Sodium Losing Material in Human Urine.* (24210)

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The purpose of this paper is to report evidence for existence of a material in urine of newborn infants and patients with congenital adrenal hyperplasia, with or without Addisonian symptoms, which induces sodium diuresis when injected into intact rats. Wilkins *et al.* described sodium diuresis following administration of ACTH to patients with congenital adrenal hyperplasia(1,2). Sodium diuresis of newborn infants given ACTH as well was reported in 1950(3). The possibility of the existence of an hormone causing sodium excretion was mentioned among other possible explanations. Prader reported that patients with congenital adrenal hyperplasia with Addisonian symptoms secreted aldosterone and that eventually secretion of aldosterone reached abnormally high levels(4). In 1955 urines from some premature infants in our study were assayed by Leutscher for aldosterone. He demonstrated excretion of 1 to 2 mg/day(5). These infants were receiving 5 to 10 meq sodium in each day's feedings. These and other infants were given ACTH and had a typical sodium diuresis. Urine from these premature infants after they were given ACTH was the source of the majority of material used in experiments herein described and of all the fractions previously reported to produce a diuresis of sodium(6).

Methods. Twenty-four hour urine collections were made from female premature infants 1 to 4 weeks old, given ACTH. Urine was collected from 2 newborn infants with congenital adrenal hyperplasia. One of these had Addisonian symptoms. The other, being treated with cortisone, was given ACTH. In addition, urine was obtained from 2 older patients with congenital adrenal hyperplasia, receiving cortisone. These patients were not given ACTH. One of these patients apparently had had Addisonian symptoms as an infant. Random 24 hour urines were collected from normal newborn infants. Urines were extracted with redistilled chloroform for the free steroid fraction. The residual urine was then adjusted to pH 4.5 and incubated 24 hours with 400 units of β glucuronidase (Ketodase)/ml at a temperature of 36°C $\pm$ 1. Concentrated hydrochloric acid was added to urine to lower the pH to 1 and the hydrolyzed urine was reextracted with chloroform. The chloroform extracts were combined, washed with sodium hydroxide, then with distilled water. Chloroform extracts were passed through a florosil column prepared according to the method of Nelson and Samuels(7). The column eluates were dried under a stream of air at room temperature, dissolved in redistilled methanol and chromatographed on paper in the toluene-propylene glycol system of Zaffaroni(8). The portion of paper containing urinary extracts was then divided into several sections determined by rate of flow of standard steroids on the same paper. The first area extended from just below the origin at the level of standard compound E to a level just below dihydro compound E. Area 2 extended from this point to the level of standard tetrahydro compound A. Area 3 extended from this to the level of standard compound S. The paper from compound S until just before compound A was divided midway into the final 2 areas. The cut paper was then eluted with methanol. Eluates were dried under an air stream and portions were redissolved in 1 ml of olive oil. The final assay was carried out by injecting olive oil containing area 3 eluates into individual intact white male rats. Control rats were injected with the olive oil vehicle or olive oil containing 100 to 1000 mg of standard steroids or eluates of other paper chromatogram areas. The 24-hour urine excretions of sodium, potassium and creatinine were measured for in-

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TABLE I. Assay of Sodium Excreting Effect of Urinary Extracts.

