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THE BINDING OF ETHACRYNIC ACID TO RABBIT KIDNEY CORTEX

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SUMMARY

1. The uptake of labeled ethacrynic acid by slices of rabbit kidney cortex at 25 °C approached a steady state level by 1 h of incubation, was linearly dependent on the external concentration of the compound, and was not affected by metabolic inhibitors.

2. The apparent steady state level of ethacrynic acid in the tissue considerably exceeded the external concentration. Evidence for binding of ethacrynic acid by tissue components was obtained as follows: (a) 40 % of the label could not be washed out; (b) the same fraction of the label was found in a well washed tissue solid sedimenting at up to 18000 × *g*.

3. The presence of the thiol reagents *N*-ethylmaleimide, $HgCl_2$, and iodoacetic acid did not diminish binding of ethacrynic acid.

4. Neither the uptake into nor the efflux from rabbit kidney cortex of ethacrynic acid was modified by the presence, in various concentrations, of materials presumably associated with sites of active transport of ions and sugars, *e.g.* ouabain, normally actively transported sugar, and Na^+.

5. It was concluded that the major part of the binding of ethacrynic acid by rabbit kidney cortex was not specific for either tissue sulfhydryl groups nor unique membrane active transport sites.

INTRODUCTION

Ethacrynic acid was designed for use as a diuretic agent[1] on the basis of the then prevalent opinion that protein bound sulfhydryl groups are involved in the active transport of electrolytes. Duggan and Noll[2] demonstrated that ethacrynic acid readily reacted with cysteine and, further, that ethacrynic acid markedly inhibited a (Na^+–K^+)-ATPase preparation from guinea pig kidney cortex. However, these authors also indicated that sulfhydryl reactivity alone was not an adequate determinant of the (Na^+–K^+)-ATPase–ethacrynic acid interaction *in vitro* because many common sulfhydryl reagents (*N*-ethylmaleimide, iodoacetate, *etc.*) did not inhibit kidney cortex (Na^+–K^+)-ATPase activity. Komorn and Cafruny[3] related the reduction of the concentration of protein-bound sulfhydryl groups in dog kidney by ethacrynic acid with

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ethacrynic acid's efficacy as a diuretic (*i.e.* reduction of Na^+ pump activity in dog kidney). More recently, Kramar and Kaiser[4] showed more directly that ethacrynic acid does function as an inhibitor of –SH dependent enzymes, although their work concerned liver mitochondrial enzymes.

Beyer and his colleagues[5] described an accumulation of [^{14}C]ethacrynic acid by rabbit kidney cortex slices which was partially inhibitable by the presence of 2,4-dinitrophenol.

The present investigation was undertaken to further elucidate the physical interaction of ethacrynic acid with rabbit kidney cortex slices and particularly the relationship of such interaction to the availability of –SH groups in the membrane.

Since ethacrynic acid functions in rabbit kidney to inhibit the active transport of sodium[6] and various sugars[7], it seemed likely that investigation of variables associated with these forms of transport with respect to the uptake of ethacrynic acid might also be fruitful. Hence, the presence of Na^+, ouabain, and an actively transported sugar was evaluated with respect to their effects, if any, on the uptake of ethacrynic acid into rabbit kidney cortex slices.

MATERIALS AND METHODS

Methods used in these experiments have been partially described before and only variations from previous descriptions are noted here.

Materials

[^{14}C]Ethacrynic acid (0.61 μCi/μmole) was kindly provided by Dr John E. Baer of the Merck Institute for Therapeutic Research. [^{3}H]Ethacrynic acid (0.12 μCi/μmole) was synthesized in the laboratory of Dr A. Kleinzeller by Mr William Ferrar and kindly provided by them. Quantitatively similar results were obtained with both preparations. The specific activity of ethacrynic acid used in all experiments was 1 μCi/ml except in the efflux experiments described below, in which it was 10 μCi/ml. All other reagents used were commercial preparations of reagent grade.

Experimental

The experiments were carried out using kidney cortex slices of healthy, adult rabbits. The methods of preparation and incubation of the kidney slices have been described[8]. The Krebs–Ringer balanced saline media were prepared as described by Kleinzeller and coworkers[8,9] except that only media with pH 7.4 were used. After incubation, in a few cases, slices were blotted and weighed (wet wt). In most cases, the slices were placed directly into small Teflon cups for drying at 80 °C under vacuum to a constant weight (dry wt). Whether the slices were weighed wet or not, after drying they were weighed and analyzed for ethacrynic acid content. For an evaluation of binding, only the dry tissue weight need be known. Hence, most of the data is presented in this form. The dry weight of tissue analyzed varied from 5–20 mg.

