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J Clin Invest. 1955;**34**(9):1410-1416. <https://doi.org/10.1172/JCI103190>.

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THE PHYSIOLOGICAL BASIS FOR A METHOD OF ASSAYING ALDOSTERONE IN EXTRACTS OF HUMAN URINE

By GRANT W. LIDDLE, JEROME CORNFELD, ALFRED G. T. CASPER, AND
FREDERIC C. BARTTER

(From the Clinic of General Medicine and Experimental Therapeutics, National Heart Institute and Office of Biometry, National Institutes of Health, Bethesda, Md.)

(Submitted for publication April 11, 1955; accepted May 13, 1955)

Within the past two years aldosterone, an extremely potent sodium-retaining steroid, has been isolated, structurally identified (1, 2) and subjected to numerous physiologic studies. This steroid has been isolated from adrenal tissue (3-5), from adrenal venous blood of animals (6, 7) and from human urine (8, 9).

Over the past five years there has accumulated convincing evidence that the clinical conditions which are characterized by pathological accumulation of sodium (*e.g.*, congestive heart failure, cirrhosis with ascites, and nephrosis) are also characterized by high urinary levels of sodium-retaining steroids (9-12). High levels of these steroids have also been found in the urine of healthy subjects in whom the stimulus to sodium conservation has been provided by a low-sodium diet (10). Evidence has been presented indicating that aldosterone is the principal steroid responsible for the sodium-retaining activity of such urine (8, 9).

In attempting to understand the pathogenesis of the edematous states, it would be of interest to know not only the actions of aldosterone itself but also the mechanisms governing its production. For this purpose a method of determining the quantities of aldosterone in biological fluids is required. The amounts of aldosterone appearing in human urine are so small that the measurement of this steroid by chemical methods alone is extremely difficult. For this reason it continues to be practical in carrying out clinical studies to rely on biological methods of assay.

The present studies were carried out in an attempt to characterize in detail the acute biological response to varying doses of crystalline aldosterone in the dog, and to determine whether such a response could be made the basis of a convenient assay procedure. Simultaneously, an effort was made to test the validity of the assumption that the sodium-retaining material found in

urine is aldosterone by determining whether its biological actions are identical with those of aldosterone. Finally, since crystalline aldosterone is not at the present time available in sufficient quantity for general use, comparable studies were performed to determine whether desoxycorticosterone is suitable as a biological standard of reference.

We have selected the dog as an assay animal because of the ease with which detailed studies of renal function can be performed in this species. In the dog it is also easy to administer test-substances intravenously, thereby avoiding confusion arising from the fact that different steroids have different rates of absorption from extravascular tissues and that this might affect their apparent relative potencies under the conditions of a given assay, a possibility recently re-emphasized by Swingle and his co-workers (13).

METHODS

Materials

1. Crystalline aldosterone, prepared from adrenal extract by Dr. H. L. Mason of Mayo Foundation.
2. Crystalline desoxycorticosterone (free alcohol), synthesized by Ciba Pharmaceutical Products, Inc.
3. Extract of human urine. Urine was collected by three subjects receiving low sodium diets: a normal man, a patient with congestive heart failure, and a patient with ascites due to cirrhosis. All specimens were refrigerated until extracted. A total of sixty 24-hour collections were combined to provide an homogeneous pool large enough to permit repeated biological assays. The urine was acidified with HCl to pH 1 and extracted continuously for 24 hours with methylene chloride. The extract was washed with aqueous sodium carbonate, dehydrated with sodium sulfate, and evaporated to dryness under a stream of nitrogen. Each of the three steroid preparations was dissolved in absolute ethanol and stored at -7° C. throughout the study.

Assay animals

Mongrel female dogs, weighing between 15 and 20 Kg. were adrenalectomized through a single anterior ab-

dominal incision. In some, an episiotomy was performed to facilitate subsequent catheterization. Cortisone acetate was given at the time of operation, 100 mg. intragastrically and 200 mg. intramuscularly. Thereafter, 5 mg. of cortisone acetate was given intramuscularly each day. The dogs were maintained on a constant synthetic diet containing 200 mEq. of sodium and 20 mEq. of potassium daily, given as one feeding approximately 18 hours before the study was to be performed on the following day. No animals were employed until at least one week after adrenalectomy. Some had been maintained on the above regimen for more than a year.

