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THE EFFECTS OF ETHACRYNIC ACID ON ACTIVE TRANSPORT OF SUGARS AND IONS AND ON OTHER METABOLIC PROCESSES IN RABBIT KIDNEY CORTEX

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SUMMARY

The effects of ethacrynic acid and several of its chemical analogues on the active transport of sugars and electrolytes, on cell volume, O_2 utilization, and tissue content of ATP were studied in slices of rabbit kidney cortex.

1. Incubation of the tissues in 0.5–3.0 mM ethacrynic acid sharply inhibited both Na^+-dependent and Na^+-dependent and Na^+-independent active transport of sugars and the maintenance of normal tissue electrolyte distribution; water content was inhibited in the same range of concentrations.

2. The magnitudes of the above inhibitory effects were functions of both the time that the tissue was exposed to the inhibitor and the medium concentration of the inhibitor.

3. Incubation of rabbit kidney cortex slices with four chemical analogues of ethacrynic acid, characterized by widely varying sulfhydryl reactivity, in the same concentration ranges revealed qualitatively similar effects on the above-mentioned transport processes. Hence, a structure–function relationship between sulfhydryl-reacting structure and inhibitory effects of ethacrynic acid and its analogues was not observed.

4. Ethacrynic acid and its analogues diminished O_2 uptake of the tissue in both Na^+-containing and Na^+-free media. The inhibition of O_2 uptake was, therefore, not merely reflective of ethacrynic acid-induced diminished energy requirements for Na^+ pumping.

5. Rapid diminution of tissue ATP content occurred during tissue incubation with ethacrynic acid.

6. The results presented are interpreted as indicating that ethacrynic acid exerts inhibitory effects on several levels of cell metabolism and thus is not a specific inhibitor of individual active transport processes.

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INTRODUCTION

The diuretic, ethacrynic acid, is known to be an inhibitor of electrolyte transport in and affect the metabolism of kidney[1–4] as well as other tissues[5–9]. These metabolic effects include increased tissue water content[1,4,8], reduction in O_2 uptake of whole tissues[1,4,10], inhibition of isolated enzymes[6 11–13], inhibition of O_2 uptake of isolated mitochondria[7,14], and the reduction in tissue content of adenine nucleotides[8].

Several investigators have postulated theoretical systems attempting to explain the mechanism of action of ethacrynic acid in the many functional derangements listed above, particularly with respect to inhibition of Na^+ transport. These propositions fall roughly into two categories. The first, proposed by Whittembury[2] and others[9], states that ethacrynic acid has specific inhibitory effects on one or more autonomous cation pumping systems. The second, proposed by Macknight[4] and others[7,8] states that ethacrynic acid works by reducing the availability of energy to specific, energy-dependent pumps used by the cell to maintain its constant internal environment.

This investigation was designed to contribute to the understanding of the effects and mechanism of action of ethacrynic acid and its chemical analogues on transport and other metabolic processes in rabbit kidney cortex. The results presented here, in addition to confirming the effect of ethacrynic acid on tissue water and electrolyte content, further show that other metabolic derangements, *e.g.* inhibition of active sugar transport and reduction in respiration, are not dependent on the inhibition of Na^+ transport. The data essentially support the proposition[4] that ethacrynic acid is a non-specific inhibitor of cellular energy production and that its manyfold effects may be largely explained on this basis.

MATERIALS AND METHODS

Many of the experimental and analytical methods used have been described previously and only variations or different methods will be treated here.

Experimental

The experiments were carried out using kidney cortex slices of adult, healthy rabbits. The methods of preparation and incubation of the kidney slices have been described[15]. The Krebs–Ringer balanced media were prepared as described by Kleinzeller[16] except that only media with pH 7.4 were used. Experimental methods for the determination of tissue content of sugar[15], water[17], and electrolytes[18], have been previously presented. As in earlier communications from this laboratory[15,16,18], sugar transport results are presented as accumulation ratio, *i.e.* the ratio of the final intracellular sugar concentration (S_i), corrected for the extracellular (inulin) space (see ref. 17), to the final medium sugar concentration (S_o), (S_i/S_o). Water content values are reported as the ratio of kg tissue water to kg of dry tissue wt (kg/kg dry wt). Oxygen uptake was measured at 25 °C by the Warburg technique. Results are expressed as average μl O_2 consumed per mg of dry tissue wt per h (Q_{O_2}) or total μl O_2 consumed per mg of dry tissue wt to a given time of incubation.

Slices used in ATP analysis were handled in the standard fashion except that immediately after incubation they were placed in 0.33 M $HClO_4$ at 0 °C, homogenized and centrifuged at 0 °C.

