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**Research Article**

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The results are interpreted to indicate that (*a*) biosynthesis of aldosterone is regulated at at least two sites in the biosynthetic pathway. The final conversion, that of corticosterone to aldosterone, is stimulated by sodium depletion. This effect persists for at least 3 hr while slices from sodium-depleted dogs are incubated in vitro. Stimulation at this site is thus relatively stable in vitro; its activation by sodium depletion is not inhibited by puromycin in the dog. Stimulation at this site can explain, at least in part, the increased effectiveness of adrenocorticotropin (ACTH) on aldosterone biogenesis during sodium depletion.

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# Sites of Action of Sodium Depletion on Aldosterone Biosynthesis in the Dog

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ABSTRACT Secretion of cortisol, corticosterone, and aldosterone was measured in vivo in normal and sodium-depleted hypophysectomized dogs. Biogenesis of steroids was then measured in vitro with outer slices of the adrenals of the same dogs. In some studies, metyrapone or puromycin was added.

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## INTRODUCTION

Depletion of body sodium is a potent physiologic stimulus to the secretion of aldosterone by the adrenal cortex. In the present experiments the in vivo and in vitro production of aldosterone and its precursors by the adrenal cortex of hypophysectomized dogs is examined to determine the steps in the biosynthetic pathway stimulated by sodium depletion. These studies indicate that multiple sites in the biosynthetic pathway are stimulated by sodium depletion. The major steps stimulated are the conversion of corticosterone to aldosterone and an earlier step through which more corticosterone is made available for biosynthesis of aldosterone.

## METHODS

Mongrel dogs were used for experiments. Hypophysectomy was performed by the buccal approach. After hypophysectomy, the dogs were either fed a high-sodium diet (220 mEq/day) or were depleted of sodium by injection of 2 ml of mercuhydrin intramuscularly and then fed a low-sodium diet (8 mEq/day). 2 days after the hypophysectomy the lumboadrenal vein was cannulated under Nembutal anesthesia (26 mg/kg) and the secretion rates of aldosterone, corticosterone, and cortisol were determined. 2 days after hypophysectomy the cortisol secretion rates were very low (from 20 to 100 mμg/min) as an index of complete hypophysectomy. The presence of a small remnant of functioning pituitary would result in higher cortisol secretion rates under Nembutal anesthesia. Immediately after the adrenal venous blood was obtained, the animals were sacrificed and the adrenals were removed. In one experiment rats were depleted of sodium

A preliminary report of some of these data has been published previously (1).

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by feeding a low-sodium diet for 4 wk. They were sacrificed and the adrenals were removed and quartered.

Dog adrenal slices were prepared from the outer surface of the adrenal cortex. Four slices, weighing about 100 mg each and closely matched in area and thickness, were taken from each adrenal and incubated in separate flasks in Krebs-Ringer bicarbonate buffer plus glucose, as described by Kaplan and Bartter (2). Rat adrenal quarters were incubated as described by Saffran and Schally (3). In eight experiments on 32 adrenal slices, the correlation coefficient of aldosterone production with weight of slice varied from 0.7 to 0.99, with an average of 0.88. Thus it is reasonable to express aldosterone values as a function of tissue weight. The Krebs-Ringer buffer was standardized by flame photometry so that the sodium concentration was 144 mEq/liter and the potassium concentration was 5.9 mEq/liter. Where indicated, progesterone or corticosterone was added in 25 $\mu$l of ethanol. Puromycin, ACTH, or metyrapone (Metopirone, CIBA Pharmaceutical Company, Summit, N. J.) was also added, where indicated, in 0.1 ml of incubation medium. To evaluate inhibition of adrenal protein synthesis by puromycin, slices of dog adrenal cortex were incubated for 2 hr with 1 $\mu$c of phenylalanine-$^{14}$C in the presence or absence of puromycin (1 mmole/liter). Radioactivity in adrenal protein (4) revealed that puromycin had inhibited protein synthesis by 95% in the concentration used.