The washout technique was used to study the efflux of [^{14}C]ethacrynic acid[10]. Slices were first loaded with labeled ethacrynic acid by aerobic incubation in label-containing media (10 μCi/ml) for 60 min after a standard preincubation period. Blotted and weighed slices were placed in fine wire mesh tubes and the washout of ethacrynic

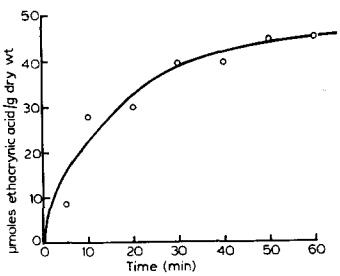
acid into a series of 50-ml tubes, each containing 10 ml of an appropriate saline vigorously aerated with O_2, was followed.

Analytical

Ethacrynic acid content was determined with either of two methods: (1) In a few experiments, the dried pellet or blotted slice was solubilized in 0.5 ml of hyamine–OH. The resultant solution was diluted to 5.5 ml with distilled water and 1 ml of this solution was counted in 10 ml of toluene–Triton X 100 scintillation fluid[11], using scintillation spectrometry (Packard Instruments, Model 3320). (2) In most experiments, 0.7 ml of 1 M NaOH was added to the dried tissue and the tissue solubilized overnight. To this was added 0.2 ml of 5 M HCl and the solution diluted to 3 ml with distilled water. 1 ml of the resultant, cloudy solution was added to 10 ml of scintillation fluid and counted as described above. This method, a modification of that of Madsen[12], produced a clear solution in the counting vial with no quenching beyond that of the water vehicle. The two methods gave equivalent results. It was assumed that ethacrynic acid was not modified by metabolic processes; no evidence is available to the contrary. Ethacrynic acid content was not determined chemically. Results are expressed in either μmole ethacrynic acid per g wet tissue weight (μmoles/g wet wt) or μmoles ethacrynic acid per g dry tissue weight (μmoles/g dry wt). Each reported value is the mean of at least three determinations with the number of determinations displayed in parentheses. A standard error of the mean (S.E.) is reported if more than five determinations were made.

RESULTS

Fig. 1 demonstrates that tissue ethacrynic acid content rises rapidly in the first 15–20 min and approaches a steady state concentration after 30–50 min. Fig. 2



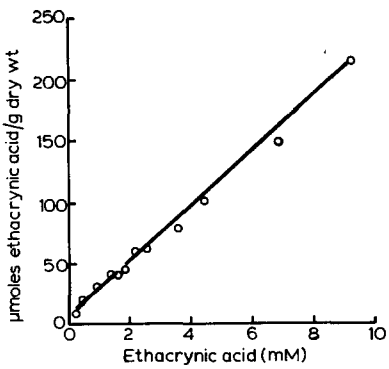


Fig. 1. The time function of ethacrynic acid bound to rabbit kidney cortex. Groups of slices were preincubated in Na^+ Krebs–Ringer media at 25 °C for 45 min and then incubated under the same conditions for the appropriate time. Incubation media contained 2 mM ethacrynic acid. After incubation, slices were placed in teflon cups and dried at 80 °C under vacuum to constant weight. Results are the means of six determinations.

Fig. 2. The amount of ethacrynic acid bound to rabbit kidney cortex as a function of medium ethacrynic acid concentration. Slices were preincubated and incubated under experimental conditions as in Fig. 1 for 60 min. Incubation media contained the indicated concentrations of ethacrynic acid. Results are the means of six determinations.

shows that the amount of ethacrynic acid taken up by kidney slices is a linear function of the final ethacrynic acid concentration in the medium after 60 min incubation. At the very high medium concentration of 10 mM, no saturation phenomena are demonstrated. Thus the amount of ethacrynic acid bound to kidney cortex slices is a function of both the concentration of ethacrynic acid in the medium and the time during which the tissue is in contact with the ethacrynic acid containing medium. Since the metabolic effects of ethacrynic acid approach maximal intensity with a medium concentration of only 2 mM, it is clear that the sites to which ethacrynic acid binds are not merely those concerned with the active transport of ions and sugars, if indeed there be such tissue sites. Ethacrynic acid is taken up against an apparent concentration gradient as follows from the data presented in Figs 1 and 2. Thus, after 60 min incubation of tissue in medium containing 2 mM ethacrynic acid, 45.8 μmoles of ethacrynic acid were found per g tissue dry wt (Fig. 1). Recalculated on the basis of tissue wet wt, considering tissue swelling, this represents 13.4 μmoles ethacrynic acid/wet wt. Under these conditions, in the extracellular (inulin) space, 1.67 μmoles of ethacrynic acid were found. Therefore, the apparent intracellular concentration of ethacrynic acid was 17.3 μmoles/ml, if ethacrynic acid was freely soluble in all the cell water. However, the uptake of ethacrynic acid is not diminished by the presence of 0.1 mM 2,4-dinitrophenol, a concentration that completely inhibits the active transport of sugars and ions[25] in rabbit kidney cortex slices (see Fig. 3). In experiments not presented in detail here ethacrynic acid uptake was not diminished in a N_2 atmosphere. Thus, this uptake is not active transport, although it fulfills at least one of the accepted criteria for active transport.