Analytical

Sugar concentrations in tissue and media were analyzed in $Ba(OH)_2$-$ZnSO_4$ extracts (see ref. 1). Electrolyte concentrations were determined by atomic absorption spectrometry of acid extracts of dried slices (see refs 16 and 19).

Tissue homogenates for ATP analysis were centrifuged at 0 °C at 12000 rev./min for 10 min in a refrigerated Sorval centrifuge. An appropriate volume of the supernatant was added to a KOH–triethanolamine buffer designed to maintain the final solution at pH 7.6. Aliquots of the buffered mixture were analyzed spectrophotometrically using Biochemica Test Combination for ATP (Cat. No. TC-J, 15979 TAAC) of the Boehringer Mannheim Co. A molar extinction coefficient of $6.2 \cdot 10^6$ cm^2/mole was used for evaluation of NADH consumed in the analytical reaction. ATP content is reported as mmoles ATP per kg protein. Protein was determined by the Biuret method[20].

Most reported values are the means of at least four determinations and $\pm$ S.E. is reported if at least four determinations were made.

Materials

2-Deoxy-D-galactose and 2-deoxy-D-glucose were used as model sugars for the group of sugars whose active transport is independent of tissue or medium Na^+ concentrations in rabbit kidney cortex slices[16]. α-Methyl-D-glucoside and D-galactose were used as model sugars for the group of sugars whose active transport is dependent on Na^+ concentration[15].

D-[1-^{14}C]Galactose, α-methyl-D-[1-^{14}C]glucoside, and 2-deoxy-D-[1-^{14}C]glucose were purchased from the New England Nuclear Corp., Boston. 2-Deoxy-D-galactose, *N*-tris(hydroxymethyl)methyl-2-amino ethane sulfonic acid (TES), L-cysteine, and 2,4-dinitrophenol were obtained from the Sigma Chemical Co. All other reagents were reagent grade.

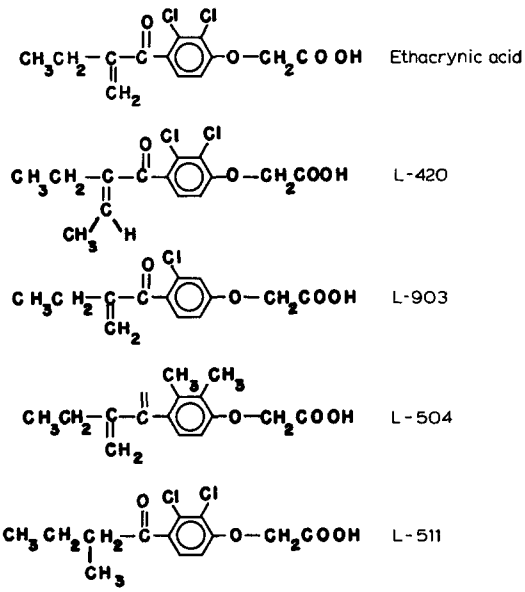

Fig. 1. Structures of ethacrynic acid and its chemical analogues.

Ethacrynic acid and its chemical analogues (structural formulae p. 130) were kindly provided by Dr John E. Baer of the Merck Institue for Therapeutic Research.

Ethacrynic acid: 2,3-dichloro-4-(2-methylenebutyryl)phenoxyacetic acid. L-589, 420(L-420): 2,3-dichloro-4-(2-ethylenebutyryl)phenoxyacetic acid. L-586, 903 (L-903): 3-chloro-4-(2-methylenebutyryl)phenoxyacetic acid. L-588, 504 (L-504); 2,3-dimethyl-4-(2-methylenebutyryl)phenoxyacetic acid. L-593, 511 (L-511): 2,3-dichloro-4-(2-methylbutyryl)phenoxyacetic acid (see Fig. 1).

It should be noted that these chemical analogues of ethacrynic acid vary considerably in their affinity for sulfhydryl groups[11].

RESULTS

Effects of ethacrynic acid on active sugar transport

In concentrations known also to markedly affect the electrolyte distribution in kidney cortex[2,4], *i.e.* 0.5–3 mM, ethacrynic acid sharply reduced the accumulation of sugars in rabbit kidney cortex regardless of whether the active transport of these sugars was dependent upon media Na^+ concentrations. Fig. 2 shows the effects of ethacrynic acid and one of its chemical analogues, L-420 (see *Materials*) on the transport of 2-deoxy-D-galactose and α-methyl-D-glucoside. At high ethacrynic acid and L-420 concentrations, *i.e.* 3 mM, the accumulation ratio approached unity, indicating a complete inhibition of active sugar transport. It was observed that in the lower concentration ranges, *i.e.* 0–1.0 mM, L-420 did not decrease active sugar transport