All steroid determinations were performed by an isotope-dilution derivative technique similar to that described by Kliman and Peterson for aldosterone (5). After the acetylation procedure, the steroid acetates were first chromatographed in a thin-layer system on silica gel with chloroform: ethanol (96:4) as the developing solution. The steroids were revealed with rhodamine G and ultraviolet light and then eluted with acetone: ethanol (1:1). Aldosterone was then purified as described originally. Corticosterone and cortisol acetates were purified by chromatography in the two systems of Kliman and Peterson before and after oxidation with chromium trioxide. 11-deoxycorticosterone (DOC) and 11-deoxycortisol acetates were purified by chromatography in the same two systems before and after reduction with 0.4 mg of sodium borohydride in 0.5 ml of methanol for 30 sec.

The specific activity of aldosterone synthesized by adrenal slices was determined in some experiments when tritium-labeled precursor had been used by first adding aldosterone-$^{14}$C as a measure of recovery. One aliquot was acetylated with nonradioactive acetic anhydride and purified. The corrected tritium counts from this aliquot represented label derived from tritium-labeled precursor. The other aliquot was acetylated with radioactive acetic anhydride and purified; this represents tritium counts from precursor and from acetylation. The specific activity of the aldosterone was then calculated from these data.

Crystalline aldosterone was obtained from CIBA. The remaining unlabeled steroids were obtained from Sigma Chemical Company, St. Louis, Mo. Phenylalanine-$^{14}$C, 4-$^{14}$C-labeled aldosterone, corticosterone, cortisol, DOC, and 11-deoxycortisol were obtained from New England Nuclear Corp., Boston, Mass. These compounds had a specific activity of 45 mc/mmole. Progesterone-$^{3}$H and corticosterone-$^{3}$H were obtained from New England Nuclear Corp. and these compounds had a specific activity of 30 c/mmole. Acetic anhydride (100 mc/mmole) was obtained from New England Nuclear Corp. and from Nuclear-Chicago Corp., Des Plaines, Ill.

All results are expressed as mean ± standard error of the mean. Statistical evaluation was made by the two-tailed $t$ test.

## RESULTS

*Precision of double-isotope dilution derivative assay of steroids.* Since evidence has been published previously to document the accuracy of the method of Kliman and Peterson for measuring nanogram quantities of steroids (5, 6), we will present data on only one problem of the method that we have encountered, namely the high "blank" that remains after corticosterone or cortisol have been purified by the three chromatographic systems used for aldosterone. The persistence of nonspecific tritium impurities from the acetylation reaction through three chromatographic steps is consistently seen either with crystalline steroids or with biological fluids. This high "blank" is eliminated by adding two additional chromatographic steps to the procedure for corticosterone and cortisol (Table I). Satisfactory precision is obtained when known amounts of steroids are assayed, and the tritium/carbon ratio is stable with additional chromatographic steps.

*Effect of sodium depletion on in vivo secretion of aldosterone, corticosterone, and cortisol.* Fig. 1 shows the in vivo secretion rates of aldosterone, corticosterone, and cortisol 2 days after hypophysectomy in sodium-replete and sodium-depleted dogs. The secretion rates of aldosterone and of corticosterone in the sodium-depleted dogs are 10-fold, that of cortisol is fivefold, the rates in sodium-replete dogs. This pattern of steroid secretion is similar to that seen after administration of renin or angiotensin in vivo (7–10), and is in agreement with previous data from the sodium-depleted dog (11, 12).

*Biosynthesis of aldosterone by adrenal slices.* To examine further the loci in the biosynthetic pathway at which sodium depletion stimulates aldosterone biosynthesis, adrenal slices were prepared from the dogs that had been studied in the in vivo experiments, and were incubated in vitro.