Assay procedure

During the assay period each dog was permitted to lie or stand on a flat table. A retention catheter was inserted; the bladder was washed with distilled water (20 cc. \times 4), and the washings were discarded. Thereafter, the urine was collected and the bladder washed at hourly intervals for five hours. Specimens were analyzed for sodium and potassium, using a flame photometer. At the end of the first hour, which served as a control period, the steroid or extract to be assayed was diluted with 3 volumes of water and injected intravenously.

Design of study

A crossover design was employed so that each of nine animals received, on a randomized schedule, each dose of each preparation. Six dose levels, spaced at twofold intervals, were employed for each of the three preparations (aldosterone, desoxycorticosterone, and urine extract), so that each dog received 18 treatments.

Three subsidiary studies, similarly designed, were also carried out as an empirical check on the reliability of the assay procedure.

RESULTS AND COMMENTS

Characteristics of the dose-response

In response to each of the three steroid preparations, there occurred both a decrease in excretion of sodium and an increase in excretion of potassium (Figure 1). Consistent changes were not noted during the first hour following treatment. At all dosages and for all three preparations, sodium excretion reached a minimum and potassium excretion a maximum during the second and third hours.

Of the five sodium and five potassium values so obtained for each treatment, some combination was sought which would best characterize the "sodium response" and the "potassium response" to treatment. The criterion used in selecting such a combination of data was that it serve to differentiate steroid-dosage levels with maximum precision. The second and third post-treatment hours were found to be of greatest value in this regard. The first and fourth post-treatment hours provided no additional information. Therefore, in quantitating responses we have pooled post-treatment hours two and three and discarded post-treatment hours one and four. It was found to be of further value to relate the post-treatment values to the preinjection control values. Thus "*potassium response*" equals *potassium for hours two and three minus potassium during control*

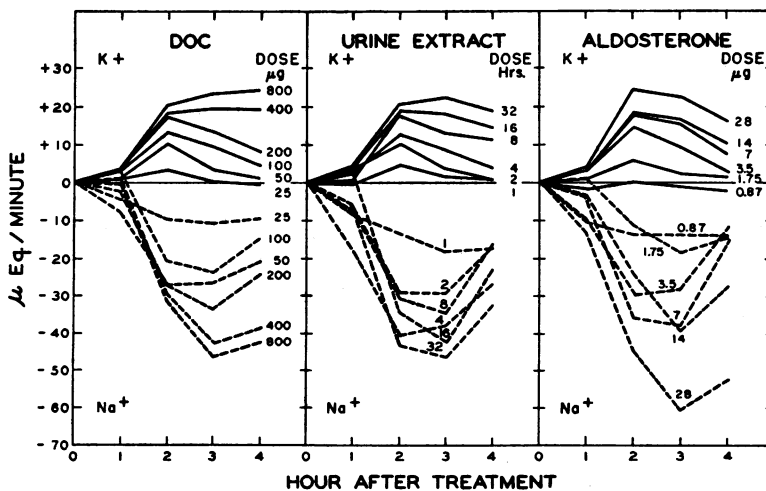


FIG. 1. HOURLY CHANGES IN EXCRETION OF SODIUM AND POTASSIUM IN RESPONSE TO DESOXYCORTICOSTERONE, URINE EXTRACT, AND ALDOSTERONE

Each line represents the average response of nine dogs. Sodium values are unweighted.

