substrate. Pyruvate required supplementation with 2 mM malate. Where addition of a membrane fraction is designated, approximately 2 mg protein of an ER membrane fraction is added. This light microsomal fraction is rich in Na–K-dependent ATPase activity. The procedure for assay of membrane Na–K-dependent ATPase activity in the presence of deoxycholate is described in ref. 16.

Cell fractions. Male New Zealand White rabbits and male albino Sprague–Dawley rats, each approximately 3 months old, were employed in this study. The procedure for isolation of kidney mitochondria has been described.[17] Preparation of the ER membrane fraction, a light microsomal fraction rich in plasma membrane fragments, is described in ref. 18. This fraction was employed for assay of cellular Na–K-dependent ATPase activity. A highly purified plasma membrane fraction,[16] consisting of large fragments of plasma membrane, was employed in the ethacrynic acid binding experiments. The procedure for counting membrane fraction radioactivity is described in ref. 19. ^{14}C-labeled ethacrynic acid is a gift of Dr. Charles Rosenblum of Merck, Sharpe & Dohme, West Point, Pa.

RESULTS

With incubated kidney slices, the inhibition by ouabain of cellular potassium maintenance and associated ATPase activity is closely linked to tissue respiration. A

decrease in the maintenance of cellular potassium is paralleled by a decline in tissue respiration.[20] The effect of ethacrynic acid on the maintenance of intracellular potassium in rabbit kidney cortex slices is presented in Table 1. No effect is obtained in the presence of 0·01 or 0·05 mM ethacrynic acid. With 0·1 mM ethacrynic acid, the maintenance of tissue potassium is inhibited without any effect on tissue respiration. A progressive decline in tissue respiration begins with 0·2 mM and higher levels of ethacrynic acid. The fall in tissue potassium is accompanied by a corresponding rise of intracellular sodium. The effect on tissue potassium is obtained even when glucose is omitted from the incubation medium. No remarkable enhancement of results is obtained by pretreatment of the rabbits with 10 mg/kg of ethacrynic acid (a diuretic dose) 30 min prior to sacrifice and removal of kidneys for slice studies. After pretreatment, a small effect on potassium was observed with 0·05 mM ethacrynic acid that appeared to be absent in the non-pretreated controls.

TABLE 1. EFFECT OF ETHACRYNIC ACID *in vitro* ON INCUBATED RABBIT KIDNEY CORTEX SLICES*

Ethacrynic acid in incubation (mM)	No. of experiments	O_2 uptake (μl O_2/mg dry wt./110 min)	Intracellular K (m-equiv./100 g dry wt. after incubation)	H_2O (ml/100 g dry wt. after incubation)
None	5	15·0 ± 0·46	23·6 ± 0·85	300 ± 16
0·1	5	15·1 ± 0·55	19·5 ± 0·77 P < 0·02	323 ± 8
0·5	5	9·2 ± 0·41	15·0 ± 0·89 P < 0·01	457 ± 10 P < 0·01

* Rabbit kidney cortex slices were incubated for 110 min in Warburg flasks at 37° in Robinson's medium (Na, 154; K, 5; Cl, 160; Ca, 3·3; PO_4, 5 and $MgSO_4$, 1·5 mM) with 10 mM glucose and 100% O_2. The results are expressed ± S.E.

The simplest concept suggested is a dissociation between the ethacrynic acid effects on cell potassium maintenance and the inhibitory effects on tissue respiration. No significant effect on cell volume, as measured by tissue water content, is observed in the slices incubated with 0·1 mM ethacrynic acid. It was also noted that after incubation of kidney cortex slices for 1 hr with 0·5 mM ethacrynic acid no significant change in membrane ATPase activity was found compared with the controls. Slices of rabbit outer medulla were similarly incubated. This tissue shows a slight increment in tissue potassium at the end of the incubation period. Respiration is depressed as in the cortex slices with 0·5 mM ethacrynic acid. No effect of ethacrynic acid on maintenance of tissue potassium was observed in renal medulla slices.