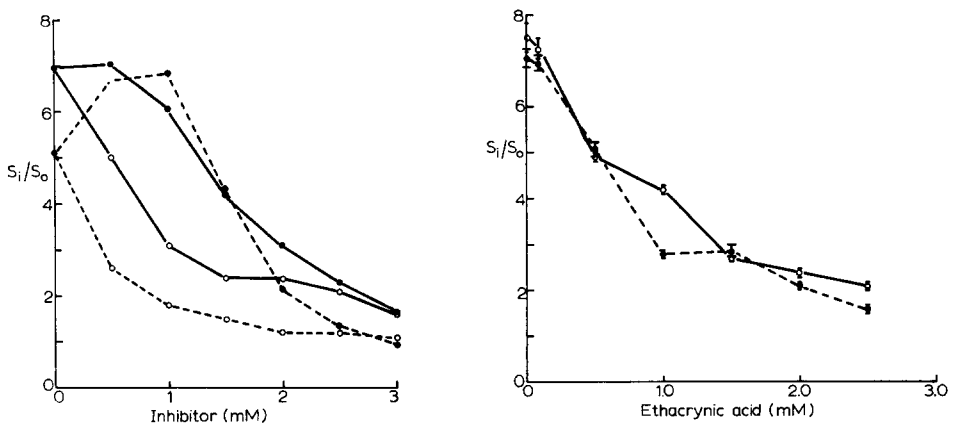

Fig. 2. Effect of ethacrynic acid and L-420 on active sugar transport in rabbit kidney cortex slices. All slices were preincubated aerobically in Na^+ Krebs–Ringer media at 25 °C for 45 min. Groups of slices were then incubated aerobically at 25 °C for 60 min in Na^+ media containing either 1 mM 2-deoxy-D-galactose (———) or α-methyl-D-glucoside (– – – –) in the presence of various concentrations of ethacrynic acid (○) or L-420 (●). Each point is the mean of at least three determinations.

Fig. 3. Effect of ethacrynic acid on 2-deoxy-D-galactose transport in Na^+-free and Na^+-containing media. All slices that were used in Na^+-free, Krebs–Ringer medium (Li^+ used to replace Na^+) were first leached for 2.5 h at 0 °C in this medium with frequent washing (see refs 16 and 21). Groups of slices were preincubated and then incubated in either Na^+-containing (○) or Na^+-free (●) media using experimental conditions as in Fig. 2. Incubation media, containing 0.5 mM 2-deoxy-D-galactose, were either inhibitor-free (controls) or contained various concentrations of ethacrynic acid. Values reported are the means of four determinations ± S.E.

whereas ethacrynic acid was an effective inhibitor from the lowest concentration shown.

Although the active transport of 2-deoxy-D-galactose has been shown to be independent of medium Na^+ concentration, it might still be proposed that ethacrynic acid produces its inhibition of active transport of this sugar *via* interference with Na^+ metabolism of the cells. However, in Na^+-impoverished slices incubated in Na^+-free media (apparent intracellular Na^+ concentration less than 3.5 mM[21]), ethacrynic acid inhibited active transport of 2-deoxy-D-galactose as well as in normal slices in Na^+-containing media (see Fig. 3). The results of Table I further demonstrate the fact that ethacrynic acid inhibited transport of sugars in rabbit kidney cortex slices indepedent of Na^+ concentrations in the system by contrasting the effect of ethacrynic acid with that of ouabain on both Na^+-dependent and Na^+-independent active sugar transport. Ouabain had little effect on the transport of the 2-deoxy sugars while

TABLE I

EFFECT OF OUABAIN AND ETHACRYNIC ACID ON BOTH Na^+-DEPENDENT AND Na^+-INDEPENDENT ACTIVE SUGAR TRANSPORT

Slices were preincubated and incubated under experimental conditions described in Fig. 2. Incubation media contained 0.5 mM sugar and were either without inhibitor (controls) or contained either 2 mM ethacrynic acid or 0.1 mM ouabain. Values are the means of at least three determinations of $S_i/S_o \pm$ S.E.

