Biochemical Pharmacology, Vol. 24, pp. 775–778. Pergamon Press. 1975. Printed in Great Britain.

DIFFERENCES IN CHARACTERISTICS OF ETHACRYNIC ACID ACCUMULATION IN KIDNEY CORTEX, MEDULLA AND PAPILLA

YEHUDA GUTMAN*, HANNA WALD and WALTER CZAZKES

Department of Pharmacology, The Hebrew University-Hadassah School of Medicine, and Laboratory of Clinical Investigation, Hadassah University Hospital, Jersulem, Israel

(*Received* 8 *July* 1974; *accepted* 25 *September* 1974)

Abstract—Ethacrynic acid (EA) accumulation *in vitro* by slices of rat kidney cortex, medulla and papilla was studied. Slice/medium (S/M) concentration ratio increased during incubation, approaching saturation after 150 min. S/M in slices from cortex reached 12 while in medullary and papillary slices it did not exceed 2. Increasing EA concentration in the medium from 10^{-7} to 5×10^{-3} M decreased the S/M in cortical slices from 12 to 2 while no significant effect was observed in slices of medulla or papilla. EA accumulation by cortical slices was drastically reduced in the absence of sodium in the medium, while no change in EA accumulation occurred in slices of medulla or papilla. In potassium-free media, EA accumulation was slightly but significantly reduced in cortical slices but no effect was observed on EA concentration in medullary or cortical slices. Probenecid reduced S/M of EA in cortical but not in medullary or papillary slices. *N*-ethylmaleimide reduced EA accumulation in slices of kidney cortex but not of medulla or papilla. These data suggest that EA accumulation in kidney cortex is through the organic acid transport process and differs from the mechanism of accumulation in medulla or papilla. Anoxia and elevation of pH to 8·5 reduced EA accumulation in slices from all three regions of the kidney.

Ethacrynic acid (EA) is a potent diuretic drug [1], probably the most effective natriuretic agent in clinical use. Several studies have indicated that its main site of action is in the ascending limb of the loop of Henle [2, 3, 4]. The mechanism of action is, however, still controversial. Inhibition of (Na + K)-dependent ATPase has been demonstrated *in vivo* and *in vitro* [5, 6], but, this action is not specific, since Mg-ATPase is also effectively inhibited by EA [5]. Moreover, some authors found no significant inhibition of kidney ATPase by EA [7, 8]. The reaction of EA with kidney cells most probably involves SH groups [9]. As a possible lead to the mechanism of EA action, localisation of EA in the kidney has been studied, as well as accumulation in kidney slices [8, 10, 11]. Most studies on accumulation of EA were carried out on slices from the kidney cortex. Since the major site of the diuretic action is in the ascending loop of Henle, which is located in the medulla, it seemd to us that comparison of binding or accumulation of EA in different regions of the kidney could help in elucidating the mechanism of action of EA. Furthermore, we have recently reported on differences in the effect of urea, sodium and lithium on (Na + K)-ATPase in the cortex, medulla and papilla of the kidney [12, 13]. Thus, differences in the properties of the sodium pump could be involved in the selective action of EA in particular sites of the kidney and may be manifested in specific binding properties for EA.

The present report shows the results of a comparison of EA accumulation in the cortex, medulla and papilla of the rat kidney and indicates a different mechanism for cortical and for medullary EA binding.

MATERIALS AND METHODS

Experiments were carried out on male albino rats of the Hebrew University strain (Sabra) weighing 180–250 g. Immediately following decapitation, the kidneys were removed and placed on ice. After sagittal section of the kidney, to expose the different regions, slices were prepared of cortex (outermost part), papilla (the pale, inner medulla, protruding into the pelvis) and medulla (outer, red-coloured medulla). The slices weighed 25–40 mg.

The incubation medium consisted of: NaCl, 112 mM; $NaHCO_3$, 15 mM; Na-acetate, 9 mM; Na_2HPO_4, 2·4 mM; NaH_2PO_4, 0·6 mM; KCl, 8 mM; $MgSO_4$, 1·2 mM; Ca-gluconate, 1·0 mM; Na_2SO_4, 0·6 mM; glucose, 5 mM. The medium was aerated with 95% O_2–5% CO_2 to give a final pH of 7·5. Two-and-a-half μCi [^{3}H]ethacrynic acid was added, and the final concentration of EA was adjusted by adding unlabelled EA at appropriate concentrations. In a few experiments, 0·25 μCi [^{3}H]ethacrynic acid was used, when accumulation of EA by slices at very low concentrations of EA in the medium were studied.