A large portion of ethacrynic acid, once taken up by the tissue, is not easily removed (see below). Ethacrynic acid uptake will hence be considered binding in this report.

Ethacrynic acid binding and tissue sulfhydryl groups

If the action of ethacrynic acid is indeed mediated through binding with tissue

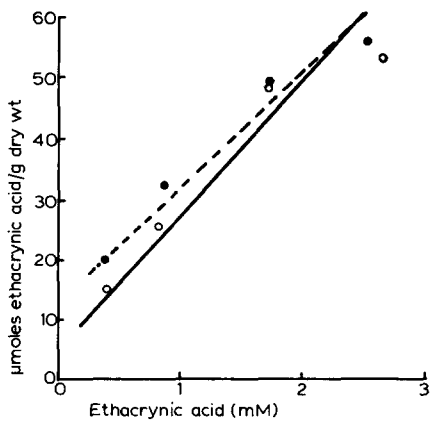


Fig. 3. Effects of 2,4-dinitrophenol on ethacrynic acid binding to rabbit kidney cortex. Slices were preincubated and incubated under the conditions of Fig. 1 for 60 min. Incubation media contained various concentrations of ethacrynic acid either without (○) or with 0.1 mM 2,4-dinitrophenol (●). Results are the means of four determinations.

TABLE I

EFFECT ON TISSUE BINDING OF ETHACRYNIC ACID BY CYSTEINE AND SUGAR

The slices were preincubated in either saline alone, or in saline containing 4 mM cysteine. The slices were then incubated for 60 min in saline containing only 2 mM ethacrynic acid, the control, or in salines containing 2 mM ethacrynic acid and 4 mM cysteine, or 2 mM ethacrynic acid and 1 mM 2-deoxygalactose.

Condition	*μmoles ethacrynic acid/g dry wt*
Control	38 ± 1.4 (6)
Preincubation in cysteine, incubation in ethacrynic acid	47 ± 1.8 (5)
Preincubation in saline, incubation in ethacrynic acid and cysteine	8 ± 0.3 (5)
Preincubation in saline, incubation in ethacrynic acid and 2-deoxygalactose	38 ± 1.6 (5)

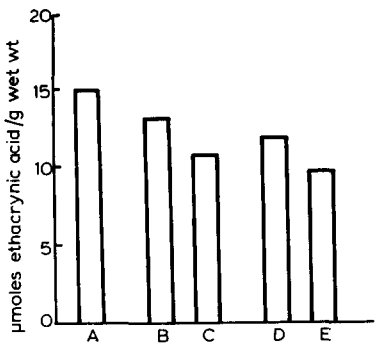


Fig. 4. Effect of L-cysteine on ethacrynic acid binding to rabbit kidney cortex. Slices were preincubated and incubated under the experimental conditions of Fig. 1. Incubation media contained 2 mM ethacrynic acid and, for various times, 4 mM L-cysteine. Cysteine was added after the slices had been incubating with ethacrynic acid for various times. Results are the means of four determinations ± S.E. The incubation conditions and results were: A, incubation for 60 min with ethacrynic acid alone, 15.00 ± 0.11; B, incubation for 45 min with ethacrynic acid alone, 13.11 ± 0.44; C, incubation for 45 min with ethacrynic acid alone followed by 15 min incubation with ethacrynic acid and cysteine 10.66 ± 0.49; D, incubation for 30 min with ethacrynic acid alone, 11.92 ± 0.69; E, incubation for 30 min with ethacrynic acid alone followed by 30 min incubation with ethacrynic acid and cysteine, 9.58 ± 0.11.