Sugar	S_i/S_o		
	Control	*Inhibitors*	
		Ethacrynic acid	*Ouabain*
(1) Na^+-dependent			
α-Methyl-D-glucoside	8.58 ± 0.70	2.40 ± 0.17	1.33 ± 0.09
D-Galactose	4.68 ± 0.32	2.02 ± 0.14	3.34 ± 0.25
(2) Na^+-independent			
2-Deoxy-D-galactose	6.67 ± 0.30	2.28 ± 0.18	7.86 (3)
2-Deoxy-D-glucose	4.54 (3)	2.50 ± 0.11	3.97 ± 0.07

TABLE II

EFFECT OF ETHACRYNIC ACID AND ITS ANALOGUES ON BOTH Na^+-INDEPENDENT AND Na^+-DEPENDENT SUGAR TRANSPORT

Slices were preincubated and incubated under experimental conditions described in Fig. 2. Incubation media contained either 1 mM 2-deoxy-D-galactose or 0.5 mM D-galactose, either without inhibitor (controls) or with 2 mM inhibitor. Values are the means of at least three determinations.

Condition	S_i/S_o	
	2-Deoxy-D-galactose	*D-Galactose*
Control	7.66 ± 0.43	8.22 (3)
Ethacrynic acid	2.22 ± 0.13	1.31 (3)
L-420	1.85 ± 0.25	
L-504	4.92 ± 0.03	5.96 (3)
L-511	2.07 ± 0.16	2.32 ± 0.17
L-903	2.86 ± 0.11	3.62 (3)

ethacrynic acid was an effective inhibitor of the active transport of all sugars tested. The other chemical analogues of ethacrynic acid which were effective for sugar transport inhibition (see Table II) were equally effective for both Na^+-dependent and Na^+-independent active sugar transport.

It has been proposed that ethacrynic acid effects its inhibitory actions on tissue function (*i.e.* Na^+ transport, $(Na^+–K^+)$-ATPase activity) *via* a mechanism of action which includes binding or association of ethacrynic acid to tissue sulfhydryl groups[11,22]. It was shown[23] that the binding of ethacrynic acid to rabbit kidney cortex slices was decreased by the concommitant incubation of the tissue with L-cysteine.

The effect of cysteine on the inhibitory action of ethacrynic acid on sugar transport was, therefore, of interest. Slices were incubated in the presence of 2-deoxy-D-galactose and ethacrynic acid or L-420, with and without 4 mM L-cysteine. The results of that experiment, shown in Table III, revealed that paralleling the reduction of ethacrynic acid binding was a reduction of the inhibition of active sugar transport by ethacrynic acid and L-420. This effect could be explained by the proposition that ethacrynic acid and L-cysteine react in solution, reducing the effective ethacrynic acid concentration. However, as shown in ref. 23, L-cysteine removes ethacrynic acid previously bound by the tissue. It was noted that L-cysteine, alone, had a slight inhibitory effect on sugar accumulation.

TABLE III

EFFECT OF CYSTEINE ON BINDING OF ETHACRYNIC ACID AND ETHACRYNIC ACID INHIBITION OF ACTIVE SUGAR TRANSPORT

Slices were preincubated and incubated under experimental conditions described in Fig. 2. Incubation media, containing 0.5 mM 2-deoxy-D-galactose, were either inhibitor free (control) or contained 2 mM inhibitor and/or 4 mM L-cysteine. Binding experiments were done with [^{3}H]ethacrynic acid as described in ref. 22. Data are reported as the means of at least four determinations ± S.E.

Condition	S_i/S_o	*μmoles ethacrynic acid/g dry wt*
Control	6.20 ± 0.2	
Ethacrynic acid	2.10 ± 0.1	38.05 ± 1.41
L-420	3.16 ± 0.01	
Ethacrynic acid + cysteine	5.32 ± 0.03	7.89 ± 0.32
L-420 + cysteine	6.22 ± 0.15	
Cysteine	4.90 ± 0.23	

Effects of ethacrynic acid on extracellular space

Any reduction in the accumulation ratio of a substance may be attributed to either (1) diminution of the rate of active transport of that substance into the space or, (2) increased leakage by diffusion of that substance out of the space, or (3) both. If increased cellular permeability of kidney cells to a sugar, brought about by ethacrynic acid, were considered the essential cause of reduction in accumulation ratio of that sugar, it might be expected that the permeability of other, similar molecules might be similarly affected. Mannitol has been shown to very sluggishly enter rabbit kidney cortex cells[15]. Fig. 4 demonstrates that incubation of slices in the presence of 2 mM ethacrynic acid does not accelerate the entry of mannitol into the intracellular

space. The intracellular space occupied by mannitol after 60 min of incubation, as represented by accumulation ratio, was 0.3 ± 0.01, *i.e.* within the range reported by Kleinzeller[15].