TABLE I

*Accuracy of the Isotope Dilution Method for Aldosterone, Corticosterone, and Cortisol*

| Steroid | No. of chromatographic steps after acetylation | Mass added to sample | Mass found | $^3H:^{14}C$ ratio |
|---|---|---|---|---|
| | | mμg | mμg* | |
| Aldosterone | 3 | 10 | 18 ± 1 (4)‡ | 5.4 |
| | 4 | 10 | 10 ± 1 (4) | 3.0 |
| | 5§ | 10 | 10 ± 1 (4) | 3.0 |
| | 4 | 23 | 21.8 ± 0.7 (4) | 6.5 |
| | 5§ | 23 | 22.0 ± 0.7 (4) | 6.5 |
| Corticosterone | 4 | 13 | 159 ± 38 (4) | 58 |
| | 5 | 13 | 15 ± 4 (4) | 4.5 |
| | 6§ | 13 | 14 ± 4 (4) | 4.2 |
| | 5 | 94 | 97 ± 2.5 (15) | 29.0 |
| | 6§ | 94 | 95 ± 2.0 (4) | 28.5 |
| Cortisol | 4 | 12 | 183 ± 24 (4) | 55 |
| | 5 | 12 | 13 ± 2 (4) | 3.9 |
| | 6§ | 12 | 14 ± 1 (4) | 4.2 |
| | 5 | 122 | 126 ± 7.0 (15) | 38 |
| | 6§ | 122 | 124 ± 6.0 (4) | 37 |

* Data are expressed as millimicrograms found ± 1 SEM.

‡ The numbers in parentheses denote number of separate determinations.

§ Chromatographed in heptane:methanol:water (4:3:1).

Fig. 2 shows histologic sections of slices from sodium-depleted and sodium-replete dogs at the end of 3 hr of incubation and is representative of multiple sections examined. The zona glomerulosa is completely included in the slice and occupies slightly more than one-third of the volume of the slice. Evidence for cellular damage is minimal, and there is no evidence for cellular hyperplasia of the zona glomerulosa 2 days after the onset of sodium depletion. Fig. 3 shows the production rate of aldosterone by slices from the sodium-replete and the sodium-depleted dog of which the sections of Fig. 2 were taken. The slices from the sodium-depleted dog produce more aldosterone in vitro than those from the sodium-replete one. After 30 min of incubation the production of aldosterone is approximately constant, with no apparent evidence of decay of the stimulus of sodium depletion pro-

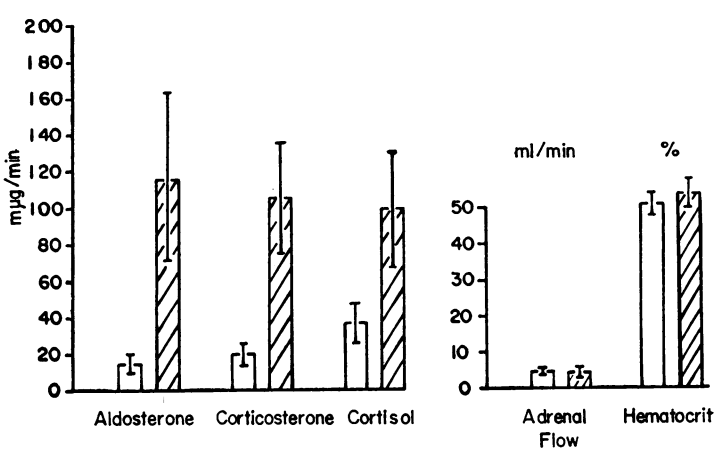


FIGURE 1 Effect of sodium depletion on secretion of aldosterone, corticosterone, and cortisol and on the rate of flow and hematocrit of adrenal venous blood in the hypophysectomized dog. The hatched bars represent sodium-depleted animals, the clear bars sodium-replete animals.

