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LOCALIZATION AND CHARACTERIZATION OF THE INHIBITORY ACTION OF ETHACRYNIC ACID ON GLYCOLYSIS*

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Abstract—Ethacrynic acid inhibits glycolysis in intact tissues and in the membrane-free cytoplasmic fraction of cells. A study of the localization of this inhibitory action in the Embden–Meyerhof pathway reveals that ethacrynic acid completely inhibits the glyceraldehyde 3-phosphate dehydrogenase activity of the cytoplasm from tumor cells, rat renal cortex and renal medulla. Phosphoglycerate kinase activity is also inhibited, but to a much lesser extent. Optimum inhibitory activity is observed when the inhibitor and enzyme are brought together prior to assay, but preincubation is not essential to demonstrate the interaction. The data strongly suggest that ethacrynic acid inhibits glyceraldehyde 3-phosphate dehydrogenase by competing with or displacing NAD at a sulfhydryl site on the enzyme. The demonstration that ethacrynic acid interferes with glycolysis and with mitochondrial oxidative phosphorylation as well as cell membrane adenosine triphosphatase leads to the conclusion that its pharmacological potency may be due to its effect at multiple sites on the cellular level. An important consequence of its action is interference with adenosine triphosphate production, thus restricting energy for sodium transport.

THE PHARMACOLOGICAL effectiveness of ethacrynic acid [2,3-dichloro-4-(2 methylenebutyryl) phenoxyacetic acid] has been attributed to an inhibition of sodium transport in the kidney.[1–4] It is widely held that this agent inhibits sodium transport by a direct action on the sodium transport system in the cell membrane. Evidence for this conclusion stems principally from the demonstrated inhibitory effect of ethacrynic acid on the transport ATPase‡ of kidney,[5] and on a specific cation pump associated with the erythrocyte membrane.[6,7] A question has been raised concerning the relevance of this mechanism of action of ethacrynic acid to explain the diuretic properties of this drug *in vivo* because of the high concentrations of ethacrynic acid required to observe inhibition *in vitro*.[5,8]

Recently, additional sites of action of ethacrynic acid have been demonstrated at the cellular and subcellular levels. A complex inhibitory effect of ethacrynic acid on isolated mitochondria[9,10] and on mitochondria of intact cells[10] has been shown to

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‡ Abbreviations used: ATPase, adenosine triphosphatase; G-3-PD glyceraldehyde 3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; ADP, adenosine diphosphate; ATP, adenosine triphosphate; FDP, fructose diphosphate; G-3-P, glyceraldehyde 3-phosphate; 3-PG, 3-phosphoglycerate; 2,3-DPG, 2,3-diphosphoglycerate.

exist. A third site of action in the Embden–Meyerhof pathway was also affected by ethacrynic acid independent of its action at the other two sites.[11] Clearly, the inhibitory effects of ethacrynic acid on mitochondrial metabolism and on glycolysis would interfere with ATP production and consequently could restrict the energy-dependent, cell membrane, ion-transport system. In the present study, attempts have been made to localize the specific site(s) of inhibition by ethacrynic acid in the glycolytic sequence and to study the interaction of the inhibitor with specific enzymes.

METHODS

Ehrlich ascites tumor cells were harvested from the peritoneal cavity of white mice 6–8 days after inoculation. The cells were collected, washed, and the soluble cytoplasmic fraction was prepared by sonication and centrifugation of particulate matter as previously described.[11] Cytosol was prepared from the cortex and from the medulla of mature, male, Sprague–Dawley rats after the method of Schneider and Hogeboom.[12] Either separated cortex or medulla was homogenized in 0·25 M sucrose (10%, w/v). The nuclear fraction was sedimented at 1000 *g*; the particulate-free cytosol was collected after a 105,000 *g* centrifugation for 60 min in the Spinco model L preparative ultracentrifuge. All procedures were carried out at 4°.

