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## HUMORAL STIMULATION OF ADRENAL CORTICAL SECRETION \*

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The existence of a humoral agent other than adrenocorticotrophin (ACTH) which stimulated the adrenal secretion of aldosterone was demonstrated by cross-circulation experiments on conscious sheep, using Na depletion of the donor as the evoking stimulus (1), and by cross-circulation experiments on anesthetized dogs, using thoracic caval constriction as the evoking stimulus (2).

The site of origin of this aldosterone-stimulating hormone (ASH) is not known. Farrell (3) has suggested that the stimulating hormone arises from the pineal gland. It has been shown, however, that there is no reduction of aldosterone secretion in response to Na deficiency after chronic pineal-ectomy (4-7). Farrell (8) has prepared an extract from the pineal gland which augments aldosterone secretion under the conditions of his experiments.

It has been shown recently that the kidney affects aldosterone secretion. The administration of renin prepared from kidney tissue increased the width of the zona glomerulosa of the rat adrenal (9, 10). The amount of renin extractable from kidneys is inversely related to sodium intake (11), and there is hypergranularity of the juxtaglomerular zone in Na deficiency (12, 13). The urinary excretion of aldosterone is increased in essential hypertension (14, 15), and the urinary excretion and adrenal secretion rate are increased in malignant hypertension and in some patients with renal hypertension (15-17). Intravenous infusion of angiotensin II increases aldosterone secretion (16, 18).

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Davis and associates (19), and Mulrow and Ganong (20) have reported that the increased aldosterone secretion usually evoked by hemorrhage in anesthetized, hypophysectomized dogs does not occur in hypophysectomized, nephrectomized dogs. Infusion of saline extracts of kidney in these bled animals caused aldosterone to rise to the level usually seen after hemorrhage in the animal with intact kidneys, and this activity has been localized to the renin fraction (21). Carpenter, Davis and Ayers (22) found no increase in aldosterone secretion in experimental benign hypertension but observed a rise in experimental malignant hypertension produced by Goldblatt clamps. Bilateral nephrectomy reduced the aldosterone secretion in caval constriction and Na deficiency (23).

In order to demonstrate that an extract contains an agent which may act as a direct physiological stimulus of aldosterone secretion, it is necessary to show that the material is active upon direct local infusion into the arterial supply of the adrenal gland (blood flow 7 to 17 ml per minute) at about 0.01 to 0.0025 of the rate at which it is active upon intravenous infusion. If a systemically active extract is inactive upon direct intra-adrenal arterial infusion, three explanations are possible: 1) the material acts pharmacologically or physiologically on receptor elements elsewhere in the body to release a substance that is directly active; or 2) the material acts relatively slowly on a precursor to produce another active substance; or 3) the material is itself changed into an active agent. The demonstration of direct stimulation of the adrenal cortex does not necessarily establish physiological hormonal correspondence, and consideration of this problem indicates the value of concurrent study of the pharmacology of the adrenal cortex.

This paper describes the effect on adrenal secretion of aldosterone, cortisol, and corticosterone when val-5-angiotensin II, val-5-angiotensin I, and adrenocorticotrophins (ACTH) were infused locally into the arterial supply of the transplanted

adrenal gland. Comparison was made with intravenous infusion and also with the effect of local concurrent lowering of  $\text{Na}^+$  and increase of  $\text{K}^+$  concentration in adrenal arterial blood, a procedure that is a potent specific stimulus of aldosterone secretion (1, 4).

Preliminary experiments are recorded also on the effect of adrenal arterial infusion of  $\beta$ -melanocyte-stimulating hormone (MSH), serotonin, bradykinin, noradrenaline, acetylcholine, lysine vasopressin, and oxytocin. These substances were infused at high but physiologically feasible rates, as a screening procedure to determine whether any might have an aldosterone-stimulating effect comparable to the output of this hormone observed in  $\text{Na}^+$  deficiency.

#### METHODS

Merino sheep with permanent, unilateral, parotid fistulae and a carotid loop on the right side were used (24). Five animals, TP 9, TP 12, Noah, Cyrano, and Osiris (weighing 43.5, 42, 41.5, 32, and 42.5 kg, respectively), had a left adrenal transplant in a combined carotid artery-jugular vein skin loop (25, 26). The autotransplants had been made 42, 40, 3, 4, and 5 months previously. The right adrenal gland was removed shortly after transplantation.

The sheep Osiris had an intermediate degree of a condition that occurred sporadically during our 4 years' experience with transplantation of the adrenal gland. Some transplants are incomplete, and although aldosterone secretion is normal and increases normally with Na deficiency, there is little if any production of cortisol normally or in response to ACTH, and substitution therapy with cortisol or cortisone must be given to preserve life. When Osiris was given ACTH, cortisol secretion rose only one-quarter as much as was found with a normal transplant. However, corticosterone secretion was not impaired to the same degree. The use of this sheep was largely restricted here to screening procedures to determine whether or not a particular agent had a strong stimulating effect on aldosterone secretion. These instances are clearly indicated in the text, and results from this animal are not included in any figures that record dose-response relations.

The sheep were kept in metabolism cages, and when experiments were made under conditions of Na repletion, it was ensured that Na intake in the preceding 24 hours exceeded the measured output.

The procedure for making infusions directly into the arterial supply of the adrenal gland and for collection of adrenal venous blood has been described in full elsewhere (1, 27). It is of great importance that the internal diameter of the polythene tube employed for adrenal venous blood collection be large enough to carry a blood flow of 25 to 30 ml per minute when the tube is at full extension

below the transplant loop; otherwise there will be a mechanical obstruction of blood flow and increased venous pressure during the collection of adrenal venous blood. The intravenous infusions were made into the right jugular vein. Full aseptic precautions were taken with all infusion procedures. The materials were prepared in isotonic saline so that the desired concentration of the substance was attained with an intra-adrenal arterial infusion rate of 0.2 to 0.8 ml per minute, i.e., 2 to 8 per cent of adrenal blood flow rate. Often the rate of infusion was increased over two or three steps to reach the desired rate, and usually the infusion was continued for 20 to 30 minutes before the adrenal venous blood specimen was collected. Control adrenal venous blood specimens taken in the intervals between testing substances were collected while normal saline was infused at 0.2 ml per minute into the adrenal arterial supply.

#### *Measurement of secretion of aldosterone and other steroids*

*Chemical assay.* The central problem in an investigation of this type is the specificity, sensitivity, and accuracy of measurement of the small amount of aldosterone in adrenal venous blood of the conscious, undisturbed, Na-replete animal. These are the requisite experimental conditions for a *valid* baseline relative to which a physiological stimulus can be assessed (1). The difficulty resides in the fact that the normal adrenal blood flow per minute under these conditions is 7 to 17 ml per minute, i.e., approaching twice the adrenal blood flow of the single gland cannulated *in situ* in the abdomen. Thus the normal baseline output of 0.2 to 1.2  $\mu\text{g}$  per hour (see below) is dispersed over 300 to 800 ml of plasma (the hematocrit of these Merino sheep = 22 to 36 per cent).

Considerations relating to some possible analytical approaches to this problem are given below.

*Soda fluorescence method.* It is stated by Bush (28) and by Neher and Wettstein (29, 30) that the lower limit of detection of aldosterone with soda fluorescence on paper is 0.2  $\mu\text{g}$ , i.e., about 0.1  $\mu\text{g}$  per  $\text{cm}^2$ . Tait and Tait (31) put the over-all sensitivity of such methods at 0.5 to 2.0  $\mu\text{g}$ . By use of a fluorimeter to increase sensitivity and eliminate what Ross (32) has termed "visual guesswork" in quantitative appraisal of Na fluorescent spots, the sensitivity can be increased to 0.1  $\mu\text{g}$  (33, 34). It follows, therefore, that if blood flow is 800 ml per hour and 30 ml of adrenal venous *plasma* is extracted [twice as much as, for example, that usually used in the method of Reich (35)], aldosterone cannot be detected visually until the adrenal secretion rate is approximately  $(600 \times 0.2)/30 = 4$   $\mu\text{g}$  per hour. This calculation assumes that the recovery of aldosterone through extraction and separation from other steroids is 100 per cent and does not take into account aldosterone associated with the discarded red blood cells (36-39). Chromatography adequate to purify aldosterone may result in the loss of up to 30 to 50 per cent (29, 34, 38, 40). It seems probable, therefore, that even if plasma flow rate is 300 to 400 ml per hour aldosterone will not be detected visually until adrenal secretion rate

approaches 4  $\mu\text{g}$  per hour. These quantitative aspects of soda fluorescence methods, as discussed in detail by Tait and Tait (31), indicate that they have little application to the problem outlined above, and a limited use only when Na deficiency is well established in this type of physiological preparation.

*Double isotope derivative method (2, 40).* This method has been used successfully for adrenal venous blood of dogs where the blood flow rate is usually about one-half or less than that found with an adrenal transplant in sheep. Because the aldosterone diacetate- $\text{C}^{14}$  recovery marker is added after acetylation, the acetylation and all steps preceding it must be 100 per cent effective. Three ml of plasma was used in the dog studies, and we have used 5 ml for studies of Na-depleted sheep (6). However, if larger quantities of plasma are used, acetylation may not always be complete. The limit of detectability for accuracy with this method is about 0.01  $\mu\text{g}$ , and considering the quantitative problem set out above, aldosterone can be detected in adrenal venous plasma when the secretion rate =  $(600 \times 0.01)/5 = 1.2 \mu\text{g}$  per hour. As the loss of aldosterone during extraction and incomplete acetylation up to the point of addition of the marker may

be 20 to 40 per cent with sheep plasma, it follows that the lower limit of certain detection is 1.5 to 2.0  $\mu\text{g}$  per hour.

*Double isotope dilution derivative method (40, 41).* This is the double isotope method of choice, since the marker that permits exact assessment of handling losses of the unknown aldosterone is added to the plasma prior to extraction, and thus the volume of plasma that may be acetylated is not so restricted as with the derivative method. Either aldosterone-4- $\text{C}^{14}$  or high specific activity 7- $\text{H}^3$ -aldosterone may be used as the recovery marker, and the alternative high specific activity  $\text{H}^3$ - or  $\text{C}^{14}$ -acetic anhydride employed. The sensitivity is 0.01  $\mu\text{g}$  and if 25 ml of plasma is extracted, aldosterone can be detected in adrenal venous plasma when secretion rate =  $(600 \times 0.01)/25 = 0.25 \mu\text{g}$  per hour. Since all extraction losses with this method are accounted for, the only unaccounted loss of aldosterone is that which occurs in the course of discarding the red blood cells. A better procedure might be to add the recovery marker to the whole blood before centrifugation, and this method is being investigated.

