Isolation of a Potent Sodium-Retaining Substance from Adrenal Venous Blood of the Dog.* (20440)

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A potent sodium-retaining substance has been isolated during the course of a study which has been designed to determine the chemical and biological characteristics of steroids in adrenal venous blood of the dog. The substance is many times more active than desoxycorticosterone acetate (DOCA) in producing sodium retention in the adrenalectomized rat. The work confirms the recent report of Simpson *et al.*(1) who isolated a sodium-retaining factor from adrenal venous blood of a dog and from a perfusate of a monkey adrenal.

Methods. Mongrel male dogs were anesthetized with sodium pentobarbital. Adrenal venous blood was collected from a cannula placed in the left lumboadrenal vein. The adrenal vein was ligated between the adrenal gland and the inferior vena cava. Blood pressure was recorded continuously and pooled dog blood was infused at a rate sufficient to maintain the blood pressure at or above 100 mm mercury during the collection period. A liter of adrenal venous blood was collected over 3 to 4 hours. Two liters of arterial blood were collected by intermittent bleeding from the femoral artery during the same period. One liter of this arterial blood was collected in a vessel containing 1500 μg of 17-hydroxycorticosterone and 500 μg of corticosterone for recovery studies. The other liter of arterial blood was analyzed in order to obtain an estimate of the concentration and the types of steroids entering the adrenal. V-A difference represents the contribution of the adrenal to steroids in adrenal venous blood. Blood was diluted with an equal volume of water and extracted twice with a volume of chloroform equal to that of the blood-water mixture. The chloroform extracts were evaporated and the residue partitioned between hexane and 70% ethanol. The 70% ethanol fraction was chromatographed in a propylene glycol-toluene system according to the method of Burton *et al.*(2). The design of chromatography was as follows: 1) the entire extract was first chromatographed for 96 hours on a paper strip impregnated with half-strength propylene glycol;‡ 2) the overflow from 1) was chromatographed for 24 hours on paper impregnated with full-strength propylene glycol; 3) the overflow from 2) was chromatographed for 4 to 6 hours on paper impregnated with full strength propylene glycol. The more polar components of 1) were rechromatographed for 240 hours. Steroid mixtures on certain areas of the paper were chromatographed several times to effect satisfactory resolution. Steroids were located on the chromatograms by 1) viewing under ultraviolet light, 2) ammoniacal silver nitrate staining of small strips cut from the main chromatogram, and 3) sulfuric acid reaction on small strips cut from the main chromatogram. Finally, each chromatogram was cut at right angles to the length of the strip into 1 or ½ inch pieces. Steroids were eluted from individual cuts with methanol. The methanol solutions were quantitatively analyzed by 1) absorption in the ultraviolet, 2) reaction with phenylhydrazine and 3) reaction with sulfuric acid. The bioassay method employed for determining alterations in urinary electrolyte excretion was adapted from the procedure described by Marcus *et al.*(3). Three modifications of this method were employed throughout the present study; 1) test rats were not

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‡ Paper is impregnated with a propylene glycol-methanol (1:1) mixture and the methanol allowed to evaporate. The highly polar steroids are more effectively resolved under these conditions in which the quantity of propylene glycol per unit weight of paper is reduced.

subjected to a sodium chloride load during the assay period, 2) urine was collected from single rats instead of rat pairs, and 3) the collection of urine was extended to 6 hours.

For each individual assay, 30 male Holtzman rats weighing 150 to 200 g were bilaterally adrenalectomized and given 0.9% sodium chloride solution to drink. On the third day following adrenalectomy, tap water was substituted for the 0.9% sodium chloride solution. On the morning of the fourth day, food and water were removed from the cages, and the rats injected 3 to 4.5 hours later. All substances tested were dissolved in olive oil and injected subcutaneously. Each rat was placed in a one-liter beaker containing a wire mesh screen which separated urine and feces. Bladder emptying was induced prior to injection of test material and at the termination of the collection period by exposing each rat to ether. Following the collection period, the feces were removed from the wire screen, the beaker was washed with a 0.5% lithium nitrate solution and the washings were diluted to 100 ml. Urinary sodium and potassium concentrations were determined with a Perkin-Elmer flame photometer (model 52A).