membrane sulfhydryl groups (–SH), then the uptake of ethacrynic acid might be expected to be reduced by the concurrent presence of excess –SH groups (*e.g.* cysteine) in the incubation medium. Table I demonstrates that cysteine reduces ethacrynic acid uptake into the tissues. It is likely that this occurred because of the greater affinity of ethacrynic acid for molecular cysteine than for kidney cortex binding sites. The data of Fig. 4 further support this view in that in these experiments already-tissue-bound ethacrynic acid was removed from the kidney slices by the subsequent addition of excess molecular cysteine. If ethacrynic acid preferentially binds the kidney tissue at membrane –SH groups, it might be expected that a thiol reagent, *e.g.* *N*-ethylmaleimide[14,15], would limit this binding. Table II shows that the incubation of kidney slices concurrently with 2 mM ethacrynic acid and *N*-ethylmaleimide

TABLE II

EFFECT ON TISSUE BINDING OF ETHACRYNIC ACID BY VARIOUS THIOL REAGENTS

Slices were preincubated in saline alone, except in one case where they were preincubated in 1 mM *N*-ethylmaleimide. The slices were incubated for 60 min with 2 mM ethacrynic acid alone, the control, or with ethacrynic acid and various concentrations of the thiol reagents: *N*-ethylmaleimide, iodoacetate, or $HgCl_2$, as designated below.

Condition	*μmoles ethacrynic acid/g dry wt*
Control	45 ± 2.0 (9)
Preincubation in 1 mM *N*-ethylmaleimide, incubation in ethacrynic acid	59 (4)
Incubation in 1 mM *N*-ethylmaleimide	46 ± 5.0 (5)
Incubation in 0.5 mM *N*-ethylmaleimide	43 ± 2.6 (5)
Incubation in 1 mM iodoacetate	49 (4)
Incubation in 0.4 mM $HgCl_2$	55 (4)

TABLE III

EFFECT OF MEDIUM COMPOSITION ON EFFLUX OF ETHACRYNIC ACID

Tissues were loaded with labeled ethacrynic acid for 60 min under standard conditions. Groups of slices were then allowed to efflux for 20 min into media of various compositions. Results are presented as the percent of ethacrynic acid remaining in the tissue after efflux with the amount of ethacrynic acid in the tissue at time 0 of the efflux taken as 100%. By Student's T Test, there is no significant variation in residual ethacrynic acid.

Condition	% *Residual ethacrynic acid*
25 °C, saline	39 (4)
0 °C, saline	44 (4)
0.1 mM 2,4-dinitrophenol	44 (4)
0.1 mM ouabain	39 (4)
2 mM 2-deoxygalactose	36 (4)
Unlabeled 2 mM ethacrynic acid	32 (4)

does not reduce ethacrynic acid uptake significantly. Interestingly, preincubation with 1 mM *N*-ethylmaleimide followed by incubation in *N*-ethylmaleimide-free, 2 mM ethacrynic acid medium results in increased ethacrynic acid uptake beyond that of the control.

Table II shows also that incubation with two other thiol reagents, iodoacetic acid[15,16] and $HgCl_2$ (refs 15, 17) did not diminish uptake of ethacrynic acid by the tissues.

The lack of transport site specificity of ethacrynic acid binding

The binding of ethacrynic acid, unlike that of ouabain[18,19] is independent of medium Na^+ concentration from 0 to 130 mM although both materials inhibit both active transport of Na^+ and $(Na^+–K^+)$-ATPase[5]. The fact that 0.1 mM ouabain did not affect bound ethacrynic acid loss indicates that ouabain and ethacrynic acid act at different sites (see Table III). Release of bound ethacrynic acid was not affected by the concurrent presence in the medium of a sugar, 2-deoxygalactose, whose

active transport in rabbit kidney cortex slices is profoundly inhibited by 2 mM ethacrynic acid[7]. That neither the binding of ethacrynic acid nor the release of bound ethacrynic acid is affected by the presence of ouabain or of an actively transported sugar indicates that the binding of ethacrynic is not site specific.

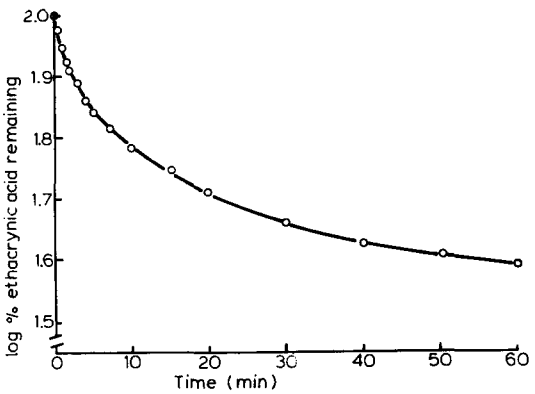


Fig. 5. Efflux of ethacrynic acid from rabbit kidney cortex. Slices were preincubated and incubated under the experimental conditions as in Fig. 1. After incubation, slices were blotted and weighed with one group being taken for analysis at that time. The remaining slices were placed in fine, wire mesh tubes and the washout of ethacrynic acid into a series of 50-ml tubes, each containing 10 ml of Na^+ Krebs–Ringer media, was followed. After immersion in the last solution, the slices were analyzed for ethacrynic acid content. Each point is the mean of the results of two experiments and the data is presented as the log % of the original (pre-washout) ethacrynic acid content remaining in the tissue at the indicated time.