In the experiments reported here the double isotope di-

TABLE I

*Accuracy and reproducibility obtained when the double isotope dilution derivative method was used to analyze aliquots from a standard aldosterone solution and a pool of sheep plasma to which aldosterone had been added*

Date	Material	Amt. of aldosterone in sample	Type of recovery marker (lab. batch no.)	Type of acetic anhydride (lab. batch no.)	Observed / expected $\times 100$ (no. of chromatographies)	Observed / expected on rechromatography from the phosphor
12/12/60	Aldosterone standard		Random $\text{H}^3$	I $\text{C}^{14}$ II	113 (3)	
7/ 3/61			7- $\text{H}^3$	I $\text{C}^{14}$ III	95 (3)	
11/ 3/61			4- $\text{C}^{14}$	P III $\text{H}^3$ III	102 (4)	113
23/ 3/61			4- $\text{C}^{14}$	P III $\text{H}^3$ IV	108 (3)	93
			4- $\text{C}^{14}$	I $\text{H}^3$ III	96 (3)	
			4- $\text{C}^{14}$	I $\text{H}^3$ III	95 (3)	105
29/ 3/61			4- $\text{C}^{14}$	I $\text{H}^3$ III	95 (3)	105
			4- $\text{C}^{14}$	I $\text{H}^3$ III	104 (3)	
27/ 6/61			4- $\text{C}^{14}$	III $\text{H}^3$ V	94 (3)	
	Plasma pool	0.20	7- $\text{H}^3$	I $\text{C}^{14}$ III	101 (4)	112
7/ 3/61			B	P III $\text{H}^3$ III	95 (3)	
11/ 3/61	B	0.20	4- $\text{C}^{14}$	P III $\text{H}^3$ III	112 (3)	
			4- $\text{C}^{14}$	P III $\text{H}^3$ III	108 (3)	
15/ 3/61	B	0.20	4- $\text{C}^{14}$	P III $\text{H}^3$ III	108 (3)	
20/ 3/61	B	0.20	4- $\text{C}^{14}$	P III $\text{H}^3$ III	100 (3)	93
23/ 3/61	A	0.10	4- $\text{C}^{14}$	P III $\text{H}^3$ III	92 (3)	
23/ 3/61	A	0.10	4- $\text{C}^{14}$	I $\text{H}^3$ III	113 (3)	
29/ 3/61	A	0.10	4- $\text{C}^{14}$	I $\text{H}^3$ III	97 (3)	
12/ 4/61	A	0.10	4- $\text{C}^{14}$	I $\text{H}^3$ II	118 (3)	
27/ 4/61	A	0.10	4- $\text{C}^{14}$	I $\text{H}^3$ IV	112 (3)	
3/ 5/61	A	0.10	4- $\text{C}^{14}$	I $\text{H}^3$ IV	104 (3)	
8/ 5/61	A	0.10	4- $\text{C}^{14}$	I $\text{H}^3$ IV	82 (3)	
22/ 5/61	A	0.10	4- $\text{C}^{14}$	I $\text{H}^3$ V	100 (3)	
29/ 5/61	A	0.10	4- $\text{C}^{14}$	I $\text{H}^3$ V	91 (3)	
6/ 6/61	A	0.10	4- $\text{C}^{14}$	I $\text{H}^3$ V	112 (3)	
20/ 6/61	A	0.10	4- $\text{C}^{14}$	I $\text{H}^3$ V	123 (3)	
	II	0.10	4- $\text{C}^{14}$	I $\text{H}^3$ V	107 (3)	
	II	0.10	4- $\text{C}^{14}$	III $\text{H}^3$ V	139 (3)	
22/ 6/61	I	0.025	4- $\text{C}^{14}$	I $\text{H}^3$ V	108 (3)	
	I	0.050	4- $\text{C}^{14}$	I $\text{H}^3$ V	116 (3)	
	II	0.100	4- $\text{C}^{14}$	I $\text{H}^3$ V	104 (3)	
27/ 6/61	I	0.025	4- $\text{C}^{14}$	I $\text{H}^3$ V	86 (3)	
	II	0.100	4- $\text{C}^{14}$	I $\text{H}^3$ V	124 (3)	
	I	0.05	4- $\text{C}^{14}$	III $\text{H}^3$ V		

TABLE II  
Specificity of the method \*

No.	H <sup>3</sup> /C <sup>14</sup> ratio at high voltage	
	Third chromatography	Fourth chromatography
83	17.8	18.8
82	3.46	3.70
89	1.51	1.60
4	1.40	1.47
5	1.07	0.95
8	4.05	3.24
35	15.5	14.4
23	1.96	1.94
77	5.26	5.34
48	6.41	5.74

\* H<sup>3</sup>C<sup>14</sup> ratios at the high voltage are shown from 10 randomly chosen samples from batches in Table I(A). The fourth chromatography was carried out after recovery from the phosphor following the routine procedure.

lution derivative assay was used exclusively for aldosterone and corticosterone.<sup>1</sup> In most experiments this method was used for cortisol (designated on the ordinates of the figures by D.I.). In others the 17-hydroxycorticosteroids were estimated by a modified Porter-Silber method (42), and this measurement has been referred to in the text as cortisol. Analysis of the same 21 specimens of adrenal venous blood for Porter-Silber chromogens and for cortisol by the double isotope dilution method showed the mean cortisol value to be 80 per cent of the Porter-Silber determination. No analyses have been made to determine the components of the additional chromogenic material. Adrenal venous blood, 25 to 45 ml, was collected over 2 to 5 minutes into an ice-cooled cylinder with heparin, and centrifugation was begun within 10 minutes. The plasma was stored at -15° C until analyzed. Aliquots of plasma, 10 to 30 ml, with the relevant markers were extracted. These were then handled essentially as described (40). The specificity of the method was evaluated by determining the constancy of the H<sup>3</sup>/C<sup>14</sup> ratio after the third and fourth chromatography. To effect the requisite specificity with sheep plasma, the oxidation step described by Kliman and Peterson (40) was retained. For canine adrenal vein plasma this has not always been necessary (40). In addition, an aliquot from a plasma pool to which a known amount of authentic aldosterone had been added was included with each batch of adrenal venous plasma analyses in this series of experiments. On occasion, a standard aldosterone sample was also included. These results, together with H<sup>3</sup>/C<sup>14</sup> ratios from a series of unknowns chosen at random, are presented in Tables I and II. A full discussion of the isotope dilution

<sup>1</sup> The isotope-labeled steroid hormones were generously provided by the Endocrine Study Section of the National Institutes of Health, Washington, and the Medical Research Council of England. We are greatly indebted to Dr. Bryan Hudson and the members of the Metabolic Unit, Alfred Hospital, for their collaboration in the preparation of aldosterone-4-C<sup>14</sup>.

method as applied to sheep plasma will be presented elsewhere by one of us (J.C.).

Additional evidence of specificity and reproducibility comes from the fact that some of the recoveries were done when 7-H<sup>3</sup>- and random H<sup>3</sup>-labeled aldosterone were used as marker and acetylation was with C<sup>14</sup>-acetic anhydride; others were done with C<sup>14</sup>-aldosterone marker and acetylation with different batches of tritium-labeled acetic anhydride.

#### Biological assay of aldosterone

During the experiments the continuous secretion of parotid saliva from the unilateral fistula was collected, and the concentration of Na and K measured with a Beckman DU spectrophotometer: normal, Na = 170, K = 5 mEq/L, Na/K = about 34. (Na deficiency of 500 mEq, e.g., causes reciprocal changes of Na and K concentration so that Na/K falls to 80/90 = 0.9.) The Na/K ratio of the saliva changes with the aldosterone concentration in peripheral blood, which follows changes of adrenal secretion rate closely (6, 43) because of the short half-life of *d*-aldosterone, e.g., 15 to 35 minutes in man (44). Thus the fistulated sheep has a built-in bioassay system which indicates the level of adrenal aldosterone secretion. Figure 1 shows a dose-response curve where *d*-aldosterone was infused intravenously into transplant (TP) 12 during Na repletion. There was a characteristic time delay of 60 to 90 minutes before the parotid salivary response, and a slightly longer time delay (80 to 120 minutes) before the salivary Na/K ratio rose after the infusion was stopped. Infusions of aldosterone directly into the arterial supply of the parotid gland have shown the same order of time delay before biological effect, indicating that time delay is largely intrinsic to the parotid secretory mechanism. The threshold of response was be-

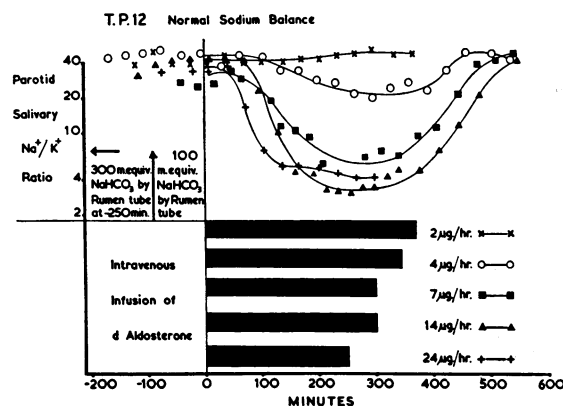


FIG. 1. ADRENAL TRANSPLANT 12 (TP 12), NA-REPLETE. The effect on the parotid salivary Na/K ratio of intravenous infusion of *d*-aldosterone at rates equivalent to 2, 4, 7, 14, and 24 µg per hour of the active *d* isomer. In this and all other experiments NaHCO<sub>3</sub> was given beforehand to eliminate the possibility that Na deficiency might supervene during experiments lasting 6 to 10 hours.

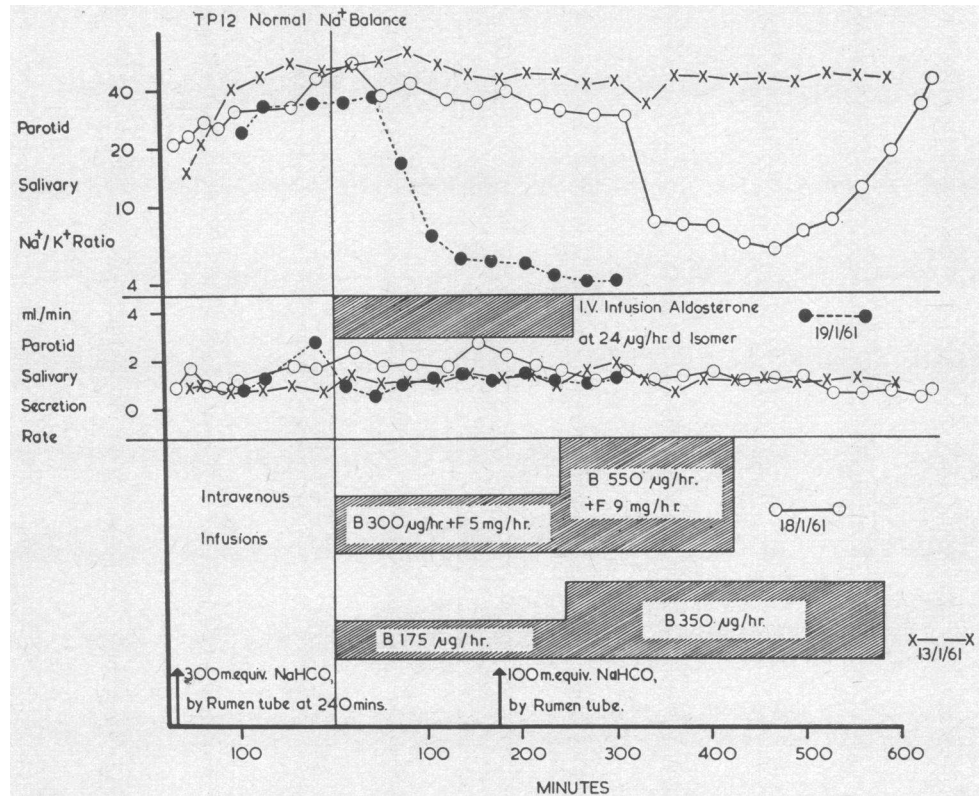


FIG. 2. TP 12, NA-REPLETE. The effect on parotid salivary Na/K ratio of intravenous infusion of: 1) corticosterone at 175 and 350  $\mu\text{g}$  per hour ( $\times$ — $\times$ ); 2) corticosterone/cortisol at 300/5,000  $\mu\text{g}$  per hour and at 550/9,000  $\mu\text{g}$  per hour ( $\circ$ — $\circ$ ); 3) aldosterone at 24  $\mu\text{g}$  per hour of *d* isomer ( $\bullet$ — $\bullet$ ). The parotid salivary secretion rate is shown also.