The inhibition of respiration in kidney slices by ethacrynic acid prompted an examination of the effects of the compound on respiration of isolated mitochondria. Administration *in vivo* of ethacrynic acid at a diuretic dose in rabbits and isolation of the kidney cortex mitochondria 30 min later did not reveal any significant inhibition of mitochondrial respiration (Table 2).

Sodium–potassium-dependent ATPase activity of the rabbit cortical tissue was measured in the presence of physiological levels of sodium and potassium (Table 2). Intracellular sodium of the cortical tissue was found to be 64 m-equiv./l. of water and

TABLE 2. EFFECT OF ETHACRYNIC ACID *in vivo* IN RABBIT RENAL CORTEX*

	No. of animals	Mitochondrial respiration (μl O_2/110 min/mg protein)	Na–K ATPase (P_i/20 min/mg protein)
Control	6	268 ± 6	4·5 ± 0·5
Ethacrynic acid	6	270 ± 14	4·35 ± 0·75

* Rabbits received a 10 mg/kg diuretic dose of ethacrynic acid intravenously in the ear vein. Thirty min later the animals were sacrificed and the renal cortex was fractionated for isolation of the mitochondria and the ER membrane fraction in which sodium–potassium-dependent ATPase activity was measured. ATPase activity was measured at pH 7·4 (tris–HCl, 0·03 M) with 4 mM Mg ATP in the presence and absence of Na (67 mM) and K (3·3 mM). Na–K-dependent ATPase is the increase in activity in the presence of added cation. The substrate for mitochondrial respiration was pyruvate and malate. Almost identical results were obtained with α-ketoglutarate as substrate.

an extracellular potassium of 3 m-equiv./l. was assumed. The ATPase activity was apparently unaffected by the administration of ethacrynic acid *in vivo*. The entire rat kidney was also analyzed for sodium–potassium-dependent ATPase activity 30 min after ethacrynic acid was administered *in vivo*. No inhibition *in vivo* occurred when a 25 mg/kg diuretic dose was administered.

The effect of ethacrynic acid when incubated with isolated rat kidney mitochondria is shown in Table 3. Gaudemer and Foucher[21] have reported that 0·1 mM ethacrynic acid *in vitro* inhibits liver mitochondrial respiration. It is seen in Table 3 that 0·05 mM ethacrynic acid inhibits kidney mitochondrial respiration *in vitro*. The inhibition of mitochondrial respiration has a delayed onset. Typically, inhibition during the first hour is of the order of 0–30 per cent and during the second hour 80–90 per cent.

Mitochondria in many mammalian kidney tubule cells are closely associated with the cell membrane. Addition *in vitro* of a membrane fraction containing sodium–

TABLE 3. EFFECT OF ETHACRYNIC ACID *in vitro* ON RAT KIDNEY MITOCHONDRIAL RESPIRATION*

Substrate	No. of experiments	Mitochondrial respiration (μl O_2/110 min/2·5 mg mitochondrial protein)	
		Control	Ethacrynic acid (0·05 mM)
No membrane addition			
Pyruvate + malate	6	409 ± 22	301 ± 14
α-Ketoglutarate	6	418 ± 29	273 ± 18
Glutamate	6	411 ± 29	127 ± 12
ER membrane addition			
Pyruvate + malate	6	639 ± 43	353 ± 70
α-Ketoglutarate	6	618 ± 44	331 ± 35
Glutamate	3	620	326
Pyruvate + malate + ADP (0·2 mM)	6	590 ± 22	530 ± 12

* The experimental incubation procedure is described in Methods. The normal level of ADP employed in the incubation is 0·1 mM. The data are presented ± S.E. In the absence of added membrane, the rate of respiration is approximately linear. Under these conditions, the ethacrynic acid inhibition of respiration occurs mostly during the last 60 min.

potassium-dependent ATPase activity stimulates mitochondrial respiration.[17] Ethacrynic acid at a 0·05 mM level greatly depresses the membrane stimulation of mitochondrial respiration (Table 3). This level of ethacrynic acid has no effect *in vitro* on the membrane ATPase activity. An interesting observation is the prevention of this ethacrynic acid inhibition by an increase in the levels of ADP; this occurs only in the presence of added membrane. It was also noted that with glutamate as substrate, in the absence of membrane addition, the inhibition of mitochondrial respiration by ethacrynic acid is greatly enhanced. A lesser number of observations with rabbit kidney cortex mitochondria *in vitro* show similar effects of ethacrynic acid. The respiration of the isolated rabbit mitochondria is less active per milligram of protein.