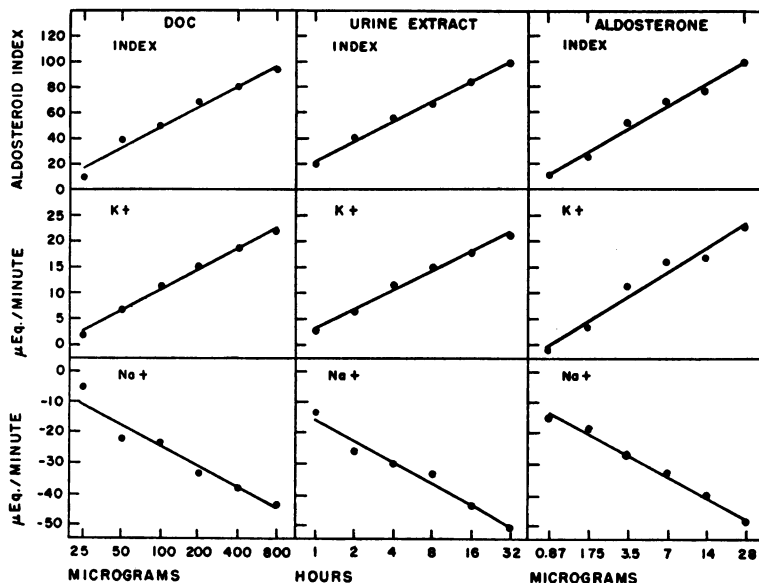


FIG. 2. "SODIUM RESPONSES," "POTASSIUM RESPONSES," AND "ALDOSTEROID INDEXES" RESULTING FROM ADMINISTRATION OF VARYING DOSES OF DESOXYCORTICOSTERONE, URINE EXTRACT, AND ALDOSTERONE

Each dot represents the average response of nine dogs. Logarithmic dosage scale.

hour, all values expressed as $\mu\text{Eq.}$ per minute. For the sodium response a further significant increase in precision was obtained (as shown by the analysis of covariance) by using, not the observed control, but rather what we have termed a weighted control value. The weighted control value derived for this set of data was $54 + 0.4$ (observed control). Thus, for any particular treatment, "sodium response" equals sodium for

hours two and three minus $[54 + 0.4$ (control sodium)], expressed as $\mu\text{Eq.}$ per minute.

The average "sodium responses" and "potassium responses" are plotted against doses of all three preparations in Figure 2. It is apparent that each response is a linear function of the logarithm of dose over the entire 32-fold range.

Under comparable assay conditions, when the alcoholic vehicle containing no steroids was ad-

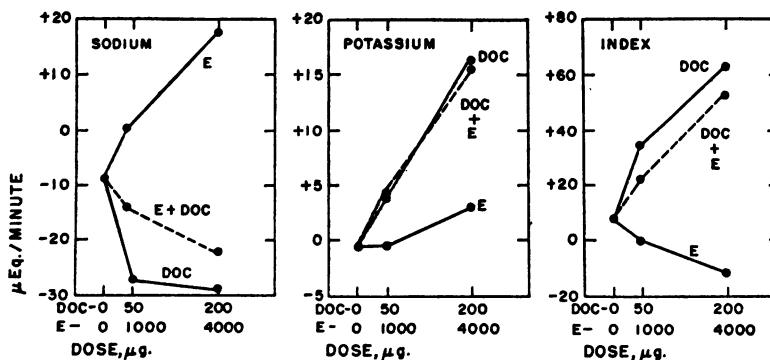


FIG. 3. "SODIUM RESPONSES," "POTASSIUM RESPONSES," AND "ALDOSTEROID INDEXES" RESULTING FROM ADMINISTRATION OF VARYING DOSES OF DESOXYCORTICOSTERONE ALONE, CORTISONE (E) ALONE AND OF THE SAME DOSES OF DESOXYCORTICOSTERONE PLUS CORTISONE

Each dot represents the average response of six dogs.

ministered (Figure 3), there was a slight tendency for the excretion of both sodium and potassium to decrease during successive hours.

For aldosterone, the slope of the line relating sodium response to dose was $-22.5 \mu\text{Eq. per minute per tenfold dilution of aldosterone}$. For desoxycorticosterone (DOC) it was -23.2 , and for urine extract it was -22.8 . The standard error of each slope was 2.9. These slopes were not significantly different from one another. Relating potassium responses to dose of each preparation, the average slope for aldosterone was 15.7, for DOC 13.5, and for the urine extract 12.3. The standard error of each slope was 1.5. Once again, these slopes did not differ significantly.