Days of assay	Area 3 eluate: Urine source	Rx	Days extract/rat	Inj. day Na excr., meq Na/mg enne.	Control (mean ± S.D.), meq Na/mg enne.	# S.D. by which exp. animals exceed control (mean)
1 (not fed)	Premature infants	ACTH	4	.19	.190 ± .024 (8)*	
		"	4	.21		
4	*Idem*	"	4	.40	.213 ± .044 (13)	>3
	"	"	4	.33		>2.5
3	"	"	2	.14	.188 ± .043 (10)	
	"	"	3	.14		
3	"	"	1	.24	.222 ± .046 (5)	
	"	"	1	.24		
4	"	"	4	.29	.22 ± .049 (11)	<2
3	"	"	2	.19	.225 ± .035 (10)	
4	" "L"	"	3	.82	.31 ± .066 (18)	>3
3	" "A"	"	2	.55	.30 ± .056 (18)	>3
			2	.48		>3
4	" "S"	"	2	.16	.160 ± .035 (12)	
			2	.18		
1	" "H_1"	"	2	.17	.138 ± .043 (10)	
(not fed)	" "H"	"	$1\frac{1}{2}$	.27		>3
2	" "H"	"	$\frac{1}{2}$	.27	.162 ± .036 (11)	=3
	" "G"	"	1	.22		
			3	.26		>2.5
2	"	"	10	.36	.238 ± .040 (5)	>3
(single 48-hr specimen)	"	"	8	.32		>2
			4	.20		
2	"	"	$1\frac{1}{2}$	.16	.182 ± .025 (10)	
			3	.26		>3
		"	3	.24		>3
4	Full term newborn infants	0	4	.16	.160 ± .035 (12)	
3	*Idem*	0	8	.22	.225 ± .035 (10)	
1 (not fed)	Newborn ♀ cong. adr. hyper. without Addisonian symptoms	ACTH	1	.52	.186 ± .094 (10)	>3
4	1-mo-old ♂ cong. adr. hyper. with Addisonian symptoms	0	1	.34	.217 ± .049 (12)	>2.5
			<1	.29		<2
2	10-yr-old ♀ cong. adr. hyper. without Addisonian symptoms	Cortisone	$\frac{1}{2}$	.22	.210 ± .052 (17)	
2 (not fed)	7-yr-old ♂ cong. adr. hyper. without Addisonian symptoms	Cortisone (inadequate) 17 KS 1	2	.24	.150 ± .044 (16)	>2

* No. of control rats.

dividual rats. Standard steroids were compounds E, F, A, B, S and DOC as well as dihydro compound F, tetrahydro compound F, tetrahydro compound E, tetrahydro B and tetrahydro A, 17 hydroxy progesterone, 11α and 11β, 17 dihydroxy progesterone and 3-(3-oxo-17β-hydroxy-4-androsten-17α-γL) proprionic acid γ lactone (SC-5523).‡ The last

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substance was shown by Kagawa *et al.* to antagonize aldosterone induced sodium retention in adrenalectomized rats(9). White male rats varied from 130 to 200 g but in any given experiment their weight did not vary more than 15 g on either side of the mean. Prior to the test assay the animals were kept in individual cages from 4 to 7 days, fed a standard rat chow and had access to distilled water *ad lib.* For the assay, rats were placed in metabolism cages over funnels draining urine into bottles containing thymol. The sides of collecting funnels were washed down with distilled water at the end of each day's collection. Rats were induced to void at beginning of assay and at end of each day's collection by application of an ether soaked sponge to the nose. Animals were given rat chow and distilled water during the test except in 4 assays where the rats were allowed only distilled water *ad lib.* to eliminate the possibility that urine was being contaminated by food particles. Sodium content of chow was 0.3%. Sodium and potassium measurements were made on internal standard flame photometer. Creatinine was measured by the method of Peters(10). Sodium and potassium excretions are reported per mg of urinary creatinine.

Results. None of the standard steroids, including SC-5233 in doses of 1 mg, nor any of the eluates from paper areas other than 3, produced any significant effect upon sodium and potassium output.

Table I lists the tests, source of urinary extract, number of days of assay, mean and standard deviation of control sodium excretion with number of control rats and sodium excretion on day of injection of individual rats tested with area 3 eluates. The pre- and post-injection excretions of sodium were similar for all rats and were equal to sodium excretion on day of injection of control rats.

The only material that significantly affected sodium excretion was the eluate of area 3, representing material found on paper chromatograms between levels of tetrahydro A and compound S. Thirty-three rats were injected with eluates of this area. This was variably associated with a slight increase in potassium excretion which was never statistically significant. Ten of these rats excreted amounts of sodium in their urine that were greater than the mean control excretions of sodium by more than 3 standard deviations. Three excreted sodium in excess of control means by 2.5 standard deviations and 2 rats had an equivocal sodium diuresis which exceeded their control means by only slightly more than 2 standard deviations.

Four eluates were divided into 2 unequal portions and each fraction injected into 1 rat. In each case the larger fraction provoked greater sodium diuresis. In 2 cases, each half of the original fraction gave an equivalent result when injected into individual rats.