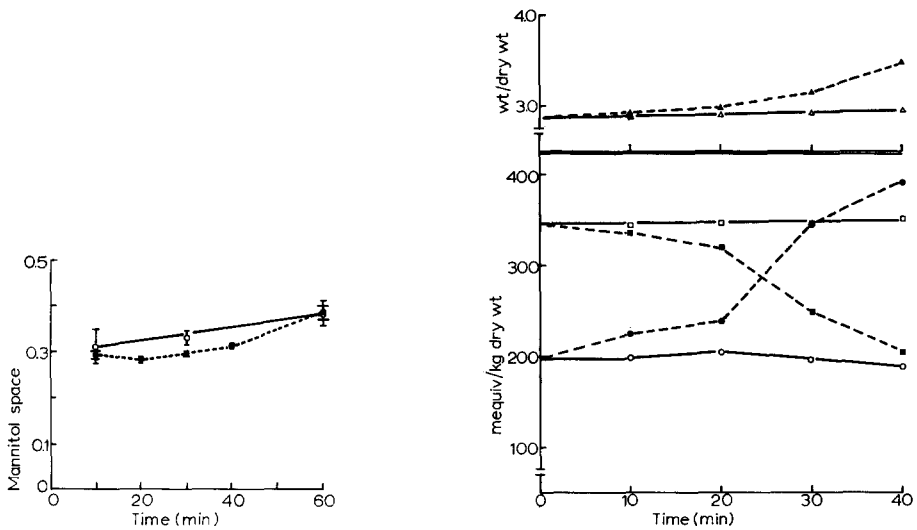

Fig. 4. Effect of ethacrynic acid on mannitol space. Slices were preincubated and incubated in Na^+ Krebs–Ringer media under experimental conditions as in Fig. 2. Incubation media, containing 1 mM mannitol were either without (control, ○) or with 2 mM ethacrynic acid (●). Each value is the mean of six determinations of mannitol space (ml/g wet wt) ± S.E.

Fig. 5. The time effect of ethacrynic acid on tissue electrolyte and water content. Slices were preincubated and incubated in Na^+ Krebs–Ringer media under experimental conditions as in Fig. 2. Incubation media were either inhibitor-free (open symbols), or contained 2 mM ethacrynic acid (filled symbols). Groups of slices were removed at the indicated intervals and analyzed for Na^+ (○, ●), K^+ (□, ■), and water (△, ▲) content. Values are the means of three determinations.

Effect of ethacrynic acid on electrolyte and water content

The incubation of ethacrynic acid and its analogues with rabbit kidney cortex slices caused the tissue to lose K^+, and to gain Na^+, Cl^- and water as previously reported by Whittembury[10] and Macknight[4]. The data of Fig. 5 show that these effects of ethacrynic acid were apparent as early as 10 min of incubation which paralleled the observation of ref. 23 on ethacrynic acid binding to rabbit kidney cortex, but were in contrast to the effects on O_2 utilization, as shown below. The effects of ethacrynic acid on tissue content of water and electrolytes are also ethacrynic acid concentration dependent. Fig. 6 contrasts the concentration dependence of L-420 with that of ethacrynic acid. It was noted that as low concentrations of L-420, *i.e.* 0–1.0 mM, there was little effect on tissue Na^+ and water content and an actual reduction in Cl^- content. This observation parallels the minimal reduction in sugar accumulation ratios in the presence of these concentrations of L-420, as described above.

The other chemical analogues of ethacrynic acid also adversely affected tissue water and electrolyte content maintenance in similar concentration ranges (see Table IV).

TABLE IV

EFFECT OF ETHACRYNIC ACID AND ITS ANALOGUES ON TISSUE ELECTROLYTE AND WATER CONTENT

Slices were preincubated and incubated under experimental conditions as in Fig. 2. Incubation media were either without (control) or with inhibitor. Tissue was analyzed for Na^+, K^+, Cl^-, and water content. Values are the means of six determinations ± S.E.

Inhibitor	[Na^+] (*mequiv/kg dry wt*)	[K^+] (*mequiv/kg dry wt*)	[Cl^-] (*mequiv/kg dry wt*)	*wt/dry wt* (*kg/kg dry wt*)
Control	203 ± 5	330 ± 8	199 ± 4	2.83 ± 0.04
Ethacrynic acid, 2 mM	487 ± 4	105 ± 4	300 ± 7	3.43 ± 0.04
L-420, 2 mM	467 ± 27	146 ± 21	320 ± 23	3.46 ± 0.08
L-504, 2 mM	372 ± 13	191 ± 10	205 ± 4	2.94 ± 0.03
L-504, 3 mM	361 ± 8	173 ± 5	213 ± 6	2.95 ± 0.04
L-511, 2 mM	497 ± 2	151 ± 5	290 ± 9	3.41 ± 0.04
L-511, 3 mM	548 ± 12	112 ± 5	365 ± 11	3.77 ± 0.08
L-903, 2 mM	434 ± 9	116 ± 4	257 ± 7	3.01 ± 0.08
L-903, 3 mM	493 ± 8	108 ± 7	281 ± 12	3.33 ± 0.07

