tion from the GI tract in relation to their effect on the calcification process in general.

Conclusions. Increasing the amount of iron by means of stomach tubing $FeSo_4$ to rats significantly increases liver iron content. When 50 mg phosphate is given simultaneously with iron, a significant reduction in liver iron content results, as well as a significant diminution in calcium and phosphorus content of newly forming incisor dentin. These results, compared with a significant reduction in blood phosphorus concentration, suggest that increasing the iron content of the diet for prolonged periods of time without significantly increasing the dietary phosphorus content may result in improperly calcified tissues.

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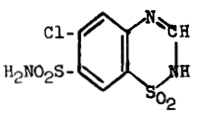
Saluretic Activity of Hydrochlorothiazide (6-Chloro-7-sulfamyl-3,4-dihydro-1,2,4-benzothiadiazine-1,1-dioxide) in the Dog. (24656)

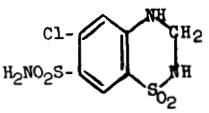
JOHN E. BAER, HORACE F. RUSSO AND KARL H. BEYER
Merck Inst. for Therapeutic Research, West Point, Pa.

Chlorothiazide,* first synthesized by Novello and Sprague(1), was shown to be a remarkably safe and potent agent for increasing excretion of sodium and chloride in animals (2,3) and in man(4). The agent has found widespread use in treatment of congestive failure, edema of pregnancy, premenstrual tension and other edematous states, and in management of hypertension. During research leading to discovery of the effectiveness of chlorothiazide as a saluretic-diuretic agent, many compounds more or less closely related to it in structure have been synthesized by Novello and Sprague and have been evaluated in these laboratories. We wish to report the preclinical evaluation of one such compound, hydrochlorothiazide,† that resembles chlorothiazide qualitatively but is considerably more potent. The structural formulae for the 2 compounds presented below make it evident that the new agent differs from chlorothiazide only in saturation of the heterocyclic portion of the molecule.



6-Chloro-7-sulfamyl-1,2,4-benzothiazine-1,1-dioxide

Chlorothiazide
Diuril*



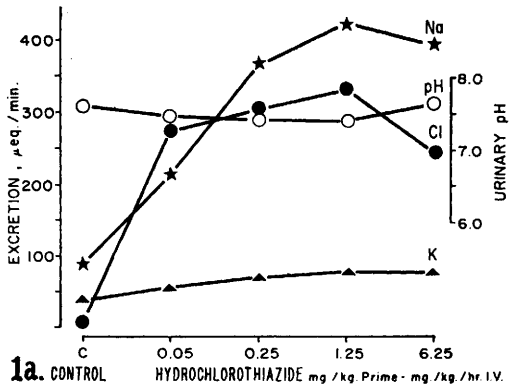
6-Chloro-7-sulfamyl-3,4-dihydro-1,2,4-benzothiadiazine-1,1-dioxide

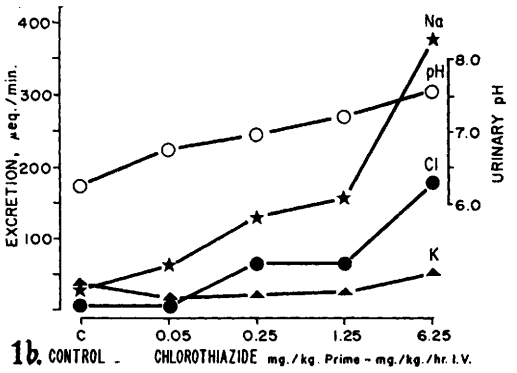
Hydrochlorothiazide
HydroDiuril†

Methods. Renal clearance studies were performed on trained unanesthetized female mongrel dogs in post-absorptive state. Water (500 ml by gavage) and isotonic mannitol-phosphate infusion at 3 ml/min. throughout experiment, assured adequate urine flow. Hydrochlorothiazide was introduced into the infusion during the "drug phase" of experiments at dosages indicated. Clearance of exogenous creatinine, administered subcutaneously, was used as a measure of glomerular filtration rate. Blood was collected at midpoint of each 10-minute clearance period. The powdered drug was given to dogs orally in gelatin capsules containing the solid compound. Fasting female mongrel dogs were intubated with 500 ml of water and urinary bladders were emptied by catheter. The animals were held in metabolism cages, and complete urine collections were made at regular intervals up to 6 hours. Hydrochlorothiazide was hydrolyzed in neutral solution (15 lb pressure for ½ hour) to a substance that can be measured by diazotization and coupling

* DIURIL is trade-mark of Merck & Co. for its brand of 6-chloro-7-sulfamyl-1,2,4-benzothiadiazine-1,1-dioxide, the nonproprietary name of which is chlorothiazide.