Incubation of tumor cytosol was carried out at 37° in an air atmosphere unless otherwise stated; incubation medium contained: NaCl, 125 mM; KCl, 10 mM; $MgSO_4$, 1·5 mM; sodium phosphate buffer, pH 7·4, 12 mM; and nicotinamide, 25 mM. Lactate was determined with an enzymatic technique[13] on the perchloric acid filtrate. Assays for G-3-PD and PGK (both forward and reverse reactions) were performed in a Gilford model 2000 recording spectrophotometer at room temperature by measuring the changes in optical density at 340 mμ according to the techniques of Schrier.[14] The incubation mixture for assay of G-3-PD in the forward direction contained: 165 μmoles tris, pH 8·2, 3·3 μmoles DL-G-3-P; 30 μmoles sodium arsenate; 20 μmoles 2-mercaptoethanol; and 4 μmoles NAD. The assay medium for measurement of G-3-PD activity in the reverse direction contained: 100 μmoles tris, pH 7·5; 15 μmoles $MgCl_2$; 60 μmoles 2-mercaptoethanol; 10 μmoles 3-PG; 1·08 μmoles NADH; 4 μmoles ATP; and 10 μg of yeast PGK (Sigma type 1-c). Assay of PGK in the forward direction was accomplished in a medium containing: 165 μmoles tris, pH 7·5; 15 μmoles $MgCl_2$; 20 μmoles 2-mercaptoethanol; 60 μmoles inorganic phosphate, pH 7·5; 4 μmoles NAD; 5 μmoles ATP; 3·3 μmoles DL-G-3-P; and 10 μg of rabbit muscle G-3-PD (capable of oxidizing 640 mμmoles G-3-P/min). The medium for assay of PGK reverse contained: 100 μmoles tris, pH 7·5; 15 μmoles $MgCl_2$; 60 μmoles 2-mercaptoethanol; 1·08 μmoles NADH; 10 μmoles 3-PG; 4 μmoles ATP; and 10 μg G-3-PD. To all assays, 10 μl of sonicate or cytosol was added in a final volume of 3 ml. Initial reaction rates (first 1–2 min, during which time the rate of change of absorption at 340 mμ remained constant) were used to calculate enzyme activities. Protein was determined by a biuret method.[15]

The substrates (fructose diphosphate, DL-glyceraldehyde-3-phosphate and 3-phosphoglycerate) and the purified enzymes (glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase) used in the assays were obtained from the Sigma Chemical Company. Alcohol dehydrogenase was purchased from Boehringer. Ethacrynic acid (kindly provided by Dr. W. H. Wilkinson of the Merck, Sharp &

Dohme Research Laboratories) was added to appropriate vessels dissolved in incubation medium or in alcohol. Whenever ethacrynic acid was added in alcohol, an equal volume of alcohol was added to control vessels. In no instance was alcohol shown to have any effect in the quantities added.

RESULTS

Influence of ethacrynic acid on glycolysis in cytosol. Earlier experiments with intact Ehrlich ascites tumor cells and red blood cells and with membrane-free preparations from these cells suggested that the site of inhibition of ethacrynic acid was proximal to the 3-phosphoglycerate site in the Embden–Meyerhof pathway.[11] Ethacrynic acid completely inhibited lactate formation from exogenous glucose in Ehrlich ascites tumor cells, but only partially inhibited lactate formation in human erythrocytes which contain 2,3-diphosphoglycerate as an additional substrate. It therefore seemed likely that the site of inhibition was between fructose diphosphate and the avenue for entry of 2,3-DPG into the glycolytic pathway. Lactate formation by the particulate-free fraction of Ehrlich ascites tumor cells was measured using either fructose diphosphate, G-3-P or 3-PG as substrate (Table 1). Since ethacrynic acid had no effect on the

TABLE 1. EFFECT OF ETHACRYNIC ACID ON GLYCOLYSIS*

	Lactate production (mμmoles/mg protein/min)	
Substrate	Control	Ethacrynic acid
Fructose diphosphate	9·21	0
Glyceraldehyde-3-PO_4	7·66	0·30
3-Phosphoglycerate	6·83	6·05