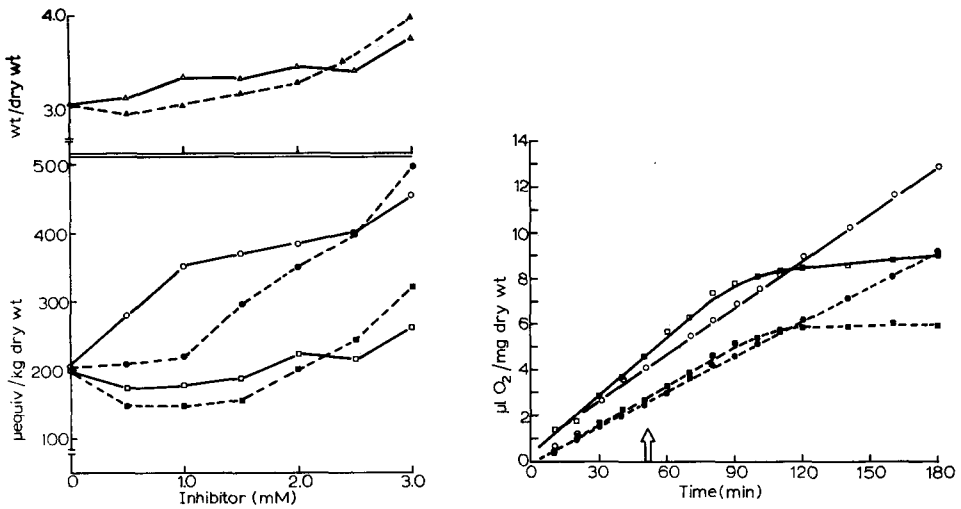

Fig. 6. The effect of varying concentrations of ethacrynic acid and L-420 on tissue water and electrolyte content. Slices were preincubated and incubated in Na^+ Krebs–Ringer media under experimental conditions described in Fig. 2. Incubation media contained either ethacrynic acid (open symbols) or L-420 (filled symbols) at various concentrations. After 60 min of incubation the tissue contents of Na^+ (○, ●), Cl^- (□, ■), and water (△, ▲) were determined. Values are the means of six determinations.

Fig. 7. Effect of ethacrynic acid on O_2 uptake of rabbit kidney cortex slices in both Na^+-containing and Na^+-free media. Slices to be used in Na^+-free (Li^+ used to replace Na^+) media were leached in Na^+-free media for 2.5 h at 0 °C with frequent washings (see refs 16 and 21). Slices were incubated in Warburg flasks containing 3 ml of the appropriate medium. The inhibitor, in the side arm, was added at the time indicated by the arrow, final concentration 2 mM. The center wells of the Warburg flasks contained 0.2 ml 2 M KOH absorbed in a fluted filter paper. Values are the means of two separate experiments and are expressed as μl O_2 absorbed per mg dry wt to the designated time. The average Qo_2 (μl O_2/mg dry wt per h) in the Na^+ medium (control) (○) was 4.75 and in the Li^+ medium (control) (●) 3.08. The Qo_2 for the third hour of ethacrynic acid in Na^+ medium (□) was 0.5 and for ethacrynic acid in Li^+ medium (■) was 0.46.

Effects of ethacrynic acid on O_2 utilization

Ethacrynic acid has been shown to diminish O_2 utilization in kidney slices[4,10] and other preparations[7,8]. Since a significant proportion of the energy requirements of kidney cortex is expended in active transport of Na^+ across tubular cells, it was assumed that this decrease in O_2 utilization in the presence of ethacrynic acid could be attributed to the inhibition of Na^+ transport[10]. Fig. 7 demonstrates that in the absence of Na^+ in the experimental system (Li^+ medium), ethacrynic acid still markedly depressed the O_2 uptake of the tissue. In experiments not reported here in detail, similar inhibition of O_2 uptake in the presence of ethacrynic acid was found when Na^+ in the system was completely replaced by Tris.

The results of a series of experiments investigating the effects of the chemical analogues of ethacrynic acid on respiration of kidney slices are presented in Fig. 8. In experiments not reported here in detail, it was found that these respiratory depression effects were, as with ethacrynic acid, present in Na^+-free systems. These results may be taken as evidence of the view that ethacrynic acid and its analogues exert an inhibitory effect on cellular metabolism beyond that of inhibition of Na^+ transport. Fig. 7 further shows that there was a time lag of 30–40 min before the ethacrynic acid had a demonstrable effect on O_2 utilization. This was in sharp contrast to the effect on the electrolyte content described above.