The incubation at 25° was carried out for 60 min, except for the experiments on the kinetics of accumulation of ethacryinc acid, which lasted 150 min.

At the end of the experiment the slices were blotted to dryness and were then extracted in boiling water for 1 hr. A sample of the incubation medium and of the tissue extract were added to 10 ml of the phosphor (prepared by dissolving 5 g PPO† and 0·3 g POPOP‡ in 1 litre of toluene and addition of 500 ml Triton X-100 and 150 ml of H_2O) and counted in a liquid scintillation spectrometer. Results are expressed as the ratio of ethacrynic acid concentration in the tissue (slice) to that in the medium (S/M).

The identity of the radioactive compound accumulated in the kidney slices after incubation with radioactive ethacrynic acid was established by chromat-

* Established Investigator of the Chief Scientist Bureau, Israel Ministry of Health.

† PPO = 2,5-diphenyloxazole.

‡ POPOP = 1,4-di-(2-(5-phenyloxazolyl))-benzene.

ography of the tissue extract with *n*-butanol–acetic acid–water 40:10:50. Over 85 per cent of the radioactivity accumulated in the slices consisted of unaltered ethacrynic acid.

Materials. Ethacrynic acid was obtained from Assia Pharmaceutical Industry, Ramat Gran, Israel; [^{3}H]ethacrynic acid, sp. act. 3·34 Ci/m-mole, was prepared at the Nuclear Research Centre, Negev (Israel Atomic Energy Commission); Probenecid and *N*-ethylmaleimide were obtained from Sigma; Ouabain was purchased from Merck.

RESULTS

Accumulation of ethacrynic acid. Figure 1 shows that ethacrynic acid accumulation was initially rapid and gradually declined. The concentration of EA by slices from the kidney cortex was considerably larger than that by slices from the medulla or the papilla. No significant differences in the rate of accumulation or in the extent of concentration of EA was observed between slices from the medulla and the papilla.

Effect of EA concentration on accumulation by kidney slices. When the concentration of EA in the medium increased, the accumulation by slices (as measured by S/M ratio) decreased considerably in the kidney cortex (from 12 to 2) while very small changes occurred in the S/M ratio of slices from kidney medulla or papilla (Fig. 2).

Effect of NEM, ouabain and of anoxia on EA accumulation. Ethacrynic acid reacts with SH groups [9]. To elucidate whether accumulation of EA involves a reaction with SH groups, kidney slices were incubated with EA in the presence of an SH reagent, *N*-ethylmaleimide (NEM). Figure 3 shows that the S/M ratio was reduced by NEM only in slices from the kidney cortex, while in slices from the medulla and papilla no change in S/M was observed. On the other hand, replacement of O_2 by N_2 in the gas mixture during incubation of the slices caused a significant reduction of the S/M ratio in slices from all three regions of the kidney (Fig. 3). However, the largest effect was on slices from the kidney cortex.

Incubation of kidney slices from all three regions of the kidney with EA in the presence of ouabain (10^{-3} M) showed no effect on the S/M ratio, either

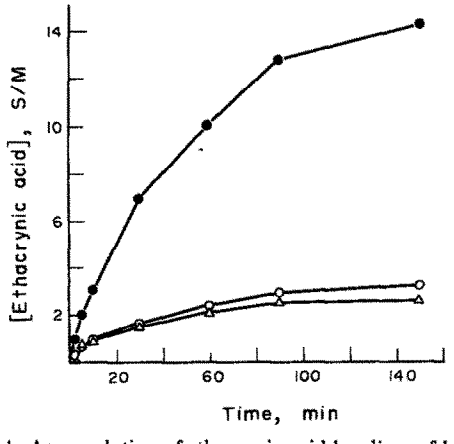

Fig. 1. Accumulation of ethacrynic acid by slices of kidney cortex, medulla and papilla. Each point is the mean of five experiments: (●) cortex; (○) medulla; (△) papilla. S/M = slice/medium.