* One ml of tumor cell cytosol (17·3 mg protein) plus 2 ml medium were preincubated in the presence and absence of 10^{-3} M ethacrynic acid. At the end of 30 min of preincubation at 37°, substrates were added and the incubation was terminated after 15 min by adding 2 ml incubate to 0·5 ml of 6% perchloric acid. All incubation vessels contained 1 mM ADP, and either FDP (3·3 mM), G-3-P (1·7 mM), or 3-PG (5 mM). When G-3-P was used as substrate, 1 mM NAD was present; when 3-PG was used, 1 mM NADH was present. The values were corrected for endogenous lactate production.

rate of lactate formation from 3-PG, inhibition by this agent was presumed to be proximal to this site, as suggested by the erythrocyte studies. In contrast, the conversion of FDP and G-3-P were completely inhibited by ethacrynic acid. These experiments focused attention on the enzymes, G-3-PD and PGK, as probable acceptors for ethacrynic acid.

Effect of ethacrynic acid on G-3-PD and PGK activities of Ehrlich ascites tumor cell cytosol. The activities of G-3-PD and PGK of Ehrlich ascites tumor cytosol were assayed in the forward and reverse directions (Table 2). Under the conditions of the assay, the activity of G-3-PD was higher in the reverse than in the forward direction, while the activity of PGK was higher in the forward direction. At a concentration of

TABLE 2. EFFECT OF ETHACRYNIC ACID ON GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AND PHOSPHOGLYCERATE KINASE*

Assay	Enzyme activity		Inhibition (%)
	Control	Ethacrynic acid	
G-3-PD (forward)	3·5	0·0	100
G-3-PD (reverse)	6·8	0·0	100
PGK (forward)	11·2	7·1	37
PGK (reverse)	4·3	1·5	65

* Cytosol (1·4 mg/ml) from ascites tumor cells was preincubated in the presence and absence of 10^{-3} M ethacrynic acid for 30 min at room temperature. An aliquot (10 μl) was assayed in each of the standard assay systems. The enzymic activity is expressed in μmoles NAD reduced/mg of cytoplasmic protein/min in the forward assays or μmoles NADH oxidized/mg of protein/min in the reverse assays.

10^{-3} M, ethacrynic acid completely inhibited G-3-PD activity regardless of the direction of the assay. Ethacrynic acid reduced the activity of the forward PGK reaction by one-third and the reverse reaction by two-thirds. In all subsequent assays, the effects of ethacrynic acid on these enzymic activities were determined only on the forward reaction of G-3-PD and the reverse reaction of PGK.

A study of the inhibitory effect of ethacrynic acid on each of these enzymes was undertaken using different concentrations of inhibitor (Fig. 1). Fifty per cent inhibition of G-3-PD was observed at 5×10^{-5} M ethacrynic acid and 100 per cent inhibition at $2{\cdot}5 \times 10^{-4}$ M ethacrynic acid. Approximately $7{\cdot}5 \times 10^{-4}$ M ethacrynic acid was required in order to observe 50 per cent inhibition of PGK, and 10^{-3} M ethacrynic acid inhibited PGK by only 63 per cent. Thus, G-3-PD appeared to be considerably

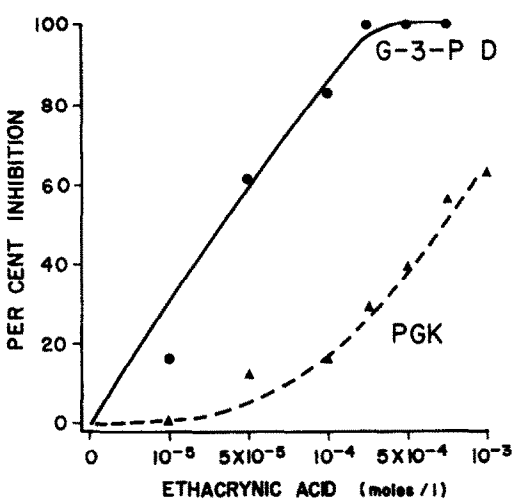

FIG. 1. Effect of varying the concentration of ethacrynic acid on the activities of G-3-PD (forward) and PGK (reverse) in ascites tumor cell cytosol. The preincubations and assays were performed as described in the footnote to Table 2.