Effect of ethacrynic acid on ATP content

Any inhibitory effect on an active, energy requiring metabolic process may be mediated *via* reduction of the tissue content of the direct source of metabolic energy for that process, *e.g.* ATP. The active transport of Na^+ in kidney cortex has

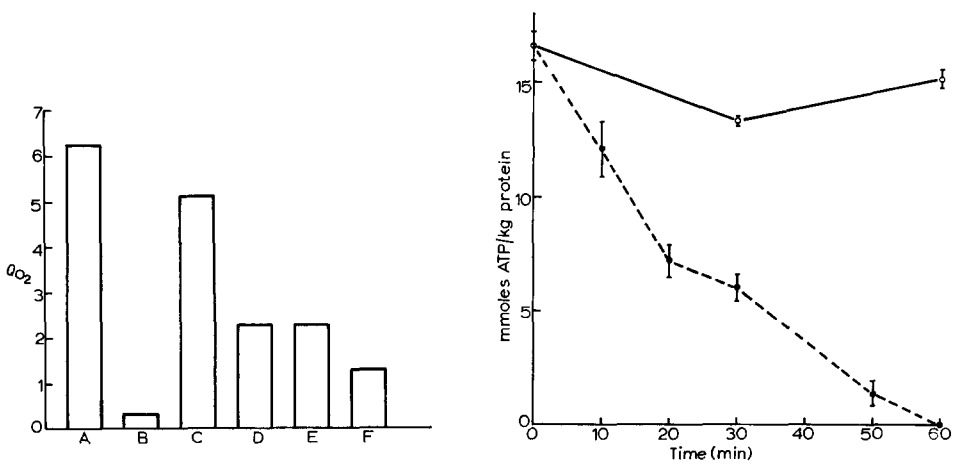

Fig. 8. Effect of various analogues of ethacrynic acid on the Q_{O_2} of rabbit kidney cortex slices. Groups of slices were incubated in Warburg flasks, as described in Fig. 7, for 120 min in either inhibitor-free Na^+ Krebs–Ringer medium (control) or in that medium containing 2 mM inhibitor. The incubation media contained: A, control; B, ethacrynic acid; C, L-420; D, L-504; E, L-511; and F, L-903.

Fig. 9. Effect of ethacrynic acid on ATP content of rabbit kidney cortex slices. Slices were preincubated and incubated under the conditions of Fig. 2 except that groups of slices were removed at the indicated times for analysis. Incubation media were either without inhibitor (control, ○) or contained 2 mM ethacrynic acid (●). Values are the means of six determinations ± S.E.

been linked to activity of (Na^+–K^+)-ATPase[11,24] and it has been shown that ethacrynic acid reduces (Na^+–K^+)-ATPase activity in kidney of several species[11]. In a series of experiments reported in Fig. 9, it was shown that incubation of rabbit kidney cortex slices in the presence of ethacrynic acid reduced ATP content of those slices. After 60 min of incubation, essentially no ATP was detectable. A comparison of Figs 7 and 9 revealed that at a time when tissue ATP content had dropped to one third of control values, the tissue continued to respire at control rates. A comparison of Figs 5 and 9 revealed that, in contrast, the reduction in transport of Na^+ was contemporaneous with the reduction in tissue ATP content.

DISCUSSION

Results of other investigators summarized above have revealed inhibitory effects of ethacrynic acid on electrolyte transport[1–9], cell volume control[1,4,8], O_2 uptake[1,4,10], isolated enzyme systems[6,11–13], active sugar transport[1], and tissue content of adenine nucleotides[8] in various tissues. In this report similar inhibitory effects have been described above in rabbit kidney cortex caused by ethacrynic acid and several of its chemical analogues. It is now difficult to understand how such a diversity of effects can be attributed to a single, master effect of ethacrynic acid on biological systems. However, ethacrynic acid enjoys wide utilization as a tool for the investigation of active transport systems. In view of the data now available, it is pertinent to discuss this latter utilization.

Ethacrynic acid inhibits O_2 uptake in both whole tissues[1,4,7,10] and subcellular fraction preparations[7]. Here it has been shown (see Fig. 7) that the degree of inhibition of O_2 utilization observed is beyond that which might be expected in a system in which only Na^+ transport is inhibited. It has also been found that ethacrynic acid brings about a rapid fall in rabbit kidney cortex ATP content (see Fig. 9). This may be a more general effect of ethacrynic acid since a similar reduction was found in rat uterus[8]. Gordon[7] has shown that in tightly coupled mitochondria preparations isolated from rat kidney and liver, ethacrynic acid inhibits respiration and acts in some respects as an uncoupling agent. It can thus be postulated that the fall in O_2 uptake brought about by ethacrynic acid is secondary to inhibition of mitochondrial ATP production.