tween 2.0 and 4.0  $\mu\text{g}$  per hour, and the maximal effect was reached with 10 to 18  $\mu\text{g}$  per hr. Figure 2 shows the effect of infusion into the same Na-replete sheep of the two principal glucocorticoids secreted by the sheep's adrenal. In contrast to the dog, the ratio of cortisol/corticosterone in sheep adrenal effluent is equal to or greater than that in man (45, 46). Intravenous infusion of corticosterone at 350  $\mu\text{g}$  per hour had no effect on salivary Na/K, nor did an infusion of corticosterone (300  $\mu\text{g}$ ) and cortisol (5 mg per hour). These figures are above the maximal adrenal output of these steroids recorded with ACTH infusion in this animal (see Figure 4) and exceed the maximum recorded from the two adrenals of the anesthetized, traumatized sheep. Thus these steroids do not significantly influence the salivary Na/K under physiological conditions. With double this rate of infusion (a pharmacological dose), there was an effect, but it was still less than the maximal effect produced by physiological rate of infusion of *d*-aldosterone (Figure 2). Thus, in the experiments recorded below, the salivary Na/K has been used to reflect a state of normal Na balance at the beginning of the experiment, and the occurrence of the specific stimulation of adrenal aldosterone secretion, provided the procedure has been continued long enough for adequate buildup of peripheral blood concen-

tration, i.e., a single intravenous injection of 5  $\mu\text{g}$  of *d*-aldosterone over 5 minutes is without effect on salivary Na/K and 15  $\mu\text{g}$  has a small evanescent effect, whereas constant infusion of 4  $\mu\text{g}$  per hour for 3 hours causes a sustained fall (Figure 1). In some experiments here, adrenal arterial infusion, which caused aldosterone output at a rate of 6 to 8  $\mu\text{g}$  per hour, was given for 20 to 30 minutes only, which was inadequate time to affect salivary Na/K ratio.

#### Substances tested by adrenal arterial infusion<sup>2</sup>

Val-5-angiotensin I, val-5-angiotensin II amide (Ciba). ACTH (Armour, 40 IU per mg).  $\alpha$ -ACTH, Dr. C. H. Li, 100 IU per mg.  $\delta_1$ -ACTH, Dr. Bell (Lederle Laboratories, 27.6 IU per mg).  $\beta$ -MSH, Dr. Jerker Porath, Uppsala. Vasopressin (Sandoz, lysine-8-vasopressin, 10 U per ml). Oxytocin (Sandoz synthetic oxytocin, 4518, vasopressor activity 5 IU per mg, oxytocin activity 450 IU per mg). Serotonin (Mann Re-

<sup>2</sup> We wish to thank Dr. C. H. Li, Dr. P. Bell (Lederle) and Dr. Jerker Porath for ACTH and MSH; Dr. Bircher (Sandoz) for vasopressin, oxytocin, and bradykinin; and Dr. F. Gross and the Ciba Co. for the continued supply of aldosterone and angiotensin.

search Laboratories, lot A5325, serotonin creatinine sulfate H<sub>2</sub>O; dosage was calculated with respect to serotonin). Bradykinin (Sandoz synthetic bradykinin, 10 µg per ml). Noradrenaline (Levophed, Winthrop Laboratories, 1 mg per ml). Acetylcholine chloride (Roche). These materials were diluted in sterile physiological saline.

dosterone (30 specimens)  $0.54 \pm 0.26$  (SD) µg per hr =  $1.4$  µg per 100 kg body weight per hour per transplanted gland; corticosterone (25 specimens)  $9.3 \pm 6.2$  (SD) µg per hour; cortisol (15 specimens)  $138 \pm 139$  (SD) µg per hour; Porter-Silber chromogens (15 specimens)  $271 \pm 210$  (SD) µg per hour.

RESULTS

*The normal rate of secretion of corticosteroids in the conscious, undisturbed, Na-replete sheep*

The findings in 30 specimens of adrenal venous blood collected under these conditions were: al-

The mean output, when one adrenal gland was cannulated in the abdomen of 6 Na-replete, anesthetized, traumatized sheep, was: aldosterone 1.6 µg per hour, corticosterone 52 µg per hour, cortisol 1.02 mg per hour. Presumably, total output

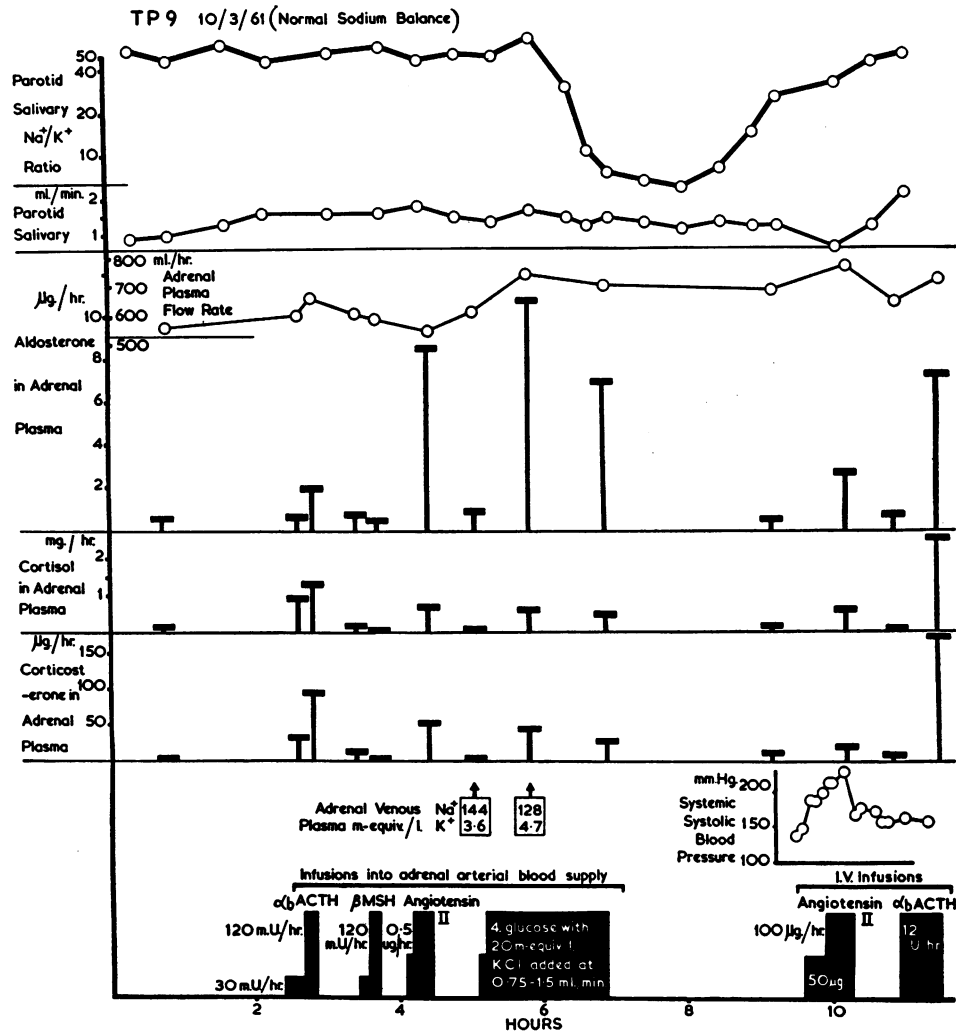


FIG. 3. TP 9, NA-REPLETE. The effect on salivary Na/K ratio, rate of parotid saliva secretion, Na and K concentration, rate of adrenal plasma flow, and adrenal secretion rate of aldosterone, corticosterone, and Porter-Silber chromogens of: 1) adrenal arterial infusion of α<sub>b</sub>-ACTH, β-MSH, angiotensin II, 4 per cent glucose with 20 mEq per L KCl added at 1.5 ml per minute; and 2) intravenous infusion of angiotensin II and α<sub>b</sub>-ACTH. The systemic blood pressure during intravenous infusion of angiotensin II is recorded also.

was double these figures. The mean blood flow was 7.8 ml per minute.

#### *Adrenal blood flow*

In the Na-replete sheep, the flow varied from 7 to 17 ml per minute. The hematocrit varied from 23 to 38 per cent so that the plasma flow ranged from 250 to 750 ml per hour. The measured blood flow rate in the individual animal on the day of the experiment was the basis of calculation of ratio of intra-adrenal arterial infusion so that a physiological or appropriate pharmacological change was contrived. For example, in Addison's disease the plasma ACTH concentration is  $8.4 \pm 7.4$  mU per 100 ml (47, 48). Given here an adrenal plasma flow of 600 ml per hour, ACTH was infused into the adrenal arterial supply at 30 to 60 mU per hour in order to test the direct effect of a local plasma concentration of the order recorded in Addison's disease. With val-5-angiotensin II the intravenous infusion of 50 to 100  $\mu\text{g}$  per hour caused systemic systolic blood pressure to rise from 130 to 180 to 220 mm Hg. After approximating that the resting cardiac output of the 30- to 40-kg sheep was 1.5 to 2.0 L per minute and adrenal blood flow was 10 to 15 ml per minute, the material was infused into the common carotid artery supplying the adrenal gland at 0.25 to 0.5  $\mu\text{g}$  per hour, the aim being to produce locally approximately the same concentration as that produced by the intravenous infusion. The plasma flow rates are recorded in individual experiments, and the effects of various infusions on blood flow will be referred to below.

#### *Alteration in the ionic composition of adrenal arterial blood*

It was of great importance to know whether a fluid to be infused would affect the Na and K concentrations of adrenal arterial blood. The concurrent decrease of Na and increase of K in adrenal arterial blood is a potent specific stimulus of aldosterone secretion which may increase 10- to 30-fold without significant change of cortisol or corticosterone secretion, although this latter is not an invariable finding. Figures 3 and 4, in which this stimulus has been used for quantitative comparison with those induced by pharmacological changes in adrenal arterial blood, show rapid rise of aldos-

terone secretion to 10.8 and 13.5  $\mu\text{g}$  per hour and, since the stimulus was continued for 60 to 110 minutes, concurrently there were changes of salivary Na/K ratio. This direct stimulation by ionic changes has been reported and discussed previously (1, 4), and attention has been drawn to the fact that concurrent change of the two ions is a more effective stimulus than is lowering Na or raising K alone.

Table III shows some further results of analysis of adrenal venous blood for corticosteroids and ionic composition before and at 20 to 30 minutes after beginning different infusions into the adrenal arterial blood supply at the rates designated. The data shown indicate the greater effect with concurrent decrease of Na and increase of K than with reduction of both Na and K or elevation of K concentration alone. Considered along with Figures 3 and 4, aldosterone hypersecretion induced was related to the extent of ionic change in the particular animal, and the effect is reproducible. No consistent effect on glucocorticoids occurred. A full report of the investigation of this mode of stimulation of aldosterone secretion will be published elsewhere.

