Lack of Correlation Between Natriuretic Activity and Inhibition of Renal Na-K-Activated ATPase.* (30536)

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Recently, several investigators have attempted to correlate the pharmacological activity of certain natriuretic agents with inhibition of an enzyme system prepared from subcellular particles of the kidney(1,2,3,4). This enzyme system is characterized by its ability to release inorganic phosphate (Pi) from ATP in the presence of adequate concentrations of sodium and potassium and is, therefore, referred to as Na-K-activated ATPase(5). This enzyme system is not confined to the kidney but is widespread in the body, with high activity in those areas where Na and K transport systems are highly developed(5). Consequently it has been suggested that Na-K-activated ATPase activity is involved in the transport of Na and K across cell membranes(3,6). If this is the case it would be expected that inhibition of this enzyme system in the kidney would result in an increased excretion of cations. Landon and Norris(3) and Taylor(4) demonstrated that the diuretic mercurials are capable of inhibiting this enzyme system *in vitro*. Jones *et al*(2) demonstrated inhibition of the system *in vivo* following administration of diuretic mercurials, but not with non-diuretic mercurials. These results support the thesis that inhibition of Na-K-activated ATPase and the production of a diuresis can be related.

In this report, 2 new natriuretic-diuretic agents (ethacrynic acid and furosemide) were examined for their ability to inhibit Na-K-activated ATPase in the rat. Ethacrynic acid is a potent natriuretic-diuretic agent in several species including man(7). Duggan and Noll(1) have demonstrated that this agent is capable of inhibiting *in vitro* the Na-K-activated ATPase prepared from guinea pig kidneys. Since ethacrynic acid is not active as a diuretic in rats(7) it was of interest to determine the effect of this agent on the Na-K-activated ATPase of rat kidney when administered *in vivo*. This effect was compared to that produced by furosemide, which is similar in activity to ethacrynic acid in the dog and human, but is also capable of producing natriuresis and diuresis in the rat(8).

Methods. The effect of ethacrynic acid and furosemide on urinary electrolyte and water excretion was evaluated by the assay procedure of Lipschitz *et al*(9). Male Sprague-Dawley rats, 275-325 g, were given an oral load of 0.9% NaCl, 25 ml/kg, at the beginning of the experiment. Ethacrynic acid (25 mg/kg) and furosemide (50 mg/kg) were dissolved in the saline load. For urine collection, 2 rats were placed in each metabolism cage and urine was collected over a 3-hour period. At the end of the collection period, the animals were killed by cervical dislocation, the bladders removed and their contents combined with the previously collected urine. The kidneys were removed and prepared for enzyme assay. Urine samples were analyzed for sodium and potassium using a Coleman flame photometer (Model 21).

The preparation and assay of the enzyme used was essentially that of Ahmed and Judah (10). A 10% homogenate of the kidneys of the rats in each metabolism cage was prepared in a medium consisting of 0.25 M mannitol, 5 mM EDTA (Na salt), 30 mM histidine-HCl (pH 6.5) and 0.1% deoxycholate (Na salt) using a Potter homogenizer (plastic pestle). The final pH was 6.8. The homogenate was centrifuged at 9,000 $\times$ *g* for 15 min in a refrigerated centrifuge and the supernatant was carefully decanted and frozen. After storage for 48 hours the material was allowed to thaw at room temperature and was then spun at 104,000 $\times$ *g* (avg) in a

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TABLE I. Effect of Furosemide and Ethacrynic Acid on Electrolyte and Water Excretion.*

	N	Sodium (mEq/kg/3 hr)	Potassium (mEq/kg/3 hr)	Urine vol (% of load/3 hr)
Control	5	1.21 (.25)	1.78 (.20)	39.1 (8.4)
Ethacrynic acid (25 mg/kg)	5	1.05 (.40)	1.60 (.28)	40.7 (6.2)
Furosemide (50 mg/kg)	3	7.69† (.08)	2.54† (.19)	249.7† (17.4)

* Values represent means ± S.E.
† Significant difference ($p < .05$).

Spinco Model L preparative ultracentrifuge, using the no. 40 rotor. The pellet was suspended in ½ the original volume in a medium containing 0.25 M mannitol, 1.0 mM EDTA and 30 mM Tris-HCl, pH 7.4. The suspension was gently homogenized by hand and then centrifuged at 20,000 × *g* for 50 minutes at 4°C. The pellet was suspended in ¼ of the original volume in the Tris-buffered medium. This suspension was gently homogenized by hand and then assayed for enzyme activity.

The enzyme system employed in this study requires the presence of both sodium and potassium to function at optimum levels(3,4, 6). For this reason no attempt was made to render the solutions sodium free by converting all salts to the tris form as has been done by others(3,4,10). Rather, the control solutions were low in sodium and free of potassium. This system is not unlike that of Bonting *et al*(5) who demonstrated that eliminating the potassium from the assay media had the same inhibitory effect as adding 0.1 mmoles of ouabain to the incubation mixture.