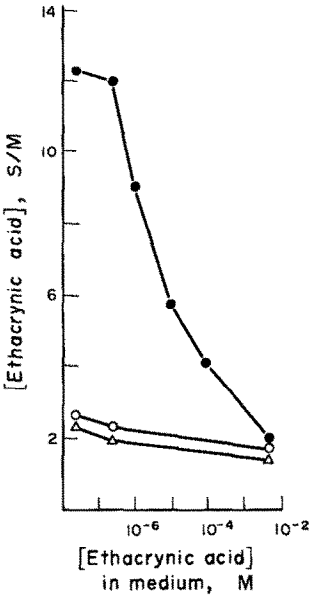

Fig. 2. Effect of ethacrynic acid concentration on accumulation by slices of kidney cortex, medulla and papilla. Each point is the mean of five experiments: (●) cortex; (○) medulla; (△) papilla. S/M = slice/medium.

when ouabain was present only during the incubation or when slices were preincubated with ouabain for 1 hr prior to addition of EA (S/M ratio in slices of kidney cortex—control 11·3, after ouabain 11·3; medulla—control 2·5, after ouabain 2·5; papilla– control 2·0, after ouabain 2·1).

Effect of sodium and potassium on EA accumulation. Since many organic acids are accumulated by kidney slices through a common transport mechanism requiring sodium, it was of interest whether EA accumulation depended on sodium or potassium concentration in the medium. Figure 4 shows that ommission of potassium from the medium caused a small but

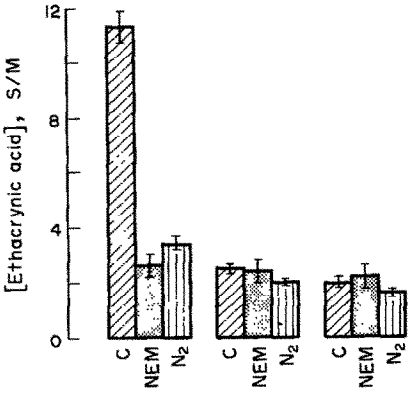

Fig. 3. Effect of *N*-ethylmaleimide and of anoxia on ethacrynic acid accumulation by slices of kidney cortex, medulla and papilla. Vertical bars denote S.E. Number of experiments—Control 5; anoxia 5; *N*-ethylmaleimide 3. For cortex, NEM–control and anoxia–control difference: $P < 0{\cdot}001$; for medulla, anoxia–control difference: $P < 0{\cdot}01$; for papilla, anoxia control difference $P < 0{\cdot}05$. C = control; NEM = *N*-ethylmaleimide; N_2 = anoxia. Left –slices from cortex; centre– slices from medulla; right– slices from papilla. S/M = slice/medium.

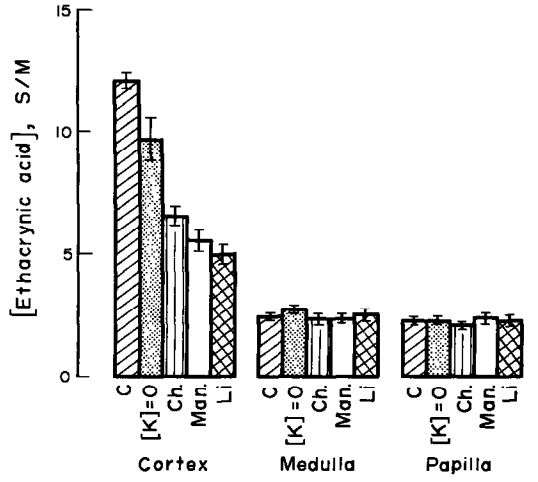

Fig. 4. Effect of sodium and potassium on ethacrynic acid accumulation by kidney cortex, medulla and papilla. Vertical bars denote S.E. Number of experiments—Control: cortex 11, medulla 13, papilla 13. In all other experiments each column is the mean of four experiments. Cortex: control–potassium-free medium difference $P < 0.005$; difference control–choline, control–mannitol and control–lithium $P < 0.001$; choline–lithium difference $P < 0.05$. C = control; Ch = sodium replaced with choline; Man = sodium replaced with mannitol; Li = sodium replaced with lithium. S/M = slice/medium.