#### *The effect of hormones and of some vascularly active substances on corticosteroid secretion of Na-replete sheep*

*Angiotensin.* Val-5-angiotensin II: The octapeptide was infused into the adrenal arterial blood supply at 0.045 to 1.0  $\mu\text{g}$  per hour. With TP 9 (Figure 3) 0.5  $\mu\text{g}$  per hour raised aldosterone secretion from 0.7 to 8.7, cortisol from 88 to 710, and corticosterone from 5 to 70  $\mu\text{g}$  per hour. With TP 12 (Figure 4) 0.5  $\mu\text{g}$  per hour caused a rise of aldosterone secretion from 0.4 to 10.3  $\mu\text{g}$  per hour without change in cortisol and corticosterone. However, in another experiment with TP 12, 0.25 and 1.0  $\mu\text{g}$  per hr of angiotensin II had no effect on aldosterone or glucocorticoid secretion. With Osiris, 0.25  $\mu\text{g}$  per hour increased aldosterone from 0.6 to 3.6  $\mu\text{g}$  per hour with a small decrease of cortisol and corticosterone secretion (see Methods). With lower rates of infusion, in TP 9 it was observed that 0.05  $\mu\text{g}$  per hour had little effect if any on corticosteroid secretion, but 0.10  $\mu\text{g}$  per hour raised aldosterone from 0.2 to 1.2  $\mu\text{g}$  per hour. Cortisol rose from 98 to 405, and corticos-



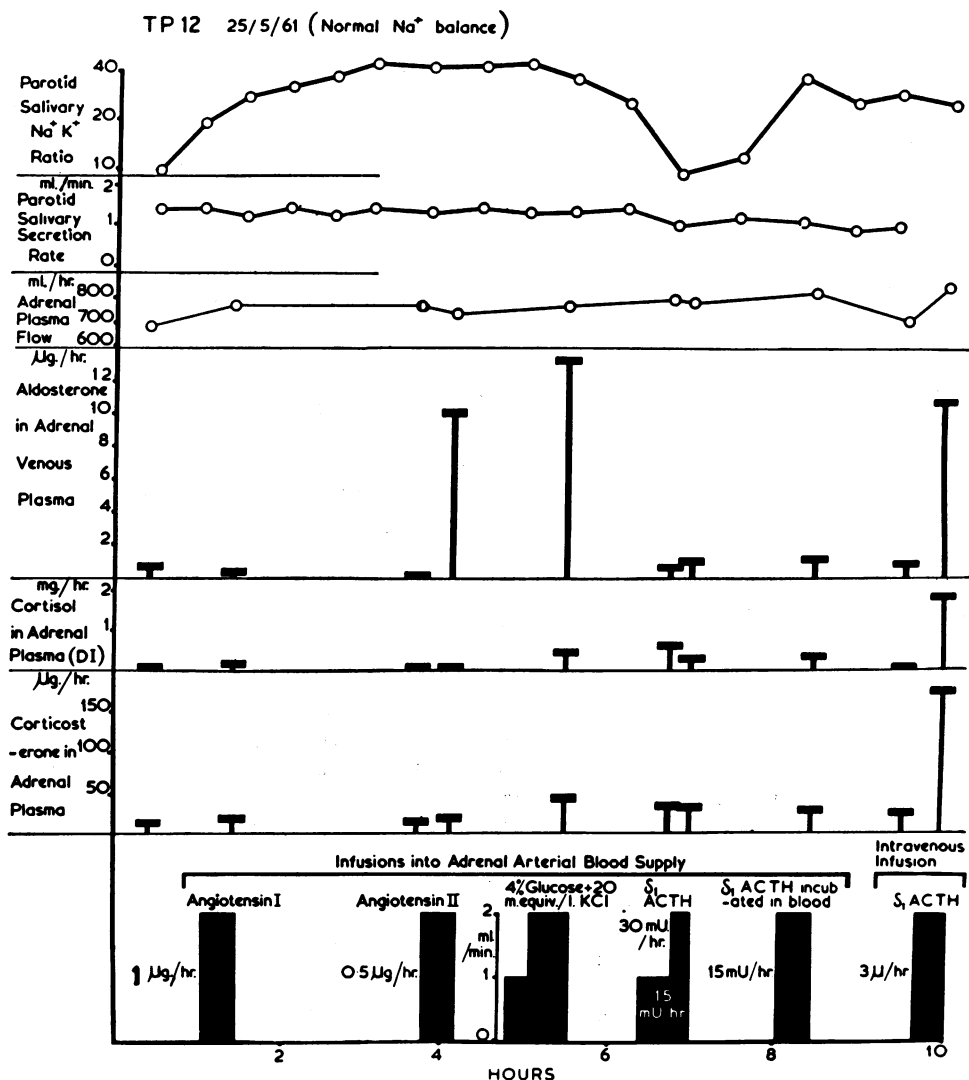


FIG. 4. TP 12, NA-REPLETE. The effect on the same physiological parameters as in Figure 3 of: 1) adrenal arterial infusion of angiotensin I, angiotensin II, 4 per cent glucose with 20 mEq per L KCl added,  $\delta_1$ -ACTH, and  $\delta_1$ -ACTH after incubation in the animal's own blood for 15 minutes at 37° C; and 2) intravenous infusion of  $\delta_1$ -ACTH. Cortisol was measured by the double isotope method. The ordinate scale is marked DI when this method was used.

terone from 4.3 to 15 µg per hour. With Noah (Figure 5) angiotensin at 0.11 to 0.22 µg per hour raised aldosterone stepwise from the control level of 0.5 to 1.1 and 2.1 µg per hour without effect on cortisol or corticosterone. With TP 12, 0.045 and 0.09 µg per hour did not affect aldosterone secretion. Figure 6 summarizes the data on the relationship between rate of aldosterone secretion and the rate of infusion and the concentration of val-5-angiotensin II calculated to have been produced in the adrenal arterial plasma. This calculation is

based on accurate knowledge of the rate of arterial infusion and the rate of adrenal blood flow but involves the assumption that the infused angiotensin II distributed only over plasma. If the octapeptide distributes over whole blood as, for example, appears to be the case with aldosterone in adrenal venous blood of sheep, the concentration would be 20 to 40 per cent less than calculated. The threshold of response was approximately 0.2 µg per L of plasma, if it is assumed that angiotensin was distributed only in the plasma. Figure

7 shows that angiotensin II infusion had no consistent effect on cortisol and corticosterone secretion. Adrenal arterial infusion of angiotensin caused no consistent change of blood flow.

Intravenous infusion of the octapeptide at rates of 50 to 100  $\mu\text{g}$  per hour raised systemic systolic blood pressure from 120 to 130 to 180 to 220 mm Hg. The effect was commensurate with dose; it began within 1 to 2 minutes of commencing the

infusion and declined precipitously 1 to 2 minutes after stopping the infusion, indicating a very short biological half-life in blood. Figure 3 (TP 9) permits comparison with intra-adrenal arterial infusion at 0.005 the rate and shows that angiotensin II was much less potent upon intravenous infusion. Here with 100  $\mu\text{g}$  per hour of angiotensin II, aldosterone rose only to 2.9  $\mu\text{g}$  per hour. Corticosterone doubled, and cortisol secretion rate

TABLE III  
*Effect on corticosteroid secretion, adrenal blood flow, and adrenal venous plasma composition of infusion of the designated solutions into the adrenal arterial blood supply*

Sheep Na <sup>+</sup> -replete	Adrenal arterial infusion	Adrenal blood flow	Packed cell vol.	Adrenal venous plasma		Corticosteroid secretion in adrenal venous plasma		
				Na <sup>+</sup>	K <sup>+</sup>	Aldos- terone	Cortisol	Corticos- terone
		<i>ml/min</i>	<i>%</i>	<i>mEq/L</i>		<i><math>\mu\text{g/hr}</math></i>	<i><math>\mu\text{g/hr}</math></i>	<i><math>\mu\text{g/hr}</math></i>
TP 12	Control	12	22	152	3.3	0.7	734	44
24/10/61	5% glucose at 0.9 ml/min	17.9	19	140	2.7	0.8	425	28
TP 12	Control	18.2	21	150	3.2	1.40	66	13
24/10/61	145 mEq/L NaCl + 15 mEq/L KCl at 0.9 ml/min	19	20	152	4.6	1.50	201	15
TP 12	Control	13	21	152	3.4	0.8	143	15
24/10/61	2% glucose + 75 mEq/L NaCl + 15 mEq/L KCl at 0.9 ml/min	13	21	146	4.7	2.0	176	13
TP 12	Control	12.1	24	145	3.1	1.7	23	12.5
28/ 7/61	1.5% glucose + 104 mEq/L NaCl + 10 mEq/L KCl at 1.8 ml/min	12.5	19	140	4.3	3.4	34	11.2
TP 12	Control	15.4	23	146	3.3	1.9	42	5
6/ 7/60	1.5% glucose + 106 mEq/L NaCl + 10 mEq/L KCl at 1.8 ml/min	15.0	22	140	4.7	5.9	12	10
TP 9	Control	17.8	32	154	3.7	1.4	66	11
29/ 8/61	4% glucose + 20 mEq/L KCl at 1.5 ml/min	17.8	29	137	5.3	14.9	248	68
TP 9	Control	13.7	36	143	4.2	0.6	580	8
16/11/60	4% glucose with 15 mEq/L KCl at 1.8 ml/min	13.5	33	127	5.3	11.3	240	4
Noah	Control	9.2	32	148	3.9	1.3	141	14
21/ 6/61	2% glucose + 75 mEq/L NaCl + 15 mEq/L KCl at 1.5 ml/min	7.5	23	136	6.8	5.3	97	16
TP 12	Control	11.6	17	151	3.0	0.7	92	6
5/12/60	4% glucose with 20 mEq/L KCl at 1.5 ml/min	17.3	14	128	5.0	7.8	189	20
Cyrano	Control	8.3	21	147	3.2	0.3	90	3
18/ 1/62	4% glucose with 14 mEq/L KCl at 1.0 ml/min	8.2	21	120	4.8	3.2	3	2
TP 9*	Control	15.1	27	149	3.6	0.8	109	11
31/ 1/62	5% glucose at 1 ml/min	18.1	26	136	3.5	2.6	38	60
	Control	16.3	27	145	3.5	0.8	109	13
	155 mEq/L NaCl + 11 mEq/L KCl at 1 ml/min	16.9	28	145	4.2	6.5	397	42
	4.5% glucose + 8.5 mEq/L KCl at 0.5 ml/min	17.1	28	141	3.7	5.7	14	11
	4.5% glucose + 8.5 mEq/L KCl at 1.0 ml/min	17.4	26	130	4.0	8.8	188	30
Osiris	Control	5.6	29	151	3.5	0.2	19	2
1/ 2/62 (see Methods)	5% glucose at 0.7 ml/min	7.1	25	136	3.1	0.1	23	2
	Control	7.8	27	148	3.5	0.1	86	3
	150 mEq/L NaCl + 10 mEq/L KCl at 0.7 ml/min	10.4	25	150	4.2	4.0	157	11
	Control	8.0	26	145	3.6	0.2	170	7
	4.5% glucose + 10 mEq/L KCl at 0.7 ml/min	9.9	24	138	4.1	5.3	151	10
	As above at 1.5 ml/min	11.0	21	129	4.3	5.6	156	13
	Control	9.3	26	154	3.9	0.4	255	10

\* In this experiment on TP 9, all corticosteroid determinations were made on whole blood by the double isotope dilution derivative method. The marker isotope was added to the blood immediately after collection.