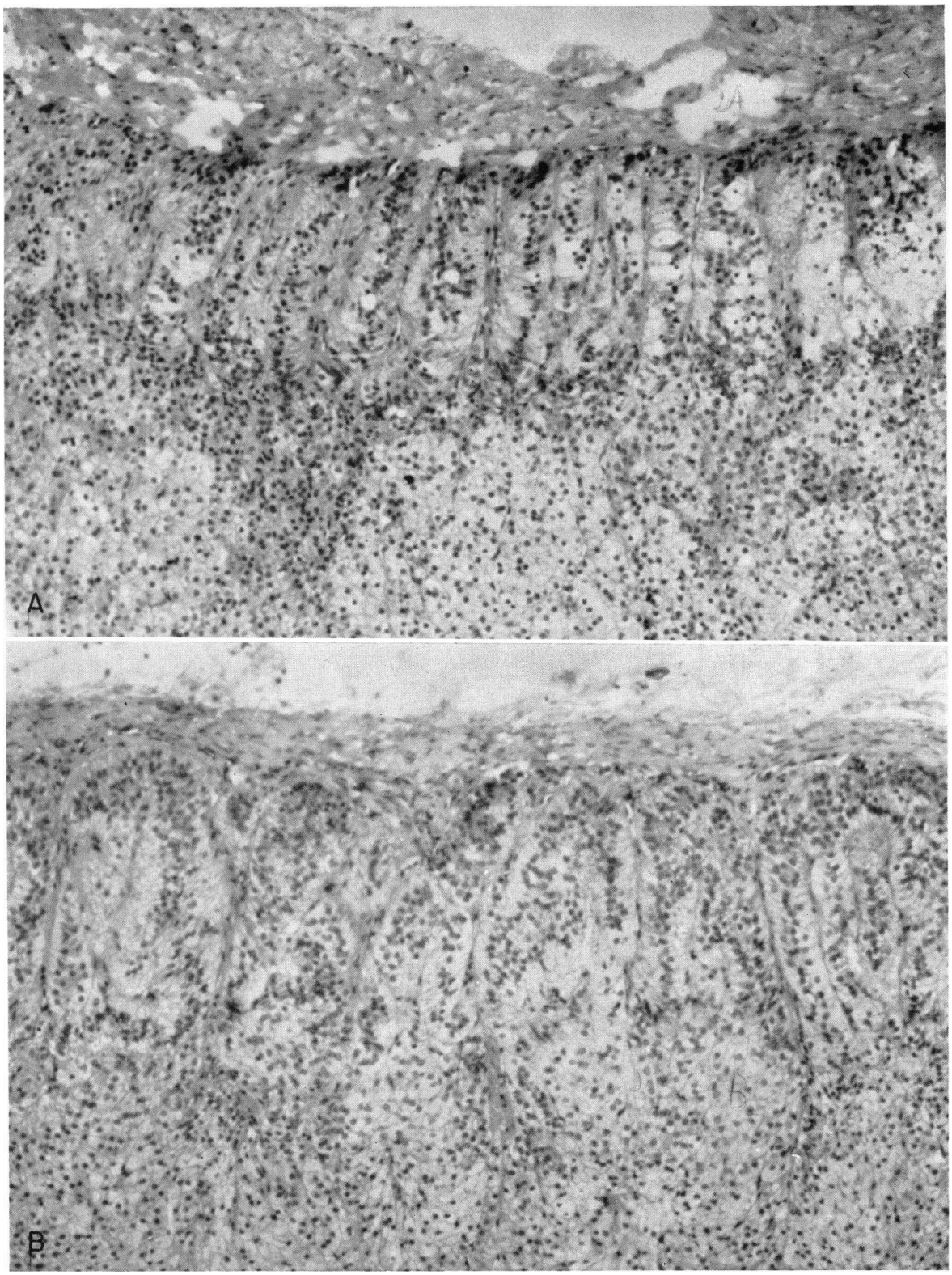


FIGURE 2 Hematoxylin and eosin sections of "outer" adrenal slices after 3 hr of incubation in vitro. Fig. 2 *A* represents a section from a sodium-replete dog, Fig. 2 *B* one from a sodium-depleted dog. These sections are from the most abnormal areas observed. × 190.