Having established the parallelism of the dose-response curves for the three steroid preparations, we may proceed to determine their ratios of potency. The ratio of potency $\frac{\text{aldosterone}}{\text{DOC}}$ when measured in terms of sodium-retaining activity was 36-fold and when measured in terms of potassium-losing activity 26-fold. Ninety-five per cent confidence limits on these estimates were 24 to 56 and 18 to 37, respectively. The ratio of potency of $\frac{\text{aldosterone, } \mu\text{g.}}{\text{urine extract, hr.}}$ when measured in terms of sodium-retaining activity was 0.87 and when measured in terms of potassium-losing activity 1.03. Ninety-five per cent confidence limits were 0.56 to 1.34 and 0.72 to 1.47, respectively. Clearly,

the relative potencies of these steroid preparations were essentially the same whether they were compared on the basis of sodium retention or potassium loss.

The experience of Desaulles, Tripod, and Schuler (14) suggesting that aldosterone is only five times as potent as DOC in its influence on potassium excretion, whereas it is thirty times as potent as DOC in its effect on sodium excretion, has no parallel in the present study.

Since the potassium and sodium responses give essentially the same estimates of relative potency, it is permissible to combine these responses into a single index, the criterion of combination being solely that it yield the maximum information about steroid dosage which can be derived from a given set of responses. Such a combination, which we have termed the aldosteroid index, is given by the method of discriminant functions (15).

As estimated from the experiment summarized in Figures 1 and 2,

$$\text{Aldosteroid index} = 2.3 \text{ ("K response")} - \text{"Na response."}$$

We show in Table I the index values for the nine dogs, six dose levels and three materials included in the present study. The average index for nine dogs is represented for each treatment in Figure 2.

Recalculation of the ratios of potency of the three steroid preparations indicates that by the present method 1 $\mu\text{g.}$ of aldosterone exhibited ac-

TABLE I
Aldosteroid index for nine dogs, three steroids each at six dose levels

Dog	A	B	D	E	G	J	K	N	R	
DOC ($\mu\text{g.}$)	25	52.4	29.2	15.8	-5.6	19.8	14.2	12.2	-14.9	-33.4
	50	60.2	41.9	30.2	10.6	83.9	50.8	28.2	14.2	23.6
	100	64.2	44.6	54.7	38.2	83.0	63.6	37.9	23.3	34.9
	200	85.6	78.5	80.2	48.9	115.0	71.7	50.0	48.2	37.2
	400	91.7	124.6	94.0	49.4	97.0	80.8	71.4	73.8	47.6
	800	90.0	136.4	104.0	63.3	123.0	108.4	81.0	63.7	81.3
Urine extract (hr.)	1	49.1	25.2	41.2	13.7	25.3	8.0	16.8	20.2	-13.8
	2	64.0	46.2	49.2	12.6	58.3	66.9	42.0	32.4	2.1
	4	83.0	75.8	65.2	25.0	59.8	86.0	62.8	29.6	24.1
	8	98.0	113.6	76.0	15.8	73.3	92.0	66.3	45.7	29.7
	16	117.6	116.6	97.0	38.2	119.2	93.2	82.1	55.8	49.8
	32	121.8	147.4	134.9	53.6	133.2	88.8	76.2	102.0	41.3
Aldosterone ($\mu\text{g.}$)	0.875	28.0	25.4	34.3	2.2	25.2	6.5	-5.2	9.6	-8.3
	1.75	53.8	41.0	12.5	14.2	40.8	27.6	25.1	13.0	21.6
	3.50	68.6	88.4	67.8	21.6	89.1	53.8	33.4	33.8	32.4
	7.00	97.4	130.0	66.1	34.6	83.6	84.5	64.7	50.0	27.8
	14.	86.6	115.8	87.1	44.0	109.3	98.0	82.5	43.7	51.8
	28.	107.4	121.8	125.4	41.0	167.8	103.4	114.0	88.0	50.2