The system employed for assay of ATPase activity was as follows: ATP (Na salt), 5 mM; MgCl, 5 mM; Tris buffer, pH 7.4, 30 mM; NaCl, 115 mM and KCl, 10 mM. In the potassium-free media, the NaCl and KCl were replaced by equimolar amounts of choline chloride. To 2 ml of either the K-containing or K-free media (previously warmed to 37°C), 0.5 ml of the tissue homogenate was added and the mixture was shaken in a Dubnoff metabolic shaker (37°C) for 15 min. The reaction was stopped with 1.0 ml of ice cold 10% trichloroacetic acid (TCA). Inorganic phosphate (Pi) liberated was estimated spectrophotometrically by the method of Bonting *et al*(5).

Two controls were run concurrently with each enzyme assay; (a) an incubate which contained no tissue and (b) a tissue-containing mixture to which TCA was added immediately. The incubate with no tissue was used as the blank in the phosphate determination. The small amount of phosphate (0.02-0.05 O.D. units) measured in the unincubated mixture was considered to be released by the TCA and this O.D. value was subtracted from the values obtained from the incubated samples. The protein content of each suspension was analyzed by the microbiuret method of Itzhaki and Gill(11). Phosphate released was converted to micromoles Pi released in 15 minutes per g protein.

The data obtained were anlyzed statistically using a one-tailed group "t" test(12). The 0.05 level of probability was used as the criterion of significance.

Results. As illustrated in Table I, ethacrynic acid, 25 mg/kg, had no effect on urinary excretion of water or electrolytes. This dosage would produce a marked natriuretic action in other species such as the dog(7). Furosemide, at a dosage of 50 mg/kg, produced a marked increase in urinary volume and the excretion of sodium and potassium.

Table II illustrates the effect of ethacrynic acid and furosemide on Na-K-activated ATPase. Ethacrynic acid significantly depressed the ratio of stimulated to unstimulated activity. This depression was due only to an inhibition of the K-containing fraction inas-

TABLE II. Effect of Furosemide and Ethacrynic Acid on Na^+-K^+-Activated ATPase.*

	N	μmoles Pi released/g protein/15 min: K^+ containing	K^+ free	K^+/K^-
Control	5	409 (22)	217 (24)	1.93 (.10)
Ethacrynic acid (25 mg/kg)	5	325 (22)	213 (16)	1.55† (.09)
Furosemide (50 mg/kg)	3	346 (25)	221 (14)	1.58† (.13)

* Values represent means ± S.E.
† Significant difference ($p < .05$).

much as the K-free fraction was not affected. Furosemide had a similar effect. It depressed the ratio of activities and did not affect the K-free fraction.

Discussion. The experiments reported herein showing that ethacrynic acid inhibits Na-K-activated ATPase *in vivo* supplement the work of Duggan and Noll(1) who demonstrated that ethacrynic acid inhibited, *in vitro,* the Na-K-activated ATPase of membranous subfractions of guinea pig kidney. Furosemide, another natriuretic-diuretic agent, was also found to inhibit Na-K-activated ATPase activity. Jones *et al*(2) have reported that the natriuretic activity of mercurials can be correlated with their ability to inhibit microsomal Na-K-activated ATPase. The results with furosemide would appear to be comparable with those of Jones *et al*(2) since this agent produced natriuresis and diuresis in the rat and was also found to inhibit microsomal Na-K-activated ATPase. However, the results obtained with ethacrynic acid would appear to question the relationship of natriuretic action and inhibition of this enzyme. Ethacrynic acid was found to produce significant inhibition of Na-K-activated ATPase without producing natriuresis. Thus it would appear that those studies seeking to correlate inhibition of microsomal ATPase and natriuretic activity are not entirely valid since inhibition of the enzyme can occur when a non-natriuretic agent is administered *in vivo.* The activity of this enzyme, which is determined *in vitro,* can apparently be inhibited by agents (administered *in vivo*) which have no demonstrable inhibitory (*i.e.,* natriuretic) activity *in vivo.*

Summary. Ethacrynic acid, which is a potent natriuretic in man and dog but inactive in the rat, and furosemide, which is a potent natriuretic in man and dog as well as in the rat, were compared for their ability to inhibit microsomal Na-K-activated ATPase following *in vivo* administration in the rat. Both agents were found to inhibit the activity of this enzyme. Furosemide produced a significant natriuretic response whereas ethacrynic acid did not. Therefore, no correlation between natriuretic activity and enzyme inhibition could be made with these agents. This study does not rule out a correlation between Na-K-activated ATPase and natriuretic activity but it does question the practice of determining Na-K-activated ATPase *in vitro* and attempting to relate this to responses *in vivo.*

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