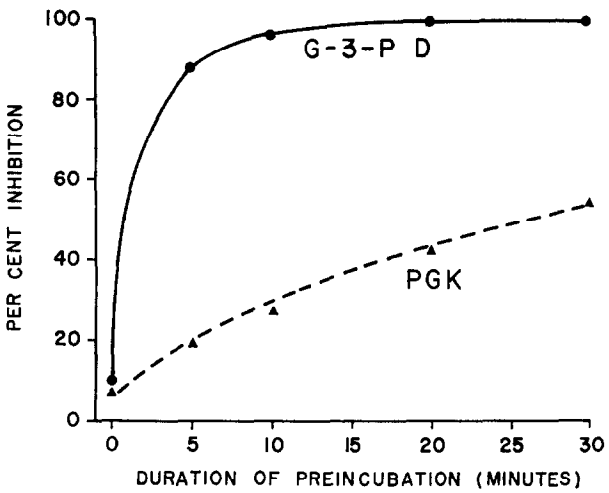

FIG. 2. Duration of preincubation and inhibitory effect of 10^{-3} M ethacrynic acid on ascites tumor cell cytosol. G-3-PD was measured in the forward direction and PGK in the reverse direction.

more sensitive to ethacrynic acid than PGK. Not surprisingly, lower concentrations of ethacrynic acid were required to inhibit G-3-PD than were found to inhibit lactate formation from FDP by cytosol of Ehrlich ascites tumor cells.[11]

In previous studies, preincubation of cytosol with ethacrynic acid was required to observe inhibition of glycolysis[11] and inhibition of ATPase activity.[5] The inhibitory effect of ethacrynic acid on G-3-PD and PGK activities was also time related (Fig. 2). Complete inhibition of G-3-PD activity occurred after 10 min of preincubation with 10^{-3} M ethacrynic acid. There was only 50 per cent inhibition of PGK activity after 30 min of preincubation.

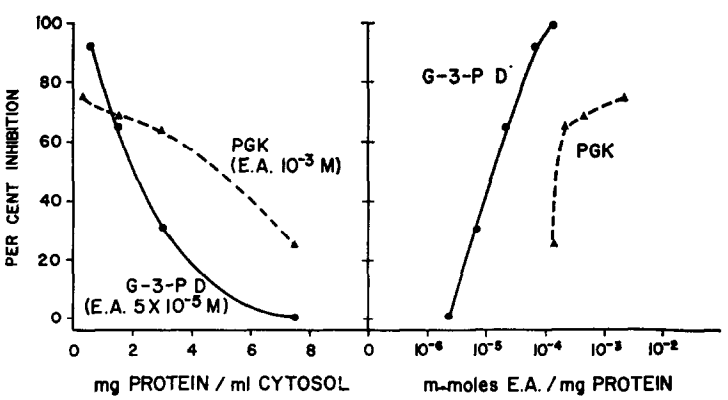

FIG. 3. Influence of enzyme concentration on the inhibitory effect of ethacrynic acid (E.A.). G-3-PD was measured in the forward direction and PGK in the reverse direction. Different dilutions of cytosol were preincubated for 30 min at room temperature in the presence of ethacrynic acid. At the end of the incubation period, aliquots were taken for enzyme assay. The final cytosol protein content in the assay cuvette (total volume 3 ml) was always brought to 16·5 μg by adjusting the volume of aliquot taken for assay.

A direct relationship between the inhibitory activity of ethacrynic acid and the amount of enzyme present was seen on assay of either G-3-PD or PGK (Fig. 3, left panel). With 5×10^{-5} M ethacrynic acid, complete inhibition of G-3-PD was observed at a low enzyme concentration and no inhibition at a high enzyme concentration.

PGK activity was similarly affected when the relative concentrations of enzyme and inhibitor were altered. However, in contrast to the result with G-3-PD, complete inhibition of PGK was not apparent at the highest concentration of ethacrynic acid used (10^{-3} M) in the presence of low enzyme concentrations. A comparison of the inhibitory activity of varying ratios of ethacrynic acid to cytosol protein (Fig. 3, right panel) showed that PGK was one to two orders of magnitude less sensitive to ethacrynic acid than G-3-PD. The shape of the PGK curve was unusual in that the enzyme was sensitive to relatively low concentrations of ethacrynic acid, but resisted inhibition at high concentrations, suggesting a complex interaction between enzyme and inhibitor.