† HYDRODIURIL is trade-mark of Merck & Co., for its brand of 6-chloro-7-sulfamyl-3,4-dihydro-1,2,4-benzothiadiazine-1,1-dioxide, the nonproprietary name of which is hydrochlorothiazide.





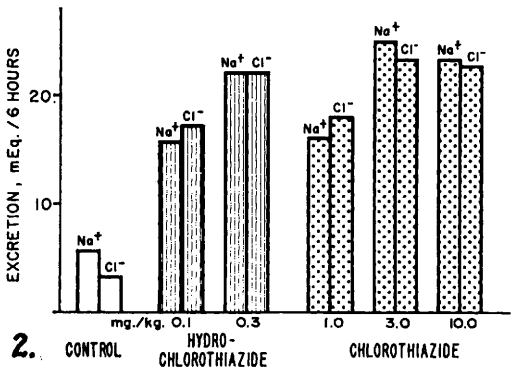


FIG. 1. Dosage response curves for (A) hydrochlorothiazide and (B) chlorothiazide on excretion of sodium, chloride, potassium and urinary pH in dogs. Duplicate successive 10-min. clearances were measured 20 min. after i.v. prime and onset of infusion at the indicated dosage level.

FIG. 2. Sodium and chloride excretion following single oral dose of hydrochlorothiazide or chlorothiazide in dogs. Avg 6-hr excretion for 6 dogs.

with N-1-naphthylethylenediamine; incorporation of this step in the procedure devised for chlorothiazide(5) permits estimation of higher concentrations of hydrochlorothiazide, but is not applicable at minimal plasma concentration required to produce saluresis.

Results. Hydrochlorothiazide caused a marked and equivalent increase in sodium and chloride excretion in dogs at lowest intravenous dosage shown in Fig. 1A. There was a lesser effect on potassium excretion even at highest dosage given, and urine pH did not reflect any alteration in bicarbonate excretion. Saluresis first increased, then reached a maximum and even decreased slightly as dosage of hydrochlorothiazide was increased to supramaximal level. In a similar experiment with chlorothiazide (Fig. 1B), little saluresis was observed at 0.05 mg/kg and maximal saluresis had not been attained at 6.25 mg/kg. Furthermore, sodium excretion exceeded that of chloride at higher doses of chlorothiazide, with resulting increase in bicarbonate excretion and in urine pH.

Hydrochlorothiazide and chlorothiazide, when given separately to a single dog at 0.05 and 0.25 mg/kg, intravenously, produced effects shown in Table I, from which it may be estimated that hydrochlorothiazide is 5 times as potent a natriuretic agent as chlorothiazide by this route in dogs. The lowest dose of hydrochlorothiazide caused an increase of 199 microequiv./min. of sodium and 163 microequiv./min. of chloride, whereas chlorothiazide caused corresponding increases of 68 and 10 microequiv./min. The increase in cation excretion that resulted from increasing the dose of hydrochlorothiazide was essentially covered by commensurate increase in chloride excretion. In the case of chlorothiazide, however, a portion of the cation was interpreted to have been covered by an increased bicarbonate excretion (increase in urine pH). The kaliuretic effect in these experiments was the same for both compounds.

The unusual effectiveness of chlorothiazide under experimental conditions of acidosis or alkalosis is duplicated by hydrochlorothiazide. The effect of small intravenous doses of hydrochlorothiazide on electrolyte excretion was determined in dogs that received 100 meq. of ammonium chloride or sodium bicarbonate daily for 5 days prior to clearance experiments shown in Table II. On sixth day, triplicate 10-minute clearances were obtained,

TABLE I. Comparative Effects of Increasing Intravenous Doses of Hydrochlorothiazide and Chlorothiazide in Dog 849.*

I.V. dose, mg/kg prime and mg/kg/hr infusion	Excretion, μequiv./min.			Na^+, % of filtered load reabsorbed†	Urine	
	Na^+	K^+	Cl^-		ml/min.	pH
Hydrochlorothiazide						
0	46	27	6	99.5	3.9	6.2
.05	245	47	169	97.4	2.9	7.4
.25	382	60	333	95.6	4.4	7.3
Chlorothiazide						
0	10	25	7	99.9	2.8	5.8
.05	78	49	17	99.1	2.7	7.4
.25	339	65	192	96.2	4.5	7.7

* Data are averages for duplicate successive 10-min. clearance periods.
† Calculated for each clearance period from measured plasma and urine concentration and glomerular filtration rate (creatinine clearance).

followed by 0.25 mg/kg priming dose and infusion of hydrochlorothiazide at 0.3 mg/kg/hr. for 20-minute equilibration period, after which 3 "drug phase" clearances were measured. In acidotic dogs (plasma HCO_3^- 10.7 and 11.5 meq./l), the increase of over 250 microequiv./min. in sodium excretion was more than covered by increase in chloride excretion. Urinary pH decreased and there was no appreciable excretion of bicarbonate. Duplicate experiments in the 2 animals were in good agreement and agreed well with similar experiments at 10 times the dose of chlorothiazide(6).