When a cell cytoplasm fraction of rabbit kidney cortex or medulla is incubated with 0·2 mM ethacrynic acid, there appears to be no effect on cytoplasmic glycolysis measured as the conversion of glucose 6-phosphate or fructose-1,6-diphosphate to lactic acid.

In another study, rat kidney plasma membranes were isolated and the plasma membrane proteins were fractionated by chromatography on gel filtration columns.[16] The distribution of organic mercurials in kidney plasma membrane proteins was

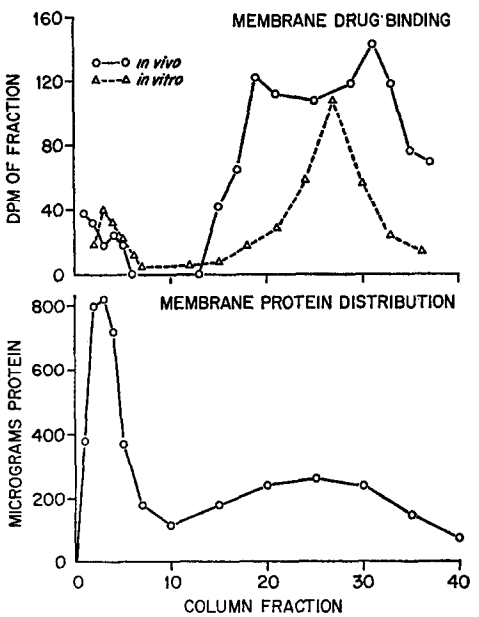

FIG. 1. Distribution of ethacrynic acid in rat kidney plasma membrane protein after gel filtration chromatography of the solubilized membrane on 6% agarose gel. The relative distribution of membrane protein is compared with distribution of the bound drug. In the study *in vivo*, 5 μc ethacrynic acid (1·1 mg) was administered to each rat intravenously. The animals were sacrificed 30 min later and kidney plasma membranes were isolated. In the study *in vitro*, the rat kidney plasma membranes were incubated for 15 min at 37°. The incubation mixture contained 1 mM ATP, 0·3 M tris buffer, pH 7·4, 67 mM NaCl, 3 mM KCl and 0·1 μc ethacrynic acid in a total volume of 6 ml. The final ethacrynic acid concentration was 1·2, $\times 10^{-6}$ M. The ethacrynic acid was labeled in the carbon-2 position with ^{14}C. The disintegrations per minute for the data *in vitro* are actually 10-fold higher, but are reduced to the scale of the data *in vivo*. It is probable that the ability of the ethacrynic acid to bind covalently to protein sulfhydryl fixes a fraction of the ethacrynic acid in the isolated membrane protein. Low molecular weight proteins are localized in the second peak of the protein effluent.

measured after administration *in vivo* of these compounds.[19] In the course of the rat kidney study, a small amount of ^{14}C-labeled ethacrynic acid was made available. The compound was administered to the rats *in vivo* and incubated with isolated plasma membranes *in vitro*. The distribution of ethacrynic acid in plasma membrane proteins of rat kidney is shown in Fig. 1. The first peak contains proteins of very high molecular weight. The first peak is the locus of the sodium–potassium-dependent ATPase activity and the apparent site of ouabain localization.[19] The second peak consists of approximately eight electrophoretically distinct low molecular weight proteins with a median molecular weight of about 40,000. It is found that 90 per cent of the membrane ethacrynic acid appears to be localized in the second peak. A representative pattern of binding *in vivo* and the pattern of binding *in vitro* are shown in Fig. 1.