Results. A. Components of adrenal venous blood. Studies were carried out on 2 male dogs weighing 18 and 15 kg. In each experiment, the following substances were present in higher concentrations in adrenal venous blood than in the arterial blood: 1) 17-hydroxycorticosterone, concentration, 259 and 294 μg/100 ml; 2) Substance 8, concentration, 4.9 and 10.4 μg/100 ml; 3) 11-desoxy-17-hydroxycorticosterone, concentration, 39 and 46 μg/100 ml; and 4) corticosterone, concentration, 116 and 120 μg/100 ml. Substance 8 chromatographed in the same region as cortisone, absorbed at 240 mμ, gave a 400 mμ maximum with phenylhydrazine under conditions employed in this laboratory but not under conditions described by Porter and Silber(4) and gave a tan-colored solution in sulfuric acid with maxima at 285 and 390 mμ. It is noteworthy that in both experiments, 11-desoxy-17-hydroxycorticosterone was secreted by the adrenal; this steroid has not been found to be secreted by the adrenal of all dogs(5). A more complete report of these analyses, which are a part of a long-range study on the nature of the steroid secretion pattern of the dog adrenal cortex, is in preparation(5). The arterial blood to which 17-hydroxycorticosterone and corticosterone had been added contained the same substances in the same concentrations as arterial blood with the exception of the added steroids, which were recovered in approximately 90 and 70% yields, respectively. This experiment indicated that none of the substances found in adrenal venous blood was derived from the major steroids by degradation during extraction and chromatography.

B. Biological activity of Substance 8. The activity of Substance 8 in inducing alterations in urinary sodium and potassium excretion was compared with that of desoxycorticosterone acetate. The results are shown in Table I. From the data presented it is evident that Substance 8 exerted a potent sodium-retaining effect. Maximal sodium retention was induced with 2.0 μg of Substance 8 per rat and with 50 μg of DOCA per rat. 0.7 μg of Substance 8 caused significant sodium retention; however, the sodium excretion that occurred in rats given 0.2 μg of Substance 8 was not significantly different from that of the control untreated animals ($p = 0.4$).

The area of the chromatogram of arterial blood in which Substance 8 would have been expected to appear contained no appreciable quantity of this material as estimated by chemical tests. Nevertheless, the eluate of this area of the chromatogram was tested for sodium-retaining activity in a dose equivalent to 73 ml of arterial blood per rat. The results in Table I show that this region of the chromatogram of arterial blood had no detectable sodium-retaining activity in the amounts of blood employed; the equivalent of 10 to 20 ml of adrenal venous blood induced significant sodium retention.

Ten and 50 μg of DOCA caused significant ($p < 0.01$) increases in potassium excretion in adrenalectomized rats (Table I). It is evident that as the dose of the hormone was increased the urinary excretion of this electrolyte increased. No such graded response in potassium excretion was observed when various doses of Substance 8 of adrenal venous

TABLE I. Urinary Sodium and Potassium Excretion with Various Doses of Substance 8 from Adrenal Venous Blood of Dogs and with Various Doses of DOCA.

Substance tested	Dose/rat	No. rats	Sodium excretion, mg/6 hr	No. rats	Potassium excretion, mg/6 hr
Olive oil (controls)	1 ml	16	5.53 ± .39*	18	7.76 ± .44
DOCA	50 μg	16	2.49 ± .20	15	10.71 ± .33
	10 "	11	3.45 ± .21	10	9.51 ± .43
	5 "	5	5.92 ± .48	5	8.79 ± .47
Substance 8 from adrenal venous blood†	3.5 μg	5	2.50 ± .57	5	9.66 ± .77
	2.0 "	5	2.33 ± .10	5	9.11 ± .61
	1.4 "	6	2.71 ± .34	6	9.80 ±1.10
	.7 "	5	3.08 ± .27	4	11.12 ± .62
	.2 "	6	4.91 ± .61	6	9.26 ± .47
Substance 8 region of arterial blood	73 ml blood	5	6.43 ± .70	5	7.60 ± .66

* Stand. error.
† Quantity estimated by reaction with phenylhydrazine.

blood was injected into rats. Furthermore, the largest dose of Substance 8 used (3.5 μg) produced only a suggestively significant increase in potassium excretion over the quantities excreted by untreated animals ($p=.05$). The relatively high variation in potassium excretion of the assay animals treated with Substance 8 precludes the drawing of final conclusions on the effect of this material on potassium excretion.