Control data for dogs that received sodium bicarbonate show that they were excreting large amounts of sodium and bicarbonate as a consequence of large dietary intake of these

TABLE II. Effect of Hydrochlorothiazide and Chlorothiazide on Electrolyte Excretion in 6 Dogs Made Acidotic with Ammonium Chloride or Alkalotic with Sodium Bicarbonate.*

	Dose		Sodium		Potassium	Chloride	Bicarbonate	Urine	Plasma	GFR,*
	Prime, mg/kg	Infusion, mg/kg/hr	Excretion, μeq/min.	% of filtered load reabsorbed	Excretion, μeq/min.			pH	pH	ml/min.
Ammonium chloride										
Control			11	99.8	15	70	.3	5.2	7.26	45.4
Hydrochlorothiazide	.25	.3	305	95.2	62	425	6	4.9		45.1
Control			21	99.8	12	96	6	5.2	7.24	59.7
Hydrochlorothiazide	.25	.3	289	96.1	63	437	6	4.7		51.2
Control			18	99.8	34	80	2	5.3	7.23	57.5
Chlorothiazide	2.5	3.0	200	97.5	53	320	6	4.7		57.1
Sodium bicarbonate										
Control			523	96.0	87	25	432	8.0	7.48	80.1
Hydrochlorothiazide	.25	.3	883	93.0	111	365	552	7.8		77.4
Control			220	97.3	78	7	209	7.9	7.49	55.1
Hydrochlorothiazide	.25	.3	727	92.8	142	316	389	7.8		68.1
Control			212	97.8	38	9	182	7.9	7.57	64.8
Chlorothiazide	2.5	3.0	598	92.8	78	194	415	8.0		55.5

* Ammonium chloride or sodium bicarbonate was administered at oral dose of 100 meq/day for 5 days; a renal clearance experiment was then performed; GFR = glomerular filtration rate.
Tabulated values are avg of triplicate successive 10 min. clearance periods.

TABLE III. Renal Clearance of Hydrochlorothiazide in the Dog.

Time, min.	Urine ml/min.	Urine pH	Hydrochlorothiazide Plasma conc., mg/l	Hydrochlorothiazide Clearance, ml/min.	GFR, ml/min.	Clearance ratio
0	3 g creatinine, subcut.					
10	Mannitol-phosphate infusion, 3 ml/min.					
30–40	.2	6.4			41.9	
40–50	.5	6.5			52.7	
	Hydrochlorothiazide: 2.5 mg/kg prime, i.v.; 3.0 mg/kg/hr					
80– 90	5.5	7.3	2.7	213	46.1	4.61
90–100	6.2	7.3	2.9	175	43.6	4.02
	Probenecid: 25 mg/kg prime, i.v.; 30 mg/kg/hr					
120–130	4.0	7.0	4.4	25	33.3	0.75
130–140	4.3	6.9	5.2	26	36.0	0.74

ions. Plasma bicarbonate was elevated (26.7 and 27.2 meq./l), urine was alkaline, and chloride excretion was minimal. Hydrochlorothiazide produced a marked increase in electrolyte excretion in alkalotic dogs. The greatest absolute increase was noted for sodium ion, although reduction in "percentage of filtered load reabsorbed" was similar to that noted in acidotic dogs. The already large bicarbonate excretion was increased somewhat, but chloride excretion rose from a negligible rate to cover nearly two-thirds of the increase in cation excretion. Duplicate experiments in 2 animals were in good agreement with each other and with similar experiments wherein a 10-fold greater dose of chlorothiazide was employed(6).

Hydrochlorothiazide caused an enhanced excretion of sodium and chloride during 6-hour period in dogs that received 0.1, 0.3, 1, or 3 mg/kg orally. A less than maximal saluretic response was obtained only at dosage less than 0.3 mg/kg. With chlorothiazide, a submaximal saluresis occurred at 1 mg/kg, and the response obtained at 3 mg/kg was maximal. Fig. 2 shows average sodium and chloride excretion for groups of 6 dogs at several significant dosages of the 2 compounds. The response to 0.1 mg/kg of hydrochlorothiazide resembled that observed at 1 mg/kg of chlorothiazide. Hydrochlorothiazide was uniformly well absorbed, as judged by the saluresis that invariably followed its oral administration to the animals.