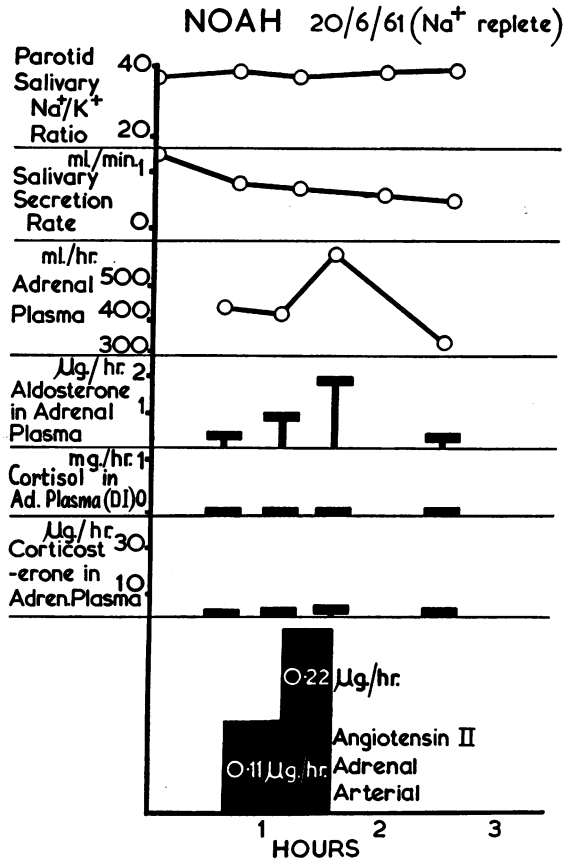


FIG. 5. NOAH, NA-REPLETE. The effect on the same physiological parameters as in Figure 3 of adrenal arterial infusion of angiotensin II.

trebled. With Osiris, the effects were comparable when the ratio of rates of intra-adrenal to intravenous infusion was 1/400, aldosterone secretion rate rising to 3.6 and 3.9  $\mu\text{g}$  per hour respectively. With TP 12 the effect of intravenous infusion (50  $\mu\text{g}$  per hour), which increased systolic blood pressure to 220 mm Hg, was compared with a prolonged intra-adrenal arterial infusion at 0.46  $\mu\text{g}$  per hour. In both instances the salivary Na/K ratio fell a small amount, indicating stimulation of aldosterone output to approximately 4  $\mu\text{g}$  per hour.

Val-5-angiotensin I: The adrenal arterial infusion of the decapeptide angiotensin I at 0.5  $\mu\text{g}$  per hour into TP 9 caused a much smaller rise of aldosterone secretion (from 0.4 to 1.7  $\mu\text{g}$  per hour) than with the same rate of infusion of angiotensin II (Figure 3), although the rate of adrenal blood flow with angiotensin I infusion was 40 per cent

less. With TP 12 (Figure 4) infusion at 1.0  $\mu\text{g}$  per hour had no effect, and in Noah 1.0  $\mu\text{g}$  per hour raised aldosterone from 0.7 to 2.9  $\mu\text{g}$  per hour without effect on cortisol and corticosterone which were secreted at 94 and 6  $\mu\text{g}$  per hour, respectively.

Angiotensin II. Prolonged infusion studies using the double isotope dilution method have shown that, with the onset of Na deficiency as a result of salivary loss, there is a progressive increase of aldosterone secretion. With TP 12, for example, aldosterone secretion at equilibrium was 0.8  $\mu\text{g}$  per hour; 4 hours later it was 2.9; at 7 and 13 hours it was 4.6 to 4.9; and at 26 hours, 7  $\mu\text{g}$  per hour. No consistent change in cortisol or corticosterone secretion occurred. The parotid salivary Na/K ratio commenced to fall at 4 hours

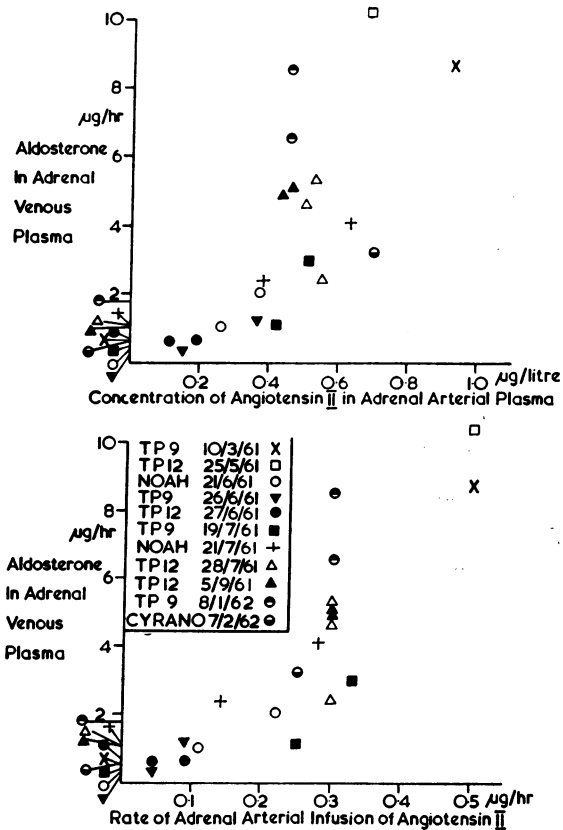


FIG. 6. NA-REPLETE SHEEP. The relation between adrenal aldosterone secretion and (top) the concentration of angiotensin II calculated to have been produced in adrenal arterial plasma by the adrenal infusion and (bottom) the rate of adrenal arterial infusion of angiotensin II. The secretion rate for the control period of each experiment is shown at zero on the abscissa.

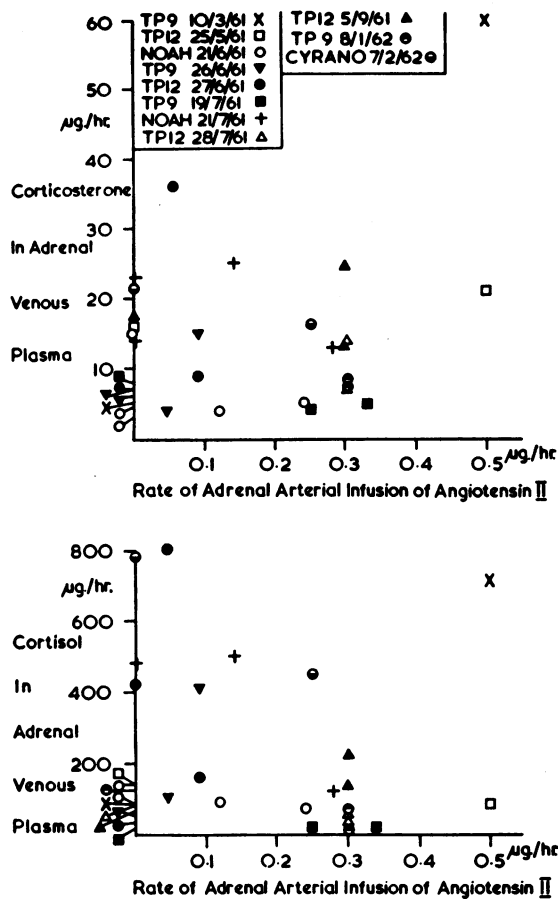


FIG. 7. NA-REPLETE SHEEP. The relation between rate of adrenal arterial infusion of angiotensin II and the adrenal secretion rate of corticosterone (top) and of cortisol (bottom). The secretion rate of the control period in each experiment is shown at zero on the abscissa.

when adrenal secretion rate reached salivary threshold, and at 26 hours was 3. The urinary Na excretion was reduced to a rate of approximately 1 mEq per day from 6 hours onward.

Five experiments were made in which a prolonged infusion of val-5-angiotensin II was given. With TP 12, angiotensin II was infused into the adrenal arterial circulation for 320 minutes at 0.32 µg per hour. After 40 minutes of infusion, aldosterone secretion rate had risen to 5 µg per hour and was 5 µg per hour at 120 minutes. By 250 to 310 minutes the rate had declined to approximately 2 µg per hour. Both cortisol and corticosterone secretion were slightly raised during the infusion. A small decrease of salivary Na/K ratio occurred. Aldosterone secretion was still increased 50 minutes after the infusion ceased. With TP 9, angio-

tensin II was infused into the adrenal arterial supply at 0.32 µg per hour for 375 minutes. Aldosterone secretion rate had increased from 1.8 to 8.5 µg per hour by 45 minutes (Figure 8), but subsequently declined and ranged from 1.3 to 3.0 µg per hour. After the infusion the rate was 0.5 µg per hour. A small decrease of salivary Na/K occurred during the infusion. There was no sustained change of cortisol or corticosterone secretion rate during the infusion. The secretion rates of these compounds were lowest when aldosterone rate was highest. The adrenal blood flow decreased 10 to 20 per cent during the infusion. The hatched blocks in the aldosterone section represent concurrent determinations made on whole blood by the double isotope dilution derivative method. The results are 25 to 35 per cent higher than with plasma analysis. The arrows on Figure 8 represent points at which the angiotensin solution was freshly prepared from a new ampule to circumvent the possibility that the decrease of aldosterone secretion was attributable to adsorption of the angiotensin on the glass. With Noah (Fig-

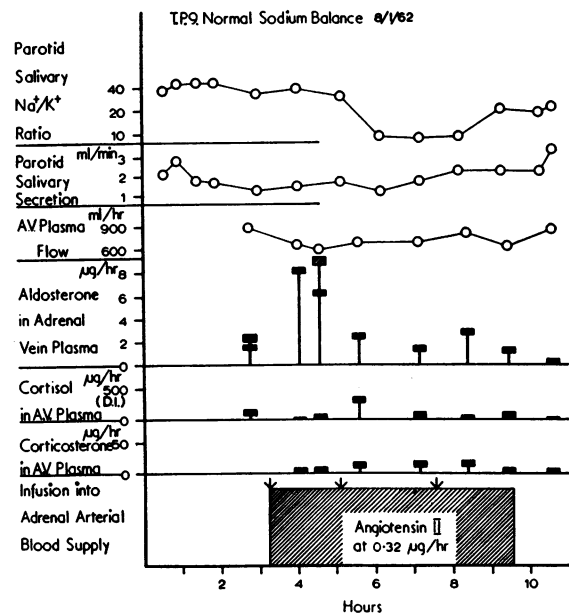


FIG. 8. TP 9, NA-REPLETE. The effect on the same physiological parameters as in Figure 3 of prolonged adrenal arterial infusion of angiotensin II at 0.32 µg per hour. The hatched blocks represent aldosterone assays made on adrenal venous whole blood by the double isotope dilution derivative method. The arrows represent times at which the angiotensin solution for adrenal arterial infusion was freshly prepared from new ampules.