TABLE II
Analysis of variance of aldosteroid index

Source of variation		Degrees of freedom		Mean square
A	Dogs	8		9,030
	Dose levels:	5		
B	Regression		1	127,859
C	Deviation from regression		4	238
D	Steroids	2		391
	Interactions			
	Dog × dose level:	40		
E	Difference in regressions		8	900
F	Variation about individual regressions		32	147
G	Dog × steroid	16		209
	Dose level × steroid:	10		
H	Difference in regressions		2	203
I	Variation about individual regressions		8	129
J	Dog × dose level × steroid	80		168
		161		

tivity comparable to that of 29.9 μg . (24.2 to 36.9) (95 per cent confidence limits) of DOC and to that of 0.96 hour (0.78 to 1.19) of urine extract. The ratio of potency of aldosterone to DOC is of the same order as that reported previously by Gross and Gysel (16), by Mach and associates (17), and by Kekwick and Pawan (18), all using different methods.

Some idea of the relative utility of alternative methods of analyzing assay data can be gained by comparing their respective "lambda" values.¹ The present method of assay and analysis gives values of λ in the neighborhood of 0.24. This implies that in estimating ratios of potency for two preparations, six observations on each preparation would be sufficient to establish with 95 per cent confidence that a given estimate is not less than one-half nor more than twice the true value.

The gain in precision which is achieved by using the "aldosteroid index" rather than either of the individual responses is shown by comparison of the corresponding values of λ : 0.24 for the index, 0.37 for the potassium response, and 0.48 for the sodium response. This implies that if one were to use the latter metameters instead of the "index" one would require from two to four times as many observations to derive the same amount of information.

Table II presents an analysis of variance of the aldosteroid indexes. The square root of the mean square for the highest order interaction (dog by

dose by steroid) supplies an estimate of the standard deviation of the indexes that would be obtained from repeated measurements on the same dog at a single dose level for a single steroid; *i.e.*, it provides the estimate of random error. Three of the mean squares (lines A, B, and E) significantly exceed this estimate of error at the .001 level; the remainder do not differ significantly from random error at the .05 level. We conclude from this analysis, first, that dose-response curves for individual dogs differ in both level and slope (lines A and E). Use of the crossover design eliminates this source of variability and, since we are interested in the relative potencies of the materials tested rather than the characteristics of the population of assay subjects, the use of a crossover design increases the precision of the assay. Second, we conclude that the differential response to different materials is the same for all dogs (line G), so that as far as the evidence of this experiment goes, each dog supplies essentially the same estimates of potency. Third, the dose-response lines for the three different steroids are parallel (line H). Fourth, there are no significant departures from linearity (lines C, F and I). Fifth, the experiment was designed so that if there were significant day effects, their main effects, would be balanced out. The mean square for days, 203, (not shown in Table II), is not significantly above random error, thus indicating that dogs make a satisfactory return to control status within 24 hours after a test.

¹ "Lambda" = standard deviation \div slope.

Reproducibility of assay results

To what extent will the aldosteroid index provide useful results when used in an experiment other than the one it was derived from? We summarize in Table III the average values of the aldosteroid indexes provided by each of four independent experiments. At the foot of the table we show the standard deviations, slopes and values of λ derived from each experiment. The values of λ vary from 0.19 to 0.26 and indicate that the utility of the aldosteroid index is not confined to the experiment from which it was derived.² The variation from experiment to experiment in the absolute levels of the indexes reflects differences in responsiveness of different dogs. The five dogs used in experiment IV were also used in experiment III. Three of these five dogs showed significant declines in responsiveness over the period from October to January. The biological significance of these declines is not at present known, but in view of the unchanged magnitude of the standard deviation and slope, it would appear to have no effect upon the precision of the present assay.

² For experiments 1 and 2 the standard deviation was estimated from the dog-dose interaction, which may, in view of the results of Table II, provide an overestimate.