significant reduction of the S/M ratio in slices from the cortex but had no effect on slices from the medulla or papilla. Replacement of sodium in the incubation medium by choline, mannitol or lithium caused a drastic fall in the S/M ratio for EA in the kidney cortex but no significant change in the medulla or papilla (Fig. 4). Even replacement of part of the sodium in the medium (from 142 to 122 mM), caused a significant reduction of S/M for EA in the cortex (from $12{\cdot}1 \pm 0{\cdot}3$ to $10{\cdot}6 \pm 0{\cdot}4$, $P < 0{\cdot}025$) but no change in medulla or papilla.

Effect of probenecid on EA accumulation. Since probenecid is an inhibitor of organic acid accumulation by kidney [14, 15], it was of interest whether probenecid affected EA accumulation. Figure 5 shows that probenecid reduced EA accumulation by kidney cortex (S/M). The effect of probenecid was already considerable

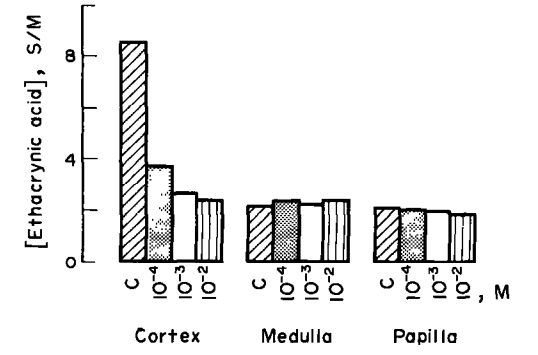

Fig. 5. Effect of probenecid on ethacrynic acid accumulation by slices of kidney cortex, medulla and papilla. Each column is mean of two or three experiments. S/M = slice/medium. C = control. 10^{-4}–10^{-2} M: concentration of probenecid in medium.

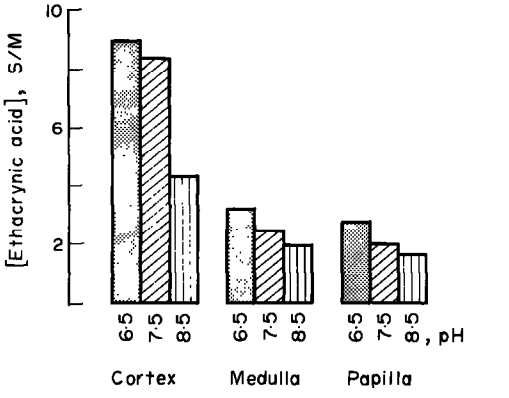

Fig. 6. Effect of pH on ethacrynic acid accumulation by slices of kidney cortex, medulla and papilla. Each column is mean of three experiments. S/M = slice/medium.

at a concentration of 10^{-4} M. However, with kidney medulla and papilla probenecid had no effect on EA accumulation even when present at a concentration of 10^{-2} M.

Effect of pH on EA accumulation. When the pH of the incubation medium was varied between 6·5 and 8·5, accumulation of EA showed a definite change in all three parts of the kidney as seen in Fig. 6; the S/M ratio of EA was highest at pH 6·5 and lowest at pH 8·5 with intermediate values at pH 7·5.

DISCUSSION

The experiments of the present report demonstrate several differences in the mechanism of accumulation of EA in the kidney cortex from that in kidney papilla and medulla. Thus, Fig. 2 shows that in kidney cortex EA accumulation is a saturable process, with S/M values decreasing progressively with the increase in EA concentration in the medium. Slices from the kidney medulla and papilla show a much lower concentrating ability and no significant saturation could be demonstrated with medium concentration of EA up to 5 mM. Blockade of SH groups with NEM significantly inhibited EA accumulation in kidney cortex slices but had no significant effect on accumulation in medulla or papilla (Fig. 3). Accumulation of EA was drastically reduced in cortical slices in the absence of Na^+, and either choline or lithium could not restore the concentrating ability (Fig. 4). However, EA accumulation in slices from medulla or papilla was not significantly affected in the absence of sodium (Fig. 4). Finally, probenecid significantly decreased EA accumulation in the kidney cortex but not in slices from medulla or papilla (Fig. 5). Thus, the accumulation of ethacrynic acid by kidney cortex is distinguished from accumulation in medulla and papilla by being a saturable process, involving reaction with SH groups and depending on the presence of sodium in the medium.