Two possible interpretations follow from the overall observation that both enzymes were more sensitive to a fixed concentration of inhibitor when the enzyme was present in dilute solutions. Dilution of the enzyme (cytosol) might result in the dilution of any nonspecific component which could conceivably bind ethacrynic acid, thus increasing the effective concentration of ethacrynic acid. Alternatively, the enhanced inhibitory action of ethacrynic acid at low enzyme concentrations could have been due simply to a higher molar ratio of inhibitor to enzyme.

Inhibition of cytoplasmic enzymes from renal cortex and medulla. Because of its pharmacological action on the kidney of several species, the effects of ethacrynic acid on G-3-PD and PGK activities of rat renal cortex and medulla were studied. The activities of these cytoplasmic enzymes were also inhibited by ethacrynic acid (Table 3). Ethacrynic acid at a concentration of 10^{-3} M completely blocked G-3-PD of both cortex and medulla; at lower concentrations of ethacrynic acid, lesser degrees of

TABLE 3. EFFECT OF ETHACRYNIC ACID ON CYTOSOL OF KIDNEY CORTEX AND MEDULLA*

	Enzyme activity			
		Ethacrynic acid (moles/l.)		
Assay	Control	10^{-3}	10^{-4}	10^{-5}
Cortex				
G-3-PD (forward)	1·7	0·0	1·2	1·7
PGK (reverse)	2·5	1·9	2·3	2·6
Medulla				
G-3-PD (forward)	1·6	0·0	1·1	1·4
PGK (reverse)	1·9	1·6	1·9	2·0

* Cytosol prepared from cortex (2·9 mg protein/ml) or medulla (2·8 mg/ml) was preincubated in the absence or presence of varying concentrations of ethacrynic acid for 30 min at 25°. A 10-μl aliquot from each vessel was assayed for G-3-PD and PGK. The values are expressed in μmoles pyridine nucleotide reduced (forward assays) or oxidized (reversed assays) per mg of protein/min.

TABLE 4. PREINCUBATION OF SONICATE WITH ETHACRYNIC ACID AND VARYING CONCENTRATIONS OF NAD*

NAD Concn. (moles/l.)	Ethacrynic acid Concn. (moles/l.)	G-3-PD activity
0	10^{-4}	0·4
10^{-5}	10^{-4}	1·5
5×10^{-5}	10^{-4}	2·8
10^{-4}	10^{-4}	3·9
5×10^{-4}	10^{-4}	4·0
10^{-3}	10^{-4}	4·2

* Ehrlich ascites tumor cell cytosol (1·6 mg/ml) was preincubated with ethacrynic acid and varying concentrations of NAD at 25° for 30 min. The cytosol was added to the medium containing appropriate constituents. A 10-μl aliquot was assayed for G-3-PD (forward) and the values are in μmoles NAD reduced/mg of protein/min.

inhibition were observed. The inhibitory action of ethacrynic acid on the kidney enzyme was roughly comparable to that observed with the tumor cell enzyme. In contrast, the PGK activity of kidney appeared to be considerably less sensitive to ethacrynic acid than did the tumor PGK.

Interaction of G-3-PD, ethacrynic acid and NAD. It is well known that NAD is bound at one site to G-3-PD through a sulfhydryl linkage.[16] In order to decide whether ethacrynic acid inhibited G-3-PD by competing or displacing NAD at the active site, Ehrlich ascites tumor cytosol was incubated with ethacrynic acid in the presence and absence of NAD (Table 4). Concentrations of NAD as low as 10^{-5} M protected the enzyme against inhibition by ethacrynic acid. Maximum protection was afforded by higher concentrations of NAD.