Efflux of ethacrynic acid

The efflux of labeled ethacrynic acid from preloaded tissue was studied. Fig. 5 shows a considerable portion of the ethacrynic acid washed out at a slow rate. With the exception of this component, compartmental analysis[20] did not reveal distinctly separable components.

The loss of ethacrynic acid from preloaded tissues into salines was not significantly affected by the presence of 2 mM unlabeled ethacrynic acid, indicating a firm association of the inhibitor with some tissue component, *i.e.* binding (see above and Table III). This conclusion was further borne out by preliminary experiments where tissue preloaded with ethacrynic acid was homogenized in iced saline and 30–40 % of the tissue ethacrynic acid remained with a well washed pellet sedimenting at forces up to 18000 × *g*.

Efflux of ethacrynic acid into the medium was not significantly affected by 0.1 mM 2,4 dinitrophenol or cold (0 °C) (see Table III).

DISCUSSION

Ethacrynic acid is avidly accumulated by rabbit kidney cortex slices *in vitro*. This accumulation is a time-dependent function and is linear over a concentration range of 25-fold. It is not inhibited by metabolic depressants, *e.g.* 2,4-dinitrophenol nor by incubation in the cold (0 °C). The lack of 2,4-dinitrophenol effect is in contrast to the observation of Beyer and his colleagues[5]. The ethacrynic acid that is quite

firmly bound appears to be associated with tissue solids rather than in intracellular solution. It would appear from these data that the accumulation of ethacrynic acid is a chemical association rather than a conventional transport process.

It has been widely proposed that the function of ethacrynic acid is mediated *via* binding with tissue sulfhydryl groups[1,3]. The data presented here indicate that although it is true that tissue uptake of ethacrynic acid is sharply reduced by the presence of excess sulfhydryl groups, this binding is not sufficient to explain ethacrynic acid's interaction with tissues. *N*-Ethylmaleimide, $HgCl_2$, and iodoacetate, all in concentrations known to inhibit active transport in kidney[13,16,21,22] and other tissues[15,23] fail to reduce tissue uptake of ethacrynic acid. An effect of these thiol reagents would be expected if ethacrynic binding were solely to transport associated membrane –SH groups.

One might argue that membrane –SH groups are not equivalently accessible to different thiol reagents[23]. The fact that three thiol reagents, having different transport inhibitory effects, presumably through interaction with tissue–SH groups[15], have no effect on ethacrynic acid uptake suggests that binding of ethacrynic acid to rabbit kidney cortex is not solely at membrane –SH groups.

It might be proposed that ethacrynic acid does bind to transport specific –SH groups but that these are in very low concentrations in the tissue membranes. Very low concentrations of ethacrynic acid were not used in these experiments because of the lack of a sufficiently high specific activity ethacrynic acid. However, at 0.33 mM ethacrynic acid, 1 mM *N*-ethylmaleimide reduced ethacrynic acid binding by less than 20%. With 2 mM ethacrynic acid and 1.0 mM *N*-ethylmaleimide, the same magnitude of reduction in binding was observed. Thus, it is unlikely that ethacrynic acid effects its actions by binding to site specific –SH groups in low concentration in cell membranes.

Other data weighing against the view that binding of ethacrynic acid is transport site specific are the facts that neither the presence of ouabain, nor the absence of Na^+, nor the presence of an actively transported sugar significantly reduced ethacrynic acid uptake. Ouabain uptake has been shown to be related to the presence of Na^+ and K^+ (refs 17, 18). Uptake of ethacrynic acid, although ethacrynic acid like ouabain inhibits (Na^+–K^+)-ATPase and Na^+ pumping[2,6,24], was not changed by variation in medium Na^+ concentration. These data were consistent with reports that ethacrynic acid and ouabain act at different sites with a different mechanism of action[6,24]. Since ethacrynic acid also inhibits non-Na^+-dependent sugar transport[7], it might be proposed that in this instance it is binding directly to a sugar transport site; no sugar effect on ethacrynic acid uptake was observed (see Table I).

Binding of ethacrynic acid to some cellular component of rabbit kidney cortex has been demonstrated in this report. This tissue component is most probably neither a group of specific membrane protein –SH side chains nor a uniquely configured membrane active transport site.

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