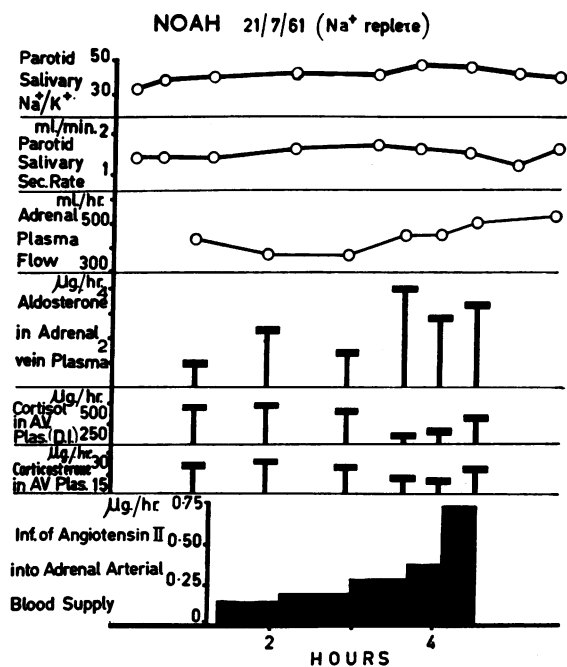


FIG. 9. NOAH, NA-REPLETE. The effect on the same physiological parameters as in Figure 3 of adrenal arterial infusion of angiotensin II at 0.14 to 0.73  $\mu\text{g}$  per hour.

ure 9) the rate of adrenal arterial infusion of angiotensin II was increased stepwise over 190 minutes. After 34 minutes at 0.14  $\mu\text{g}$  per hour aldosterone secretion was 2.4  $\mu\text{g}$  per hour, but at 93 minutes it was 1.5  $\mu\text{g}$  per hour, although the angiotensin II infusion rate was 0.19  $\mu\text{g}$  per hour. Further substantial increase of the rate of angiotensin II infusion increased aldosterone, but the effect was not sustained. At 135 minutes, with an angiotensin II infusion rate of 0.28  $\mu\text{g}$  per hour, aldosterone output was 4.1  $\mu\text{g}$  per hour; whereas at 190 minutes, with infusion at 0.7  $\mu\text{g}$  per hour, aldosterone secretion was less—3.4  $\mu\text{g}$  per hour. With Wyatt, a normal sheep, angiotensin II was infused intravenously at 100 to 400  $\mu\text{g}$  per hour for 24 hours (Figure 10). To ensure that Na deficiency did not develop during the course of the long experiment, 150 mEq of  $\text{NaHCO}_3$  was given orally after 8 and 20 hours of infusion. Intravenous angiotensin II at 100  $\mu\text{g}$  per hour raised systemic systolic blood pressure from 120 to 150 mm Hg, and the sharp drop of salivary secretion rate probably reflected the vasoconstriction. After 200 minutes the infusion rate was increased to 200

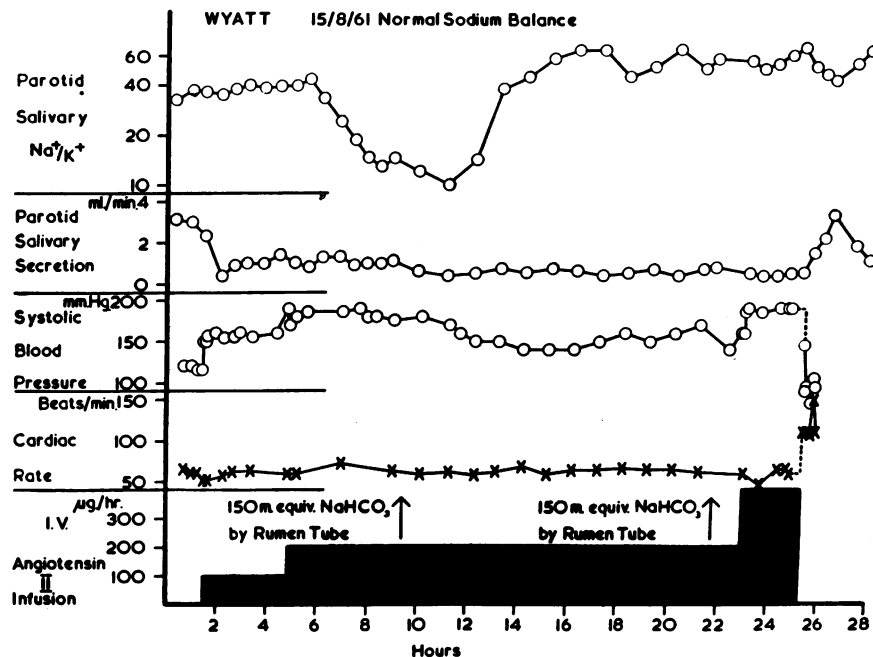


FIG. 10. WYATT, NA-REPLETE. The effect on parotid salivary Na/K ratio, saliva secretion rate, systemic systolic blood pressure, and cardiac rate, of intravenous infusion of angiotensin II at 100 to 400  $\mu\text{g}$  per hour for 24 hours.

$\mu\text{g}$  per hour, and systolic blood pressure rose to 180 mm Hg. Approximately 60 minutes later the salivary Na/K ratio fell and reached 10, but rose again to normal 6 to 7 hours later. The blood pressure decreased slowly also, and between hours 10 and 20 of infusion was approximately 150 mm Hg. During the last 150 minutes of the experiment the infusion rate was doubled again, and systolic blood pressure rose to 200 mm Hg without change in any other variable measured. Upon ceasing angiotensin II infusion, blood pressure dropped within 2 to 3 minutes to 100 mm Hg, and parotid secretion rate and cardiac rate rose rapidly. With Sexton, a Na-replete sheep, angiotensin II was infused intravenously at 50  $\mu\text{g}$  per hour for 24 hours. The mean systolic blood pressure during the first 6 hours of infusion was 185, and during the next 18 hours was 165 mm Hg. There was a small decrease of salivary Na/K ratio 150

minutes after beginning the infusion, but the ratio returned to normal 6 hours later and remained normal during the remainder of the experiment. The extent of fall indicated an aldosterone secretion rate of 4 to 5  $\mu\text{g}$  per hour. In both long-term infusions a new solution of angiotensin was prepared every 3 to 4 hours. Thus intravenous infusion of a large dose of angiotensin II for a day did not cause a sustained adrenal secretion of aldosterone above the threshold of parotid response (about 5  $\mu\text{g}$  per hour in a Na-replete animal), although a large pressor effect was maintained. Adrenal secretion rate was raised above this level during 5 to 6 hours of the experiment.

*ACTH.* Fourteen experiments with adrenal arterial infusion were done. The results with various rates of infusion of  $\alpha\text{-ACTH}$  (Li),  $\delta\text{-ACTH}$  (Bell), and Armour ACTH are given in Table IV. The rates of adrenal blood flow and

TABLE IV  
*Effect on corticosteroid secretion and adrenal blood flow of adrenal arterial infusion of corticotrophins*

Sheep	Adrenal arterial infusion	Infusion rate	Calculated adrenal plasma ACTH conc.		Adrenal blood flow	Packed cell volume	Corticosteroid secretion rate		
			<i>mU/hr</i>	<i>mU/100 ml</i>			Aldosterone	Cortisol	Corticosterone
							$\mu\text{g/hr}$	$\mu\text{g/hr}$	$\mu\text{g/hr}$
TP 12 27/6/61	Control				11.4	23	0.7	30	7
	$\alpha\text{-ACTH}$	4.4	1.0	9.5	24	0.5	127	12	
	$\alpha\text{-ACTH}$	17.6	4.4	17.8	23	0.8	298	20	
TP 9 10/3/61	Control				14.0	34	0.6	174	6
	$\alpha\text{-ACTH}$	30.0	4.5	14.7	32	0.9	994	31	
	$\alpha\text{-ACTH}$	120.0	19.0	16.0	32	2.2	1,380	96	
	40 min postinfusion			14.8	32	0.9	158	13	
TP 9 26/6/61	Control				11.0	28	0.3	43	6
	$\delta\text{-ACTH}$	13.5	3.2	11.5	28	0.7	919	46	
TP 9 18/5/61	Control				8.3	33	0.4	35	4
	$\delta\text{-ACTH}$	30.0	11.0	7.6	31	4.4	1,370	111	
	$\delta\text{-ACTH}$	60.0	8.5	17.0	30	7.7	1,720	114	
	50 min postinfusion			16.4	33	1.2	1,069	47	
	100 min postinfusion			14.3	35	0.5	168	6	
TP 12 25/5/61	Control				16.9	25	0.4	136	16
	$\delta\text{-ACTH}$	15.0	2.0	17.4	24	0.8	632	36	
	$\delta\text{-ACTH}$	30.0	3.7	16.3	24	1.2	374	35	
	Control			15.8	25	1.1	28	28	
Noah 23/5/61	Control				15.0	23	1.0	230	8
	$\delta\text{-ACTH}$	30.0	4.4	14.8	23	0.7	450	18	
Osiris 21/3/61 (see Methods)	Control				6.1	31	0.7	730	22
	Armour ACTH	55.0	14.5	7.2	28	1.7	670	31	
	Armour ACTH	220.0	67.0	7.1	24	1.9	560	28	
TP 9 26/6/61 (water depleted)	Control				6.9	28	0.5	480	19
	Control				8.1	31	0.6	920	33
	$\delta\text{-ACTH}$	1.5	0.45	8.2	30	1.0	1,050	47	
	$\delta\text{-ACTH}$	6.0	1.9	7.3	29	1.4	1,400	64	
	120 min postinfusion			6.8	29	0.2	220	7	

corticosteroid secretion under control conditions and during infusion are shown. The table shows also the calculated concentration of ACTH produced in the adrenal arterial plasma. It should be emphasized that, although infusion rate and adrenal blood flow were accurately known, the figures derived involve the assumption that the infused ACTH was distributed only in plasma. If the material was distributed evenly over whole blood, the plasma concentration accordingly was about 20 to 35 per cent lower. Preferential adsorption on cells would involve a greater error, and the possibility of plasma binding must be considered; this may be the case also with angiotensin. The results indicate that *at lower rates of infusion, ACTH affects cortisol and corticosterone only*. At higher rates, which may result in ACTH concentrations in plasma of the order reported in Addison's disease (47), significant increases of aldosterone secretion also occurred. Aldosterone secretion was changed very little until cortisol secretion exceeded 1,000  $\mu\text{g}$  per hour. There was a commensurate relation between the infusion rate of ACTH and the increase of cortisol and corticosterone observed. The two observations at the bottom of the table represent abnormal circumstances. With Osiris (see Methods), the limited glucocorticoid response to very high doses of ACTH is shown. In the experiment on TP 9 the sheep was severely depleted of water (plasma Na 172 mEq per L) and was very restless and excited. Cortisol and corticosterone, but not aldosterone, were raised, presumably as a result of endogenous ACTH release. The adrenal arterial infusion of a small amount of  $\delta_1$ -ACTH caused a further rise of cortisol and corticosterone, but had little effect on aldosterone. After these observations the sheep was permitted access to water and drank 5.0 L in 3 minutes. Two hours later cortisol and corticosterone secretion rates were reduced to normal, and plasma Na was 162 mEq per L. An hour later plasma Na was 152 mEq per L.

Further evidence comparing the relative effects of angiotensin II, electrolyte changes, and ACTH on aldosterone secretion, when tested by intra-adrenal arterial infusion over an interval adequate for full development of a salivary response (see Methods), is shown in Figure 11. With TP 9, concurrent decrease of Na and increase of K con-

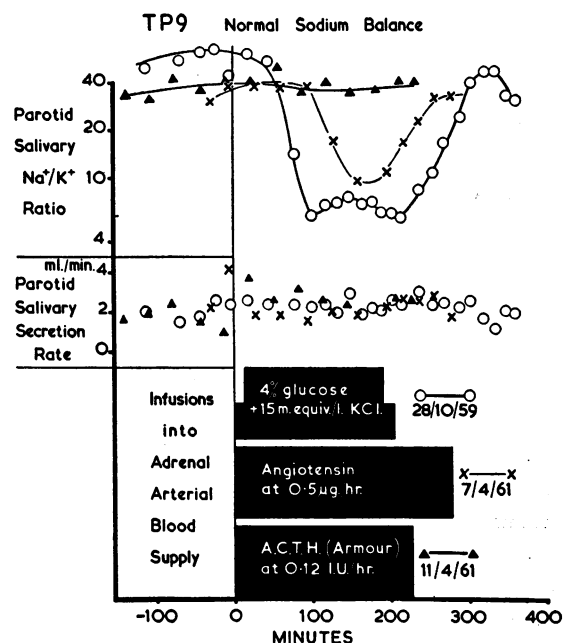


FIG. 11. TP 9, NA-REPLETE. The effect on parotid salivary Na/K ratio and salivary secretion rate of adrenal arterial infusion of: 1) 4 per cent glucose with 15 mEq per L KCl added at 1.5 ml per minute; 2) angiotensin II at 0.5  $\mu\text{g}$  per hour; and 3) ACTH (Armour) at 120 mU per hour.

centration caused a sustained lowering of salivary Na/K equivalent to infusion of over 10  $\mu\text{g}$  per hour of aldosterone. The effect reversed 120 minutes after stopping the infusion. With val-5-angiotensin II, infusion at 0.5  $\mu\text{g}$  per hour caused the Na/K ratio to fall, but it rose to normal again despite continued infusion, indicating that adrenal secretion of aldosterone was not sustained at a rate above salivary Na/K threshold for this animal (3.8  $\mu\text{g}$  per hour). With Armour ACTH at 120 mU per hour, the aldosterone secretion was not stimulated above this threshold of 3.8  $\mu\text{g}$  per hour, although ACTH concentration in the adrenal arterial plasma may have risen to approximately 20 mU per 100 ml.