Discussion. The findings in this study are in essential agreement with those of Simpson *et al.*(1), who found a substance of high electrolyte activity in dog and monkey adrenal venous blood. The substance described by Simpson *et al.* chromatographed in the same region as cortisone but was not further characterized. Substance 8, the sodium-retaining steroid described in this communication, chromatographed in the same area as cortisone. It appeared to be homogeneous and had the following characteristics: absorbed at 240 mμ in the ultraviolet, reduced ammoniacal $AgNO_3$, reacted with phenylhydrazine to yield a 400 mμ maximum under the conditions of the test as applied in this laboratory(5) and reacted with sulfuric acid to give a tan solution with maxima at 285 and 390 mμ (*cf.* cortisone maxima 280, 340 and 410 mμ). Substance 8 did not give a blue color with iodine reagent (cortisone reacts to give a blue color). The last two findings distinguish the substance from cortisone. It is not possible at this time to state unequivocally that Substance 8 is pure; the chemical characteristics presented may not be those of the sodium-retaining factor but those of a contaminant. If the sodium-retaining steroid is associated with one or more substances, its potency is even greater than that described for Substance 8 which is approximately 25 times as active as DOCA.

The data presented in this report suggest that the sodium-retaining substance from adrenal venous blood has less influence upon potassium excretion than DOCA when the potassium effect is expressed in relation to the sodium effect. Experiments are in progress to determine the influence of this substance on potassium excretion over a wider dose range than employed in the present work. Simpson *et al.*(1) have expressed the activity of their sodium-retaining factor in terms of a ratio of Na^{24} to K^{42}; they have not presented individual figures for Na^{24} and for K^{42}. The results presented in this report were based on total sodium and total potassium excretions and definitely establish the fact that Substance 8 induces sodium retention in the adrenalectomized rat.

Summary. A substance has been isolated from the adrenal venous blood of dogs which is approximately 25 times as potent as DOCA in producing sodium retention in adrenalectomized rats. It has not been established with certainty that this substance is pure. The material is slightly less polar than cortisone, exhibits an absorption maximum at 240 mμ, does not react with the phenylhydrazine reagent described by Porter and Silber, and

does react with a phenylhydrazine reagent developed in this laboratory. The substance shows absorption maxima at 285 and 390 mμ when mixed with concentrated sulfuric acid.

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Factors Influencing Renin Diuresis and Proteinuria.* (20441)

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Renin, injected subcutaneously, elicits a variety of abnormal phenomena associated with acute, widespread necrotizing vascular lesions in bilaterally nephrectomized dogs(1) and in uninephrectomized rats pretreated with adrenal cortical steroids(2-5). Administration of excess salt is an essential conditioning factor to the vascular disease elicited by renin. This association and the nature of the lesions suggested that renin might act deleteriously through its effects on electrolyte distribution and on permeability of vessel walls.

In this connection, renin is known to cause increased diuresis and proteinuria in rabbits (6-8) and rats(9-11); the diuresis is associated with changes in electrolyte output(7, 12); the proteinuria is attributed in part to increased permeability of glomerular capillaries(11). The association of renin diuresis with proteinuria was therefore studied as were also the factors which condition these two phenomena since it seemed that such a study might aid in further defining the pathogenetic mechanisms of renin-induced vascular disease.

The particular aspects investigated were: 1) effects of subcutaneous versus intraperitoneal administration; this also involved a comparison of two renin preparations and observation on the effects of subtotal hepatectomy; 2) effects of providing water, sodium chloride and potassium chloride as drinking fluids; 3) effects of adrenalectomy and replacement therapy; 4) effects of hypophysectomy and of ACTH and somatotrophin and 5) effects of large doses of angiotonin.

Procedure. Except as indicated below, the procedure was that male albino rats of uniform strain were placed in metabolism cages 48 hours before injection of renin and kept there for 48 hours after cessation of treatment; urine flow and proteinuria (determined by the Shevky-Stafford method) were measured daily. This general plan, in which each animal is his own control, was required because of the wide variability of urine flow and proteinuria found in these rats as in those studied by Addis *et al.*(9); in some experiments, observations were repeated several times before trends or differences could be established. Since antibodies might interfere with the responses to renin, all animals were discarded from the study after a course of renin treatment.

1. *Route of administration.* The route of administration and the nature of the renin preparation used were of particular interest because of the report(13) that renin given intraperitoneally is much more diuretic than when given subcutaneously. Preliminary experiments had indicated to us that the reverse was the case; the difference in these observations might result from differences in the non-renin content of the preparations used. Consequently, 2 porcine renin preparations were

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