Renal clearance of hydrochlorothiazide exceeds glomerular filtration rate (Table III). To obtain a plasma concentration of hydrochlorothiazide that could be determined analytically, a much larger dosage of hydrochlorothiazide was employed than is required to produce saluresis. This relatively large dose of the agent did not affect glomerular filtration rate, as measured by exogenous creatinine clearance. Hydrochlorothiazide itself was excreted at 4 times glomerular filtration rate, the large renal clearance indicating secretion of the drug by renal tubules. Administration of probenecid‡ in the third phase of experiment, reduced clearance of hydrochlorothiazide (uncorrected for plasma binding) to slightly less than glomerular filtration rate.

Discussion. Enhanced saluretic activity that results from saturation of the heterocyclic ring of chlorothiazide appears to be consequent to an increase in potency, rather than to a qualitatively different type of activity. Thus, no really qualitative difference is seen in the spectrum of electrolyte excretion of chlorothiazide and hydrochlorothiazide over the more than 100-fold dosage range shown in Fig. 1. Likewise, in severe acidosis or alkalosis, as induced by ammonium chloride or sodium bicarbonate ingestion, the electrolyte excretion pattern, and in fact the actual data, for hydrochlorothiazide (Table II) resemble closely those of experiments at 10 times the dose of chlorothiazide(6). Similarly, in oral experiments, a half-maximal excretion of sodium is accompanied by equivalent chloruresis, but this response is obtained

‡ BENEMID is trade-mark of Merck & Co., for its brand of p-(di-n-propylsulfamyl)-benzoic acid, the nonproprietary name of which is probenecid.

at a dosage of 0.1 mg/kg of hydrochlorothiazide and 1 mg/kg of chlorothiazide.

Both chlorothiazide and hydrochlorothiazide are secreted by renal tubules, being cleared at rates in excess of glomerular filtration rate. Both compounds, thus, will be removed rapidly from blood of normal dogs following a single intravenous dose. The mechanism whereby both chlorothiazide and hydrochlorothiazide are excreted in excess of glomerular filtration presumably involves that utilized by penicillin, p-aminohippurate and certain other organic acids, inasmuch as it is suppressed by administration of probenecid, an agent known to depress selectively renal tubular secretion of certain organic acids without affecting electrolyte excretion and without interference in renal transport of numerous other substances(7).

Summary. Hydrochlorothiazide is a potent saluretic agent. It resembles chlorothiazide qualitatively, but is several times more active, intravenously and orally, in the dog.

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A Metabolite of 1-Hydrazinophthalazine (Hydralazine).* (24657)

CARL D. DOUGLASS AND ROSE HOGAN

Dept. of Biochemistry, School of Medicine, University of Arkansas, Little Rock

The antihypertensive drug 1-hydrazinophthalazine (hydralazine, apresoline) has been shown to interfere in the process of biological acetylation of sulfanilamide(1). The mechanism for this interference appeared to be competition between substrate and drug for the active acetyl group. It was further shown that free hydralazine disappeared from pigeon liver extracts when all components of the acetylation system were present. These facts suggested the possibility that 1-acetyl-2-phthalazyl hydrazine (acetyl hydralazine) should be formed as a conjugate of hydralazine. We here report the identification of this material as a product of hydralazine metabolism *in vitro* and *in vivo*.

Materials and methods. In vitro incubations and extractions. An adult pigeon was killed by decapitation and its liver rapidly dissected out and placed in cold Robinson's solution(2) containing 20 mg of glucose per 100 ml. Ten grams of liver slices were prepared with the Stadie-Riggs microtome. The slices were divided into 2 equal portions and each washed twice with Robinson's solution and finally suspended in 10 ml of the solution containing 200 μmoles of sodium acetate and in one case 100 μmoles of hydralazine. The latter materials had been previously made up in a small portion of Robinson's solution and neutralized before incroporation into the suspending medium. The mixtures were incubated with shaking at 37° for 4 hours with air as the gas phase. The contents of the vessels were then ground in a Potter-Elvehjem type homogenizer with a close-fitting teflon pestle. Three drops of glacial acetic acid were added to each homogenate and the mixtures deproteinized by allowing them to stand for 5 minutes in a boiling water bath. After centrifuging, the pH of each supernatant was adjusted to 9-10 with

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