Adrenal arterial infusion of ACTH caused a considerable rise in adrenal blood flow in some instances (Table IV).

Intravenous infusion of  $\alpha_b$ -ACTH at 12 U per hour to TP 9 (Figure 3) increased aldosterone from 0.9 to 7.5, cortisol from 50 to 2,640, and corticosterone from 11 to 178  $\mu\text{g}$  per hour. With  $\delta_1$ -ACTH, intravenous infusion in TP 12 at 3 U per hour gave essentially the same result (Figure 4).

Aldosterone secretion rose from 1.1 to 11.1, cortisol from 28 to 1,850, and corticosterone from 28 to 177  $\mu\text{g}$  per hour. With Osiris, a very high dose of 45 U per hour raised aldosterone secretion from 0.7 to 5.3  $\mu\text{g}$  per hour, but cortisol rose from 106 to 640 only, and corticosterone from 8 to 54  $\mu\text{g}$  per hour (see Methods). In contrast to the result with val-5-angiotensin II on the same day (TP 9, Figure 3), the intravenous infusion of  $\alpha_b$ -ACTH at a rate 100 times greater than adrenal arterial infusion was a much more effective stimulus of aldosterone secretion. With  $\delta_1$ -ACTH in TP 12, with a ratio of adrenal arterial to intravenous infusion rate of 1/100, the intravenous dosage was also much more potent on all steroid components measured. One experiment was made (Figure 4) in which ACTH was incubated in the animal's own peripheral blood for 15 minutes at 37° C and infused into the adrenal arterial blood supply at 15 mU per hour. The effect did not differ significantly from that produced when 15 mU per hour was infused without incubation.

*$\beta$ -MSH, lysine vasopressin, oxytocin, noradrenaline, serotonin, bradykinin, and acetylcholine.* Some preliminary experiments that examine the

aldosterone-stimulating effects of adrenal arterial infusion of these substances are given in Table V and Figure 3.  $\beta$ -MSH was without effect when the weight of material infused was equivalent to 120 mU per hour of Dr. Li's  $\alpha_b$ -corticotrophin (i.e., 1.2  $\mu\text{g}$  per hour; Figure 3). Verney (49) showed that an intravenous infusion of 4 mU per hour of vasopressin will inhibit an established water diuresis in a dog. Here 12 mU per hour of lysine vasopressin had little effect on corticosteroid secretion, and a very large infusion rate of 48 mU per hour increased aldosterone from 0.3 to 1.2  $\mu\text{g}$  per hour only. A rise in cortisol secretion also occurred. An infusion of oxytocin at 25 mU per hour, with respect to vasopressor activity in the synthetic material used, had little effect on corticosteroid secretion. Both oxytocin and vasopressin reduced adrenal blood flow by half at the rates infused. Serotonin infusion had little effect on corticosteroid secretion. The infusion could have produced an adrenal blood concentration approximately five times greater than that reported in peripheral blood in man (50). Serotonin caused a 10 to 30 per cent increase of adrenal blood flow. In Osiris, bradykinin infusion at 1  $\mu\text{g}$  per hour

TABLE V  
Effect on corticosteroid secretion and blood flow of adrenal arterial infusion of the substances tabulated

Sheep	Adrenal arterial infusion	Adrenal infusion rate/hr	Adrenal blood flow ml/min	Packed cell volume %	Corticosteroid secretion in adrenal venous plasma		
					Aldos- terone $\mu\text{g/hr}$	Cortisol $\mu\text{g/hr}$	Corticos- terone $\mu\text{g/hr}$
Osiris 28/ 3/61	Control		7.6	29	0.6	642	22
	Val-5-angiotensin II	0.25 $\mu\text{g}$	.60	26	3.6	500	10
	Serotonin	250.00 $\mu\text{g}$	10.6	26	0.6	606	9
	Bradykinin	1.00 $\mu\text{g}$	13.2	23	1.8	207	12
	Oxytocin	25.00 mU (vaso- pressor activity)	6.0	23	0.7	451	17
	Noradrenaline	1.25 $\mu\text{g}$	11.6	24	1.3	169	23
TP 12 7/ 3/61	Noradrenaline	5.00 $\mu\text{g}$	10.9	22	0.9	97	14
	Control		11.7	22	0.5	577	10
	Noradrenaline	2.40 $\mu\text{g}$	7.9	20	0.6	710	22
	Control		12.1	22	0.3	308	7
Noah 16/ 3/61	Serotonin	240.00 $\mu\text{g}$	13.5	25	0.5	565	16
	Control		11.6	28	0.4	507	21
	Bradykinin	0.50 $\mu\text{g}$	12.2	28	0.3	137	5
	Vasopressin	12.00 mU	9.2	28	0.4	78	9
	Vasopressin	48.00 mU	5.5	27	1.2	362	11
TP 9 28/11/61	Control		9.3	26	0.2	62	2
	Control		14.6	32	0.7	107	8
	Acetylcholine	60.00 $\mu\text{g}$	21.0	31	1.3	342	17
TP 9 29/ 8/61	Acetylcholine	120.00 $\mu\text{g}$	21.0	31	1.9	176	5
	Control		20.5	32	0.5	39	5
	Acetylcholine	115.00 $\mu\text{g}$	24.5	31	0.6	90	18



increased aldosterone secretion from 0.6 to 1.8  $\mu\text{g}$  per hour. Cortisol secretion fell. Adrenal blood flow increased by 25 per cent. Acetylcholine infusion at 60 to 120  $\mu\text{g}$  per hour increased aldosterone secretion on one occasion in TP 9 without consistent effect on cortisol or corticosterone. Blood flow increased 20 to 35 per cent. Noradrenaline had little effect on corticosteroid secretion. When Osiris (see Methods) was used, the effects were compared with an infusion of angiotensin II which reproduced the usual increase of aldosterone secretion rate seen with a normal transplant (see Figure 6).

#### DISCUSSION

A basic condition for this investigation, and an important finding, has been the direct determination for the first time of the normal rate of aldosterone secretion of the Na-replete, conscious, undisturbed sheep (adrenal blood flow 420 to 1,020 ml per hour). The results of 30 analyses on four animals show a secretion rate of  $0.54 \pm 0.26$  (SD)  $\mu\text{g}$  per hour = 1.4  $\mu\text{g}$  per 100 kg body weight per hour per transplanted gland. For reasons given in the Methods section, the use of the double isotope dilution derivative method (40, 41) has been obligatory for this measurement. The determination provided the important condition of a definite baseline relative to which the effects of the various stimulating agents could be assessed. This secretion rate is approximately one-quarter to one-tenth the rate found upon direct sampling of adrenal vein blood in the anesthetized, surgically operated animal. Davis (5) reports secretion rates of this order in the anesthetized, hypophysectomized dog, and we have made similar findings in acute experiments on Na-replete midcollicular decerebrate, hypophysectomized sheep (i.e., about 0.5  $\mu\text{g}$  per hour).

Many sheep with the sole adrenal gland auto-transplanted to the neck have lived for 3 to 4 years and have withstood Na deficiency of 500 to 900 mEq in a manner clinically indistinguishable from a normal sheep, whereas a Na-deficient, adrenally-insufficient animal dies in 48 to 72 hours. When removed 1 to 36 months after transplantation, the gland was normal or greater than normal size; the cortex of the gland of the seven animals examined was histologically normal. As shown in Results, the functional capacity of the transplanted adrenal

to secrete cortisol and corticosterone in response to ACTH stimulation is approximately double the output of these steroids from one adrenal in the Na-replete, anesthetized, traumatized animal. The observed capacity of the transplanted adrenal to produce aldosterone in response to severe Na deficiency (8 to 18  $\mu\text{g}$  per hour) equals that of the adrenal gland cannulated acutely in the abdomen of the Na-deficient sheep in the comparable instance when the contralateral adrenal gland has been removed some weeks or months previously [mean of 14 Na-deficient, anesthetized, unilaterally adrenalectomized animals: aldosterone  $9.5 \pm 3.6$  (SD), corticosterone  $104 \pm 57$ , cortisol  $1,370 \pm 530$   $\mu\text{g}$  per hour, mean blood flow  $9.5 \pm 3.5$  ml per minute]. The evidence indicates that the quantitative response of the transplanted adrenal to ACTH or Na deficiency, and presumably therefore to aldosterone-stimulating hormone, approximates the sum of the two abdominal adrenals, or the single abdominal adrenal of the animal unilaterally adrenalectomized some weeks previously. The time characteristics of response to change of Na balance are the same as that of the normal animal (6, 51). Postmortem examination has shown three other instances like Osiris (see Methods) where only a portion of the gland has survived transplantation. *In vivo* this is usually apparent from palpation of the adrenal transplant in the loop and is confirmed by finding limited capacity to produce cortisol in response to ACTH. The significance, in relation to theories of genesis of the adrenal cortex, of the occasional instance where apparently only the glomerulosa survives transplantation have been discussed elsewhere (52).

The effects of the various hormones and peptides tested have been compared with local concurrent lowering of Na and increase of K concentration within physiological range in adrenal arterial blood. This ionic change is a potent, specific stimulus of aldosterone secretion. Significant rise of aldosterone occurred when plasma Na was decreased 5 to 10 mEq per L and K concentration was raised 0.5 to 1.0 mEq per L. Some experiments showing aldosterone stimulation with increase of K concentration only and no effect with decrease of both Na and K concentration also are recorded here. The results reported here with the double isotope dilution method confirm our earlier reports of the direct effect of ionic change

of arterial blood on the adrenal cortex (1, 4). This direct effect has also been shown by Urquhart, Davis and Higgins (personal communication). Ionic changes of the order produced here experimentally may occur in a variety of clinical conditions—e.g., diabetic coma, renal disease, intestinal obstruction, paralytic ileus, diarrhea, burns, and heart failure (53). The fact that aldosterone secretion can be stimulated without ionic change, as, for example, with thoracic caval constriction, has focused attention on questions of volume change. This has caused some oversight of a major physiological mechanism of stimulation of aldosterone secretion which probably obtains in a wide variety of medical and surgical emergencies as well as in chronic clinical conditions causing this type of ionic imbalance.