There is, however, another source of cellular ATP, but here, too, ethacrynic acid is an effective inhibitor. Several investigators[6,25] have shown that ethacrynic acid inhibits glycolysis in a number of different tissues. Hence, the availability of ATP for use by transport systems is strikingly affected by ethacrynic acid.

It has been shown here (see Figs 5 and 6) and elsewhere[2–5,8] that ethacrynic acid inhibits active Na^+ transport in several different cell types. It is widely held[11,24] that (Na^+–K^+)-ATPase is intimately associated with Na^+ pumping in kidney. This enzyme system is known to be sensitive to ethacrynic acid[11]. The inhibitory effect of ethacrynic acid on Na^+ transport can just as well be explained by its inhibition of ATP production as by its direct inhibitory effect on (Na^+–K^+)-ATPase. The data do not allow the separation of these disparate mechanisms of action.

Sugar transport has been shown in this report to be markedly inhibited by ethacrynic acid (see Figs 2 and 3 and Tables I and II). The simplest explanation for these effects is, as discussed above, the inhibition of production of metabolic energy

in the form of ATP. This would explain the effects of ethacrynic acid on both Na^+-independent and Na^+-dependent active sugar transport, although the latter inhibition might be mediated *via* interference with Na^+ transport.

Ethacrynic acid has been considered a specific inhibitor of an individual Na^+ pumping system in red cells[9] and in kidney[10]. Since it has been shown above that ethacrynic acid apparently directly affects the source of metabolic energy for the active transport of any solute, this inhibitor cannot be used to characterize an active transport system.

Duggan and Noll[11] correlated the sulfhydryl reactivity of ethacrynic acid and its analogues to the diuretic efficacy of these compounds. These authors concluded that the inhibitory effects of ethacrynic acid were mediated *via* binding to tissue sulfhydryl groups. In this report it has been shown (see Table II and Fig. 8) that four chemical analogues of ethacrynic acid have qualitatively similar effects on the metabolic activities of rabbit kidney cortex which were studied, although the sulfhydryl reactivity of these compounds varied widely[11]. L-511, which has no methylene group capable of reacting with tissue sulfhydryl groups (see Fig. 1), and did not react with thioglycolic acid *in vitro*[11], was an effective inhibitor of both Na^+ and sugar transport (see Tables II and III). L-420 had much less sulfhydryl reactivity than ethacrynic acid[11], yet it, too, was a very effective inhibitor of Na^+ and sugar transport in concentrations of 2–3 mM. Further, all of the chemical analogues of ethacrynic acid inhibited O_2 uptake (see Fig. 8), though none as effectively as ethacrynic acid.

There is no doubt that ethacrynic acid reacts with sulfhydryl groups both *in vitro*[11] and *in vivo*[22]. This conclusion is further supported by the experiments reported here where incubation with cysteine limited the inhibitory effect of ethacrynic acid on sugar transport (see Table III and the results on the binding of ethacrynic acid to rabbit kidney cortex slices presented in ref. 23). However, the data presented here may be used to question the view that the sulfhydryl reactivity of these compounds is the necessary and sufficient characteristic to explain their inhibitory effects on metabolism. Ethacrynic acid was originally synthesized with the object of its being a sulfhydryl reagent and hence an inhibitor of Na^+ transport[26]. However, the *in vitro* effects of its own non-sulfhydryl-reactive chemical analogues make moot the proposition that ethacrynic acid exerts its varied inhibitory effects solely *via* interaction with tissue sulfhydryl groups. No other obvious structural-functional correlates have been revealed by this work.

A vexing and unanswered question is that of the time lag of onset of inhibition of O_2 uptake in the presence of ethacrynic acid and its analogues. Since ATP content rapidly falls in the presence of ethacrynic acid it is apparent that the time lag does not reflect the time necessary for ethacrynic acid to penetrate to the mitochondrial sites of major O_2 utilization and ATP production. At this time it can only be stated that the actions of ethacrynic acid on O_2 uptake are complex and not yet fully explained. However, the sum total of the results presented above do not necessarily argue against the possibility that ethacrynic acid, in some of its effects, at the beginning of its action acts at the cell membrane and then as it penetrates more and more to the inside, continues blocking cell metabolism.

The lack of specificity in the metabolic effects of ethacrynic acid suggests that this compound is a blunt tool for the investigation of transport processes.

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