Interference by contaminating steroids

Earlier experience indicated that under the conditions of this assay procedure hydrocortisone and cortisone in doses of more than 1 mg. caused increases in excretion of sodium (19). Data presented in Figure 3 illustrate the manner in which this acute sodium-losing action of cortisone can modify results obtained by the present method. Fortunately the amounts of cortisone or hydrocortisone required to produce this interference are so large that they would probably never be encountered in extracts of human urine except during treatment with large doses of 17-hydroxycorticosteroids or ACTH or in patients with Cushing's syndrome. The extract of urine employed in the present study contained no more than 3 $\mu\text{g.}$ of 17-hydroxycorticosteroids per "hour" of extract, as estimated by the Porter-Silber reaction. Contamination of this degree is far below that necessary to modify assay results. The close similarity between the responses to this urine extract and responses to crystalline aldosterone has been interpreted as indicating that biologically detectable contamination did not occur. Whenever heavy contamination of an extract with 17-hydroxycorticosteroids is a possibility, however, it would appear advisable to remove them before proceeding with the bioassay.

TABLE III
Aldosteroid indexes at different dose levels and for different steroids in four independent experiments

Dose level $\mu\text{g. DOC}^*$	Exp. I	Exp. II	Exp. III			Exp. IV		
	April	July	October			January		
	DOC	DOC	DOC	Aldosterone*	Urine extract*	DOC	Aldosterone*	Urine extract*
0	14.9	22.1	—	—	—	—	—	—
25	—	—	10.0	13.1	20.6	—	—	—
50	39.6	59.5	38.2	27.7	41.5	28.4	20.3	34.1
100	66.3	80.5	49.7	54.2	56.8	—	—	—
200	88.1	105.4	68.4	71.0	67.3	63.8	63.2	71.9
400	99.2	124.3	81.2	79.9	85.5	—	—	—
800	114.2	131.6	94.7	102.1	99.9	—	—	—
1,600	112.5	—	—	—	—	—	—	—
Standard deviation(s)	14.1	16.0	—	13.0	—	—	11.9	—
Slope (b)	60.5	62.5	—	54.7	—	—	63.0	—
$\lambda = s/b$	0.23	0.26	—	0.24	—	—	0.19	—

Exp. I—Cross-over on 7 dogs, each tested for 7 days.

Exp. II—Cross-over on 6 dogs, each tested for 6 days.

Exp. III—Cross-over on 9 dogs, each tested for 18 days (individual results in Table I).

Exp. IV—Cross-over on 5 dogs, each tested for 8 days.

* Responses to 0.875 $\mu\text{g.}$ aldosterone and 1 hr. of urine extract are on line 25 $\mu\text{g. DOC.}$

SUMMARY

A method for assaying aldosterone and like steroids has been described. This method entails the use of adrenalectomized female dogs, maintained on a high sodium diet. The effects of varying doses of aldosterone on sodium and potassium excretion have been presented. Administration of crystalline aldosterone results in a rise in potassium excretion and a fall in sodium excretion, both of which are rectilinear functions of the log-dose of aldosterone over the range from 1 to 30 micrograms. An "index" has been derived for the purpose of reducing the complex response to a single dimension, while achieving maximum precision ($\lambda = 0.24$) in differentiating dosage levels. With this method the response to aldosterone closely resembles the response to crystalline desoxycorticosterone which is approximately 1/30 as potent as aldosterone. Extracts of urine from patients with congestive heart failure, cirrhosis with ascites, and from healthy subjects receiving a low sodium diet, have been found to possess activity indistinguishable from that of aldosterone. The responses to aldosterone are easily distinguishable from the responses to hydrocortisone-like steroids by the present method.

ACKNOWLEDGMENTS

The authors acknowledge with gratitude the generous provision of steroids for this study by Dr. H. L. Mason of Mayo Foundation and by Dr. Robert Gaunt of Ciba Pharmaceutical Products, Inc., as well as the advice and assistance of Dr. Leroy Duncan, Dr. Maurice Pechet, Dr. James O. Davis, Mrs. Gaynelle Greene, Mrs. Helen Reeves, and Miss June Richard.

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