The experiments have confirmed the results (16, 54) in man showing the stimulation of aldosterone secretion by intravenous infusion of angiotensin II. The data, in addition, have shown that *the action of angiotensin is definitely on the adrenal cortex* and not through an intermediate pathway such as the pituitary. As reported for low rates of intravenous angiotensin infusion in dogs (55), the effect was predominantly on aldosterone secretion within the dose range used for adrenal arterial infusion. Increases in cortisol or corticosterone secretion were sometimes observed, but the effect was not consistent, and there was no change in some instances where a large increase of aldosterone occurred. A comprehensive investigation of the effect of intravenous infusion of angiotensin was not made, but the above result suggests that the findings of a consistent augmentation of glucocorticoid secretion might be attributable to intermediate pathways and not to direct action on the adrenal. The effect of the synthetic octapeptide was much greater than that of the synthetic decapeptide angiotensin I. It cannot be determined whether the small effect seen with the decapeptide was due to partial conversion to octapeptide during the approximately 6- to 10-second interval we estimate for transit between the intracarotid needle and the adrenal cortical capillaries, or to direct action of the decapeptide. The rate of conversion of decapeptide to octapeptide is believed to be rapid in intact animals (56), and the occurrence of this conversion *in vivo* is suggested

by experiments in sheep showing a rapid pressor response to intravenous infusion of angiotensin I. The aldosterone-stimulating effect of highest rates of adrenal arterial infusion of angiotensin II examined here was less than that of concurrent lowering Na and increasing K concentration of adrenal arterial blood. Angiotensin II was much more potent on direct intra-adrenal arterial infusion than when infused intravenously at a rate 200 times greater. This finding was directly in contrast to that made with ACTH. A likely explanation is a very short biological half-life of angiotensin. As with the dog and man, intravenous infusion of angiotensin in the sheep caused a large rise in blood pressure within 2 minutes, and the pressure fell precipitately within 2 minutes of stopping an infusion (57, 58).

Three major possibilities that can be raised at this stage are: 1) that angiotensin II is a normal physiological mechanism of stimulation of aldosterone secretion—i.e., angiotensin II is the aldosterone-stimulating hormone, ASH; 2) that angiotensin II may be the cause, or an important contributory cause, of hypersecretion of aldosterone in some pathological conditions such as malignant hypertension, but plays no role in the physiological response of the adrenal to Na deficiency; and 3) that the chemical composition of angiotensin II resembles ASH, and this is the explanation of its action.

The evidence cited in the introduction implicating the kidney in aldosterone control is generally consistent with the suggestion that the renin-angiotensin system in the normal vehicle of humoral stimulation of aldosterone secretion (59). The proposition is, however, far from proven. In confirmation of other studies (5, 20) we have shown that intravenous infusion of saline extracts of kidney causes a large pressor response and stimulation of aldosterone secretion.

Our experiments indicated a relation between the concentration of angiotensin II in adrenal arterial plasma and adrenal secretion of aldosterone. The threshold of aldosterone response was in the region of 0.20 to 0.25  $\mu\text{g}$  of angiotensin II per L of plasma. Emphasizing what has been said earlier, this figure assumes that angiotensin II was distributed only in plasma. If it were distributed evenly over whole blood, the angiotensin II con-

centration in adrenal plasma would be reduced by approximately 30 per cent, i.e., a threshold concentration of 0.14 to 0.18  $\mu\text{g}$  per L.

These findings on threshold concentration of angiotensin II in adrenal arterial plasma for stimulation of aldosterone secretion emphasize the important implications of accurate measurement of angiotensin II concentration in peripheral blood. Making allowance for the 50 per cent recovery cited, the figures of Kahn, Skeggs, Shumway and Wisenbaugh (60) show a mean angiotensin II concentration in plasma of approximately 0.02  $\mu\text{g}$  per L with normotension, 0.04  $\mu\text{g}$  per L in benign hypertension, and 0.3  $\mu\text{g}$  per L in malignant hypertension. Genest and co-workers (61) report blood angiotensin concentrations of 0.3 to 2.0  $\mu\text{g}$  per L with intravenous infusion of val-5-angiotensin II at 100 to 360  $\mu\text{g}$  per hour. Biron and colleagues (62) found the mean angiotensin concentration in 18 normal subjects to be 0.11  $\mu\text{g}$  per L of blood, and only some patients with essential and renal hypertension showed substantial increases of 2 to 8  $\mu\text{g}$  per L. There was no significant change in malignant hypertension or in Conn's syndrome. The results differ considerably from those of Kahn and associates (60), and no conclusion is possible at this stage as to whether angiotensin II plays a causative role in the high aldosterone secretion reported in malignant hypertension.

With onset of Na deficiency the rise of aldosterone secretion is progressive, whereas the experiments with prolonged infusion of angiotensin in five different sheep indicated considerable decrease of aldosterone stimulation with time, although the pressor effect was maintained [Page and Bumpus (56)]. This, however, is not necessarily evidence that angiotensin II is not ASH, since in Na deficiency, angiotensin might act synergistically with other factors, such as change in the ionic composition of the adrenal cortex itself. Simple infusion of angiotensin into the adrenal arterial supply of the Na-replete sheep may not reproduce the several necessary conditions holding in Na deficiency. Also, naturally occurring sheep angiotensin may not be the valine-5 form, as is the case with bovine angiotensin. In relation to pressor activity, it has been shown by Gross and Turrian (57) that valine-5 and isoleucine-5-

angiotensin II have the same effect. Further work is required to establish a clear quantitative picture of the angiotensin II-aldosterone dose-response relation, the extent to which the response changes with a very prolonged infusion, and whether the intracellular changes of Na deficiency augment the adrenal sensitivity to angiotensin II.

In the experiments recorded here, adrenal arterial infusion of ACTH at rates adequate to increase cortisol and corticosterone to 1,000 and 45  $\mu\text{g}$  per hour, respectively (a 5- to 20-fold increase on basal), had little effect on aldosterone. The calculated plasma ACTH concentration produced was about 1 to 4 mU per 100 ml. Higher rates of ACTH infusion, calculated to produce a plasma ACTH concentration of the order reported in Addison's disease (47) or in the stressed dog (63), resulted in significant rise of aldosterone secretion.

$\delta_1$ -ACTH was a more effective stimulus of aldosterone secretion than  $\alpha_b$ -ACTH, when compared on a weight-for-weight basis. The difference was greater when assessed relative to the cited potency of the two preparations, but this may reflect standardization resulting from the adrenal ascorbic acid-depletion test ( $\text{Li}\alpha_b$ ) in which most preparations assay about 100 U per mg and from the *in vitro* corticosteroid assay in which Bell  $\delta_1$  gives about 28 U per mg (64). Using visual appraisal of Na fluorescence (see Methods), McDonald and Reich (65) reported that no aldosterone was detected in 45 ml of adrenal venous plasma obtained during intravenous infusion of ACTH at 5.6 U per hour in a Na-replete sheep, except in one experiment in which a trace was found 60 to 90 minutes after beginning. Here, with intravenous infusion of ACTH at 3 to 12 U per hour, aldosterone secretion rates of 7.5 to 11.1  $\mu\text{g}$  per hour were observed in the presence of a cortisol secretion rate of 1,850 to 2,640  $\mu\text{g}$  per hour and corticosterone rates of 170 to 180  $\mu\text{g}$  per hour.

The difference of biological half-life between angiotensin II and ACTH may be the explanation of the contrast of potency between intra-adrenal and intravenous infusions with the two substances. The intravenous infusion of ACTH caused much higher corticosteroid output than adrenal arterial infusion at 0.01 the rate. With adrenal arterial

infusion of ACTH, recirculation effects would have been minimal. If the half-life of ACTH in sheep blood is 4 to 18 minutes as reported for man and rat (66, 67) rather than the 1-minute estimate for rats (68), recirculation with the intravenous infusion of 3 to 12 U per hour most likely would have caused a very high peripheral blood concentration by 30 to 40 minutes when the adrenal venous blood specimen was collected, e.g., 20 to 100 mU per 100 ml (66).

We have confirmed for direct adrenal arterial action the earlier reports (69) indicating that the action of ACTH at low dosage is primarily on glucocorticoids and that significant aldosterone-stimulating effect occurs only with high dosage. In Na deficiency in the conscious animal where aldosterone secretion is 5 to 15  $\mu\text{g}$  per hour and cortisol and corticosterone secretion are unchanged or below 500 and 30  $\mu\text{g}$  per hour respectively, ACTH does not contribute *directly* to the aldosterone hypersecretion. Its constant presence at basal level may, however, be permissive of full, functional capacity of adrenal response to the physiological stimulus of Na deficiency (70, 71). On the other hand, in anesthetized, acutely traumatized animals in which glucocorticoid secretion approaches maximum, it is probable that ACTH secretion is a contributory cause of the aldosterone hypersecretion observed. It is possible that the role of ACTH in the *physiological* stimulation of aldosterone secretion may have received some overemphasis because much of the investigation has been carried out on traumatized anesthetized animals.

In a number of our experiments eight to twelve collections of 30 to 40 ml of adrenal venous blood were made over the course of 4 to 8 hours. It is interesting that this caused no significant increase of aldosterone secretion in the sheep. It is necessary to remove this total amount or more (500 to 800 ml) over an interval of 5 to 10 minutes to get a significant effect.

The preliminary experiments reported here did not suggest that any of a number of other hormone and vascularly active agents was a strong stimulus of aldosterone secretion. The absence of effect with  $\beta$ -MSH confirms the report of Davis and co-workers (72). Vasopressin caused a small increase of aldosterone at very high dosage and

also, as found by Hilton (73), an increase of cortisol. Bradykinin infusion at 1  $\mu\text{g}$  per hour caused a definite increase of aldosterone without change of glucocorticoid secretion and this, together with the effect of acetylcholine, requires further investigation. Noradrenaline and serotonin had little effect. The latter substance has been reported (74) to cause formation of increased amounts of blue tetrazolium-reducing material by the rabbit adrenal *in vitro*.

#### SUMMARY

1. Adrenal venous blood collections from conscious, undisturbed, Na-replete sheep with adrenal transplants were analyzed for aldosterone, corticosterone, and cortisol by the double isotope dilution derivative method. The normal secretion rates were: aldosterone  $0.54 \pm 0.26$  (SD)  $\mu\text{g}$  per hour, corticosterone  $9.3 \pm 6.2$ , cortisol  $138 \pm 139$ . The use of the double isotope dilution derivative method was obligatory for determination of aldosterone and corticosterone, since the small steroid output of the conscious animal was dispersed in an adrenal blood flow of 420 to 1,020 ml per hour.

2. Adrenal arterial infusion of val-5-angiotensin II produced a large stimulation of aldosterone secretion. The threshold of adrenal response was approximately 0.2  $\mu\text{g}$  of angiotensin II per L of adrenal vein plasma. No consistent effect on cortisol or corticosterone was observed. When angiotensin II infusion was prolonged for 3 to 24 hours, a decline in adrenal aldosterone output occurred, whereas the pressor response was maintained. Angiotensin I was a much less effective stimulus of aldosterone secretion upon adrenal arterial infusion.

3. Adrenal arterial infusion of  $\alpha_{\text{b}}$ -ACTH (Li) and  $\delta_1$ -ACTH (Bell) caused large increases of cortisol and corticosterone secretion. There was little increase in aldosterone secretion rate until cortisol and corticosterone secretion rates exceeded 1,000 and 45  $\mu\text{g}$  per hour, respectively (i.e., 5 to 20 times basal levels). The highest rate of ACTH infusion which was calculated to give adrenal ACTH concentrations in plasma of the order reported in Addison's disease (about 10 mU per 100 ml) increased aldosterone secretion to 2 to 7  $\mu\text{g}$  per hour. Intravenous infusion of ACTH at a rate 100-fold greater than adrenal

arterial infusion caused a much larger effect on aldosterone and glucocorticoid secretion. This contrasted with angiotensin II where adrenal arterial infusion was a more effective stimulus.

4. The quantitative effect of these substances on aldosterone secretion was compared with that produced by concurrent local reduction of Na and increase of K concentration in adrenal arterial blood, a potent, direct-acting, and specific stimulus of aldosterone secretion.

5. Adrenal arterial infusion of lysine vasopressin, acetylcholine, and bradykinin caused a minimal increase in aldosterone secretion. Noradrenaline, serotonin, oxytocin, and  $\beta$ -melanocyte-stimulating hormone had little effect.

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