Further evidence to suggest that ethacrynic acid interfered with G-3-PD by interacting at a site of attachment of NAD was obtained by assaying G-3-PD activity with high concentrations of ethacrynic acid (Fig. 4, left panel). In these experiments, the cytosol and ethacrynic acid were brought together without benefit of preincubation. The degree of enzyme inhibition was exquisitely sensitive to the order of addition of ethacrynic acid and NAD to the assay mixture. When NAD was added first and immediately followed by ethacrynic acid, there was no decline in G-3-PD activity. When ethacrynic acid was added just before the NAD, virtually all enzyme activity was blocked. Moreover, the profound degree of inhibition by ethacrynic acid was independent of the concentration of mercaptoethanol in the assay system. A reduction in the inhibitory action of ethacrynic acid was noted only when the mercaptoethanol was decreased to concentrations where enzyme activity was suboptimal in the absence of ethacrynic acid. Ethacrynic acid did not affect enzyme activity by altering the NAD because addition of alcohol dehydrogenase to ethacrynic acid-inhibited systems resulted in an appreciable reduction of the pyridine nucleotide (Fig. 4, right panel).

Assessment of the inhibitory activity of ethacrynic acid on G-3-PD measured in the reverse direction gave similar results (Fig. 5). Ethacrynic acid was without effect if NADH was added first, but completely inhibited G-3-PD activity when presented to the enzyme prior to addition of the reduced pyridine nucleotide.

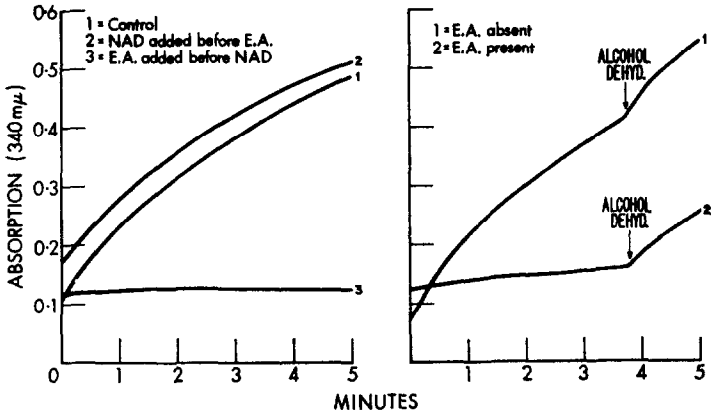

FIG. 4. Influence of NAD and ethacrynic acid (10^{-3} M) on forward G-3-PD activity from tumor cell cytosol (17·0 μg protein/cuvette). Left panel: curve 1, control rate in the absence of ethacrynic acid; curve 2, rate of NAD reduction when NAD was added to the usual reaction mixture prior to ethacrynic acid; curve 3, ethacrynic acid added to the assay system prior to addition of NAD. Right panel: curve 1, control rate in the absence of ethacrynic acid; curve 2, ethacrynic acid added to assay system prior to NAD addition. Ten μl alcohol was present in each cuvette at the beginning of the assay. Five μl yeast alcohol dehydrogenase (1·5 μg protein) in Tris buffer, pH 8·2, was added as indicated.

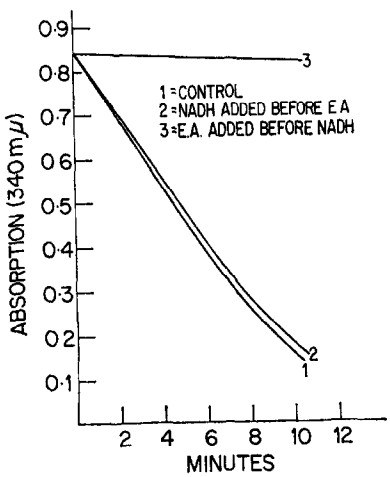

FIG. 5. Influence of NADH and ethacrynic acid (10^{-5} M) on reverse G-3-PD activity from tumor cell cytosol (5·2 μg protein/cuvette). Curve 1, control rate without ethacrynic acid; curve 2, rate of NADH oxidation when NADH was added to the reaction mixture prior to ethacrynic acid; curve 3, ethacrynic acid added to the assay prior to addition of NADH.

DISCUSSION

Studies *in vitro* indicate that ethacrynic acid inhibits metabolism at three independent sites on the cellular level: (1) cell membrane transport ATPase, (2) mitochondrial oxidative phosphorylation, and (3) glycolysis.