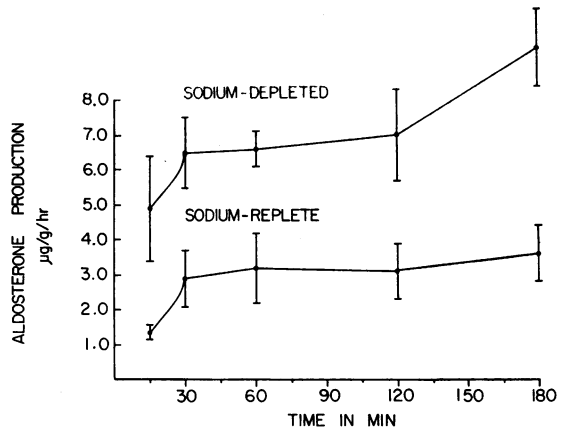


FIGURE 3 Effect of sodium depletion on secretion of aldosterone by dog adrenal slices in vitro. Eight adrenal slices, each from sodium-replete and sodium-depleted dogs, were incubated for 3 hr. At each point in the figure the medium was changed and production of aldosterone was measured.

TABLE II

*Effect of Sodium Depletion on Steroid Production by Dog Adrenal Slices In Vitro*

| Expt. No. | Experimental condition of dogs | Aldosterone production | Corticosterone production | Cortisol production |
|---|---|---|---|---|
| | | *µg/g per hr* | *µg/g per hr* | *µg/g per hr* |
| 1 | Sodium-replete (12)* | 3.7 ±0.5 | 3.2 ±0.26 | 1.9 ±0.4 |
| | Sodium-depleted (12) | 7.3 ±1.1§ | 1.6 ±0.6§ | 1.6 ±0.2 |
| 2‡ | Sodium-replete (4) | 5.5 ±0.2 | 1.5 ±0.1 | 1.6 ±0.2 |
| | Sodium-depleted (4) | 11.4 ±0.5‖ | 1.0 ±0.1§ | 1.6 ±0.3 |
| 3 | Sodium-replete (4) | 0.8 ±0.1 | | |
| | Sodium-depleted (4)¶ | 9.5 ±0.8‖ | | |

* The numbers in parentheses denote the number of flasks incubated.
‡ In this experiment the adrenal tissue was homogenized at the end of incubation and the homogenate was assayed.
§ $P < 0.02$, ‖ $P < 0.001$ } Between sodium-replete and sodium-depleted.
¶ This dog was on low sodium diet for 8 wk and was not given a mercurial diuretic.

duced in vivo. The slight rise in aldosterone production during the 3rd hr of incubation in the sodium-depleted group is not statistically significant.

Table II shows the production of aldosterone, corticosterone, and cortisol by slices from sodium-replete and sodium-depleted dogs. As in Fig. 3, the results of experiments involving 10 additional dogs show that the rate of biosynthesis of aldosterone by slices from sodium-depleted dogs is about twice that by slices from sodium-replete ones. In experiment 3 of Table II sodium depletion was produced by feeding a diet low in sodium for 2 wk to rule out the possibility that the observed effects were a result of the mercurial diuretic. In experiment 2 of Table II the adrenal slices were homogenized in the medium at the end of the incubation and the steroid assays were done on the homogenate to rule out the possibility that changes observed in these experiments were the result of changes in release of hormones and not of synthesis.

The pattern of steroid production seen in vitro (Table II) is different than that observed in vivo (Fig. 1). The increase in corticosterone and cortisol secretion during sodium depletion that was observed in vivo is not apparent in vitro. The in vivo results suggest that an early reaction in the pathway of aldosterone biosynthesis is stimulated by sodium depletion. The increase in cortisol secretion indicates that the stimulus of sodium depletion affects the zona fasiculata to a minor extent, and suggests that the early step occurs before 17-hydroxylation, i.e., before formation of progesterone (Fig. 4). The in vitro results suggest that this early stimulus had decayed during the prolonged in vitro incubation after removal from the in vivo stimulus.