The dose of [^{14}C]ethacrynic acid *in vivo* was 5 μc or 1·1 mg administered to 300 g rats. Ethacrynic acid bound to plasma membrane and the microsomal fraction was approximately 0·13 nmole ethacrynic acid/mg of protein. Maximal binding *in vivo* in the second peak of the column-fractionated plasma membrane was 1 nmole ethacrynic acid/mg of protein. Maximal binding to the second peak *in vitro* was 2–2·5 nmoles ethacrynic acid/mg of protein. Ethacrynic acid binding to the cytoplasmic supernatant *in vivo* was 0·57 nmole/mg of protein.

The cytoplasmic concentration was estimated from the measured radioactivity as $1{\cdot}1 \times 10^{-7}$ M. A diuretic dose that is less than 10-fold higher than the tracer dose employed would not be likely to bring the cytoplasmic level much above 1×10^{-6} M. The possibility that this level of ethacrynic acid might significantly inhibit mitochondrial respiration appears unlikely, since 10^{-5} M is the lowest level with any effect on mitochondria *in vitro*. With mitochondria *in vitro*, it should be noted that the onset of inhibition was very slow.

DISCUSSION

Ethacrynic acid administered *in vivo* results in a diuretic response, i.e. a decrease in net sodium salt reabsorption by the kidney. In several previous studies *in vitro*, a variety of cellular effects have been produced with ethacrynic acid which would be likely to impair salt reabsorption. Ethacrynic acid inhibits energy metabolism.[2,11,12] It impairs cell volume regulation.[1,2] It depresses sodium–potassium-dependent ATPase activity.[6–9] It blocks cation-exchange diffusion and alters permeability.[14]

In the present study, the observations with lower levels *in vitro* and with administration *in vivo* modify this picture for rat and rabbit kidney. The observation that ethacrynic acid incubated with kidney cortex slices depresses tissue potassium levels in the absence of any effect on respiration can be interpreted as an effect on passive permeability of ions. The available evidence does not lend support to the concept of an inhibition of energy metabolism as a likely mode of action of ethacrynic acid *in vivo*. No inhibition of mitochondrial respiration is detected after administration *in vivo*. Mitochondrial respiration is inhibited by 0·05 mM ethacrynic acid *in vitro*. The compound measured *in vivo* does not approach this level in the cytoplasm of kidney cells. Incubation of 0·2 mM ethacrynic acid with a cell cytoplasm fraction is without effect on the conversion of glucose 1,6-diphosphate or fructose 1,6-diphosphate to lactic acid.

Administration *in vivo* of ethacrynic acid and the lower levels employed in the

incubations *in vitro* do not cause an inhibition of sodium–potassium-dependent ATPase activity in rabbit renal cortex. Ethacrynic acid does interact with cell membranes of renal tissue either *in vivo* or *in vitro*. However, only about 10 per cent of membrane-bound ethacrynic acid associates with those macromolecules of the plasma membrane which include the ATPase system. Nechay *et al.*[22] in a previous study measured binding of ethacrynic acid to dog kidney microsomal membranes and concluded that the degree of binding detectable appeared to be inadequate to inhibit the ATPase activity. Cole *et al.*[23] administered an analogue of ethacrynic acid with diuretic activity to dogs and failed to find any inhibition of sodium–potassium-dependent ATPase activity.

The possibility of ethacrynic acid dissociation from cell fractions during the isolation procedure is very real. The binding to the small protein fraction of plasma membrane, however, appears to be consistent both *in vivo* and *in vitro*. Direct addition of ethacrynic acid *in vitro* to cell fractions fails to inhibit glycolysis, inhibits mitochondria after a considerable delay and the ATPase activity only at very high levels. The small animal species chosen in this study are less sensitive to ethacrynic acid than is the dog, but they do appear to give a diuretic response in the absence of a demonstrable ATPase inhibition.

Wolf *et al.*[24] infused both ouabain and ethacrynic acid into the dog kidney. With ouabain there is a parallel decrease in oxygen utilization and sodium reabsorption; this is attributed to inhibition of a sodium pump linked to cellular respiration. Ethacrynic acid affected sodium reabsorption without a corresponding reduction in cellular respiration. A permeability change was postulated as a possible mode of action. The present observed association of ethacrynic acid with low molecular weight protein fraction of kidney plasma membranes is compatible with the hypothesis of a cell membrane site of action, possibly one that modifies permeability of the membrane.

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