It is of interest that ethacrynic acid inhibits the transport ATPase,[5] mitochondrial metabolism[10] and glycolysis in the kidney of the rat, a species that is insensitive to the pharmacological effects of this agent. In selective intact organisms, ethacrynic acid acts

as a potent diuretic agent with a rapid onset of action when relatively small quantities are administered.[1–4] Several authors[5,8] have called attention to the high concentrations of inhibitor required, and the efficacy of preincubation to obtain maximum effects *in vitro*. Therefore, the response *in vivo* appears to be in conflict with the data obtained from studies of ethacrynic acid *in vitro*. The finding that ethacrynic acid acts at multiple sites provides one explanation for this apparent discrepancy. That is, low concentrations of ethacrynic acid that would exert relatively little effect at each independent site could have a significant additive influence in the intact cell. To this mechanism, one must add the multiplier effect of ethacrynic acid on ion transport. Not only could ethacrynic acid block transport ATPase directly, but diminished ATP synthesis by inhibition of glycolysis and mitochondrial oxidation would reduce the activity of the transport ATPase still further.

In the present study, localization of ethacrynic acid action in the Embden–Meyerhof pathway has permitted us to study some of the characteristics of the interaction of inhibitor with soluble enzyme. Under appropriate conditions, it is possible to obtain appreciable inhibition of cytoplasmic G-3-PD at low concentrations of ethacrynic acid (Fig. 3). The requirement for preincubation of inhibitor with enzyme is not absolute, for an immediate response is seen upon direct assay of G-3-PD with ethacrynic acid (Figs. 4, 5). Although ethacrynic acid inhibits both G-3-PD and PGK, the present effort is devoted principally to an examination of the interaction of ethacrynic acid with G-3-PD. The degree of inhibition is considerably greater for G-3-PD than for PGK with regard to concentration of inhibitor required for maximum inhibition of each of the enzymes (Fig. 1) or the minimal concentration required to detect an inhibitory effect (Fig. 2). For kidney enzymes, the differences are even greater (Table 3); ethacrynic acid has almost no effect on PGK.

Ethacrynic acid has been shown to bind thiols *in vitro*[5] and *in vivo*.[17] Since G-3-PD is known to have sulfhydryl groups at its active site and is inhibited by certain sulfhydryl-binding agents, it is possible that ethacrynic acid also inhibits by interacting with the thiol groups. Clearly, the enzyme displays a selectivity toward sulfhydryl binding agents. For example, iodoacetate is a potent inhibitor of G-3-PD, while iodoacetamide is not.[16] In other enzyme systems, iodoacetamide has been shown to exert a greater affinity for sulfhydryl groups than iodoacetate.[18] It has recently been proposed that NAD exerts a directing influence on the binding of iodoacetate, but not iodoacetamide, to G-3-PD.[19] In contrast to the binding of iodoacetate to G-3-PD, the binding of ethacrynic acid to the enzyme is far greater in the absence of NAD, suggesting a different mechanism of interaction. Indeed it seems likely from the present experiments that ethacrynic acid might compete for the same sulfhydryl group that acts as one receptor site for NAD because of the protective effect of NAD for the enzyme in the presence of ethacrynic acid (Table 4 and Fig. 4, left panel).

Considerable attention has been drawn to the effect *in vitro* of ethacrynic acid on ion transport. Ethacrynic acid inhibits monovalent cation transport in erythrocytes,[6,7] muscle,[20–22] kidney,[23–25] platelets[26] toad bladder,[27,28] small intestine[29] and toad oocytes.[30] In almost all instances, it has been assumed that the primary effect of ethacrynic acid is exerted on an ion transport pump. Only Sachs and Welt[7] demonstrate that ethacrynic acid does not reduce the concentration of the energy source (ATP) in human erythrocytes while inhibiting ion transport. This finding is somewhat surprising in view of the fact that ethacrynic acid does interfere with glycolysis from

exogenous substrate in human erythrocytes.[11] MacKnight[24] and Herms and Kersting[25] observed a profound effect of ethacrynic acid on ion transport, but ascribed this to interference with energy generation, in keeping with other observations of the influence of ethacrynic acid on kidney slices.[31] Clearly, ethacrynic acid can act at more than one site in intact tissues and caution should be observed in interpreting effects on systems that depend upon mitochondrial oxidation or glycolysis for a source of energy.

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