The mean sodium to creatinine excretion on day of injection of 30 rats injected with area 3 eluates was 0.249 with a standard error of the mean of $\pm$ .016. The mean sodium to creatinine excretion of 280 control rat days combined was 0.192 $\pm$ .003. The difference between the 2 means is significant at the 99% level of confidence. (This calculation excludes 2 experiments carried out in 1956 when control sodium to creatinine excretion was significantly higher than subsequent control values.)

The actual creatinine excretions of rats given the extract of the area between tetrahydro A and compound S tended to be slightly but not significantly higher than the mean control excretion of creatinine. As may be seen in the chart, withholding food for the day of test did not seem to affect results other than to lower slightly the mean sodium to creatinine ratio.

A representative experiment is illustrated in Fig. 1. Two area 3 eluates were given to 2 individual rats. Ten control rats were injected with either pure olive oil or standard steroid material in olive oil. Sodium excretion is given for pre- and post-control days and day of injection of the 2 rats given area 3 eluate and mean sodium excretion is given for the 2 control days and day of injection for the 10 control rats. Dotted lines represent mean excretion for 30 control rat days $\pm$ 2 standard deviations on either side of the mean.

Discussion. The results of these tests dem-

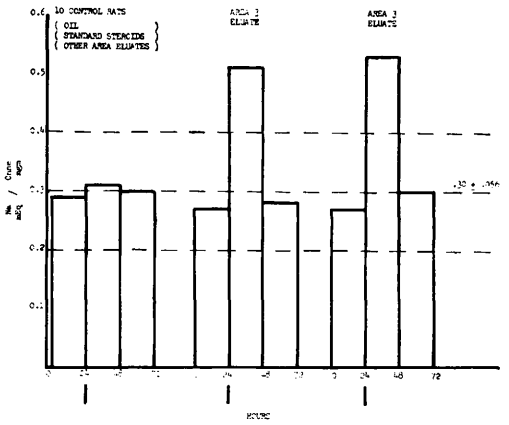

FIG. 1. Results of representative assay of area 3 eluates. Sodium excretion recorded per mg of urinary creatinine. First 3 bars represent mean excretion of 10 control rats for the 3 days of assay. Next 3 bars represent sodium excretion of an individual rat given a single area 3 eluate. Last 3 bars represent excretion of another individual rat so treated. Pips at bottom indicate time of inj. of either olive oil or standard steroid in olive oil in the case of 10 control rats and time of inj. of area 3 eluates in the 2 individual rats so treated. Dotted lines represent mean control excretion of sodium for the 30 control rat days ± 2 S.D. on either side of the mean.

onstrate the existence of material extractable from human urine under stated conditions which, when injected into intact rats, causes increased urinary sodium excretion. Evidence concerning the nature of this material will be published later.

There was no real attempt to quantitate the material used but when varying aliquots of the same material were injected into rats, the larger dose produced more sodium diuresis than the smaller and repeated injections of the same material gave reproducible results.

The material is more plentiful after ACTH administration and since it also resembles other urinary corticoids in its solubility, etc., it is suggested to us that it is a product of the adrenal cortex. However, this does not eliminate the possibility that the adrenal cortex is secreting commonly found steroids which are then metabolized along unusual lines by the liver, etc. Most of the material is found in the free fraction of urine of newborn infants. The significance of this is probably negligible since it has been shown that newborn infants conjugate steroids poorly and the majority of corticoids excreted are in the free fraction(11).

Our failure to demonstrate a sodium losing effect with each area 3 eluate may be the result of many possible factors acting singly or in combination. It may be explained by decreased efficiency of recovery of the material or decreased excretion of the material. The necessity of using intact rats complicates the experiments and the rats' own adrenals could be putting out antagonistic steroids under stress of injection, etc. The materials injected also may be contaminated with small amounts of active sodium retaining hormones. However, the principal sodium retaining hormone, aldosterone, should be well separated on the paper chromatographic systems used.

Summary. A substance has been obtained by chromatographic separation of extracts of urine obtained from premature infants and patients with congenital adrenal hyperplasia which when injected into intact rats produces an increase in urinary sodium excretion. The material is found more plentifully after ACTH administration to the patient but it has not been proven to be a secretory product of the adrenal cortex. It has minimal or no effect upon potassium excretion. It does not cause its effect by increasing glomerular filtration, at least as evidenced by creatinine excretion.

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