We have further investigated whether preloading of the cells with sodium (by preincubation in a potassium-free, glucose-free medium under anoxia) could affect EA accumulation, but no decrease of EA concentration in these slices was observed. Thus, it was not intracellular but extracellular sodium which was essential for the accumulation process.

The lack of interaction of ouabain and EA binding corroborates a recent report on EA binding to kidney cell membranes [8], However, Charnock and Almeida have reported inhibition by ouabain of EA accumulation [10]. The difference in results from the present report may be due to the difference of species studied (rabbit vs rat).

Two features of EA accumulation were common to slices from all regions of the kidney: reduced EA concentration under anoxia (Fig. 3), indicating that accumulation is energy dependent and increased EA accumulation when pH was lowered (Fig. 6). This corroborates previous reports of reduced transport of organic acids by the kidney [14], particularly probenecid in alkalosis [15].

The characteristics of EA accumulation resemble in many respects those of organic acid accumulation [14]: It is a saturable process, limited to the kidney cortex, requires the presence of sodium and shows competition with other organic acids, probenecid being the prototype [14, 15, 17, 18]. Our results suggest that EA accumulation by kidney cortex may be completely unrelated to its action as a diuretic drug. Ethacrynic acid shows maximal accumulation in cortical slices; however, the (Na + K)-dependent ATPase from the papilla and medulla is more sensitive to inhibition by EA (to be published) and the main natriuretic activity is located in the ascending limb of Henle [2, 3, 4, 16].

Acknowledgement—This paper is part of a Ph.D. thesis of H. W. Supported by a research grant of the Israel Ministry of Health to Dr. Y. Gutman.

REFERENCES

1. K. H. Beyer, J. E. Baer, J. K. Michaelson and H. F. Russo, *J. Pharmac. exp. Ther.* **147,** 1 (1965).
2. M. Goldberg, *Ann. N.Y. Acad. Sci.* **139,** 443 (1966).
3. A. F. Lant, W. I. Baba and G. M. Wilson, *Clin. Sci.* **33,** 11 (1967).
4. J. H. Laragh, P. J. Cannon, W. B. Stason and H. O. Heinemann, *Ann. N.Y. Acad. Sci.* **139,** 453 (1966).
5. P. W. Davis, *Biochem. Pharmac.* **19,** 1983 (1970).
6. B. R. Nechay and R. R. Contreras, *J. Pharmac. exp. Ther.* **183,** 127 (1972).
7. B. R. Nechay, R. F. Palmer, D. A. Chinoy and V. A. Posey, *J. Pharmac. exp. Ther.* **157,** 599 (1967).
8. C. Inagaki, M. Martinez-Maldonado and A. Schwartz, *Archs. Biochem. Biophys.* **158,** 421 (1973).
9. R. M. Komorn and E. J. Cafruny, *Science, N.Y.* **143,** 133 (1964).
10. J. S. Charnock and A. F. Almeida, *Biochem. Pharmac.* **21,** 647 (1972).
11. E. J. Landon and D. F. Fitzpatrick, *Biochem. Pharmac.* **21,** 1561 (1972).
12. Y. Gutman, H. Wald and W. Czaczkes, *Pflügers Arch.* **345,** 81 (1973).
13. Y. Gutman, S. Hochman and H. Wald, *Biochim. biophys. Acta* **298,** 284 (1973).
14. A. Despopoulos, *J. theoret. Biol.* **8,** 163 (1965).
15. W. O. Berndt and E. C. Beechwood, *Am. J. Physiol.* **208,** 642 (1965).
16. W. N. Suki, G. Eknoyan and M. Martinez-Maldonado, *Ann. Rev. Pharmac.* **13,** 91 (1973).
17. G. Vogel, F. Lauterbach and W. Kroger, *Archs. Ges. Physiol.* **283,** 151 (1965).
18. S. G. Schultz and P. F. Curran, *Physiol. Rev.* **50,** 637 (1970).