Evidence of some increased activity of the early

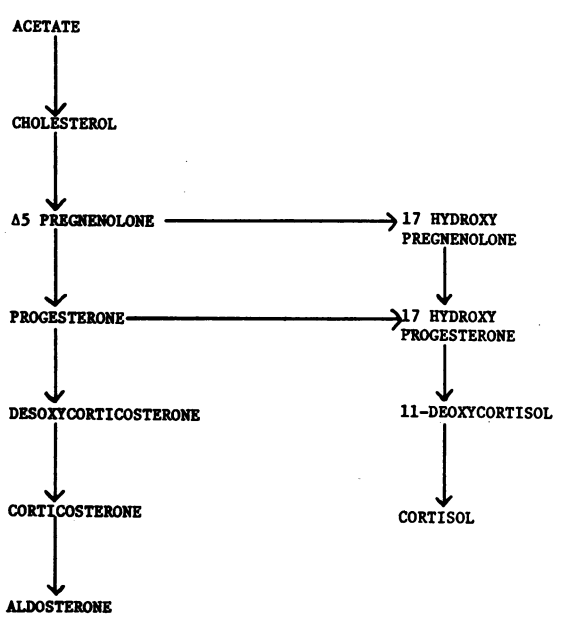


FIGURE 4 Biosynthetic pathways for aldosterone and cortisol in the adrenal cortex.

TABLE III

*Effect of Sodium Depletion on Production of DOC and 11-Deoxycortisol by Adrenal Slices Blocked with Metyrapone ($4 \times 10^{-4}$ moles/liter) In Vitro*

| Experimental condition | DOC production | 11-Deoxycortisol production | Aldosterone | Corticosterone | Cortisol |
|---|---|---|---|---|---|
| | *μg/g per hr* | *μg/g per hr* | *μg/g per hr* | *μg/g per hr* | *μg/g per hr* |
| Sodium-replete (4)* | 2.9 ± 0.38 | | 0.1 | <0.1 | <0.1 |
| Sodium-depleted (4) | 6.0 ± 0.57‡ | | 0.5 | <0.1 | <0.1 |
| Sodium-replete (4) | 6.3 ± 0.3 | 2.7 ± 0.6 | | | |
| Sodium-depleted (4) | 10.1 ± 0.9‡ | 1.2 ± 0.4 | | | |
| Sodium-replete (4) | 3.7 ± 0.5 | 2.3 ± 0.3 | | | |
| Sodium-depleted (4) | 6.7 ± 0.7§ | 2.2 ± 0.5 | | | |

* Number of slices incubated.
‡ $P < 0.001$, sodium depleted vs. sodium-replete.
§ $P$ 0.02, sodium depleted vs. sodium-replete.

part of the pathway during sodium depletion was obtained by adding metyrapone to slices from sodium-replete and sodium-depleted dogs. The concentration of metyrapone used ($4 \times 10^{-4}$ mole/liter) inhibits selectively 11- and 18-hydroxylation (13–21). Accordingly, the major steroids produced are DOC and 11-deoxycortisol. Slices from sodium-depleted dogs produced significantly more DOC than slices from sodium-replete ones in the presence of metyrapone (Table III). The production of 11-deoxycortisol is not increased in the slices from sodium-depleted animals. These data indicate that a step before the production of DOC is stimulated by sodium depletion, but further localization of the exact site of this effect is not feasible with these slice techniques because of the contamination of the zona glomerulosa cells with cortisol-producing cells, and because the stimulation of the early part of the pathway has decayed considerably.

The production of corticosterone, a major precursor of aldosterone, is consistently less in slices from sodium-depleted dogs than from sodium-replete ones (Table II). Because aldosterone synthesis is higher in slices from sodium-depleted animals, this finding suggests that the conversion of corticosterone to aldosterone is increased by sodium depletion. To confirm this possibility the conversion of tritium-labeled corticosterone into aldosterone was studied (Table IV). Slices from sodium-depleted animals converted twice as much tritium-labeled corticosterone into aldosterone as

TABLE IV

*Effect of Addition of Corticosterone-$^3H$ to Adrenal Slices from Sodium-Replete and Sodium-Depleted Dogs*

| Experimental condition | Additions | $^3H$ in aldosterone | Specific activity of aldosterone |
|---|---|---|---|
| | | *dpm/100 mg per 2 hr* | *dpm/μg* |
| Sodium-replete (12)* | Corticosterone-$^3H$, *1.37 μc* | $2.9 \pm 0.2 \times 10^4$ | |
| Sodium-depleted (12) | | $5.1 \pm 0.5 \times 10^4$‡ | |
| Sodium-replete (4) | Corticosterone-$^3H$, *1.5 μc* | $3.8 \pm 0.48 \times 10^4$ | $1.4 \times 10^5$ |
| Sodium-depleted (4) | | $6.6 \pm 0.8 \times 10^4$‡ | $0.49 \times 10^5$ |
| Sodium-replete (4) | Corticosterone-$^3H$, *1.5 μc* plus | $2.4 \pm 0.3 \times 10^4$ | |
| Sodium-depleted (4) | 10 μg of corticosterone | $5.3 \pm 0.3 \times 10^4$‡ | |
| Sodium-replete | Corticosterone-$^3H$, *1.5 μc* | $2.5 \pm 0.2 \times 10^4$ | |
| Sodium-replete | Corticosterone-$^3H$, *1.5 μc* plus 1 U of ACTH | $2.1 \pm 0.3 \times 10^4$ | |

* The numbers in parentheses denote the number of flasks incubated. There are four flasks incubated for each dog.
‡ $P < 0.001$ as compared to sodium-replete control.

TABLE V

*Effect of Addition of Nonradioactive Steroid Precursors to Production of Aldosterone, Corticosterone, and Cortisol by Adrenal Slices from Sodium-Replete and Sodium-Depleted Dogs*

| Experimental condition | Steroid added to flasks | Aldosterone | Corticosterone | Cortisol |
|---|---|---|---|---|
| | | *μg/g per hr* | *μg/g per hr* | *μg/g per hr* |
| Sodium-replete (12)* | Progesterone, *25 μg* | 5.0 ± 0.46 | 10.6 ± 1.1 | 17.2 ± 1.0 |
| Sodium-depleted (12) | | 10.3 ± 1.0‡ | 13.0 ± 0.9 | 18.1 ± 1.7 |
| Sodium-replete (12) | Corticosterone, *25 μg* | 7.2 ± 0.8 | 22§ | 3.3 ± 0.5 |
| Sodium-depleted (12) | | 12.8 ± 2.4‖ | 21§ | 4.9 ± 0.8 |

* The numbers in parentheses denote the number of slices incubated. There are four flasks incubated for one dog.
‡ $P < 0.001$
§ These figures represent recovery of added precursor rather than production.
‖ $P < 0.02$.

slices from sodium-replete ones. Although the concentration of corticosterone was lower in the incubation medium of slices from sodium-depleted animals, the specific activity of aldosterone synthesized in the presence of tritium-labeled corticosterone was lower in slices from sodium-depleted animals. This indicates that the higher incorporation of label into aldosterone could not be a result of lower "pool" size alone. The addition of carrier corticosterone to the tritium-labeled corticosterone did not greatly influence the increased incorporation of labeled corticosterone into aldosterone during sodium depletion. ACTH did not increase the conversion of tritium-labeled corticosterone into aldosterone.

When nonradioactive precursors were added to the incubation medium (Table V), the finding of increased activity of steps in aldosterone biosynthesis past progesterone during sodium depletion was confirmed. Saturating quantities of progesterone added to the incubation medium stimulated aldosterone production more in adrenal slices from sodium-depleted dogs (Table V). Aldosterone production was also stimulated more in slices from sodium-depleted animals in the presence of saturating quantities of corticosterone. The production of corticosterone in the presence of progesterone was about the same in slices from sodium-replete and sodium-depleted animals (Table V). This suggests that no or very little increase occurred in activity of the biosynthetic steps between progesterone and corticosterone during sodium depletion, and that mainly the conversion of corticosterone to aldosterone was stimulated in this experiment.

*Effect of puromycin on the conversion of corticosterone to aldosterone.* Inhibitors of protein synthesis have been shown to block the action of ACTH (22–24) in stimulating the synthesis of corticosterone or cortisol and to inhibit the synthesis of aldosterone (25, 26). It was of interest, therefore, to determine whether puromycin would block the synthesis of aldosterone by inhibiting the conversion of corticosterone to aldosterone or by inhibiting only the earlier step activated during sodium depletion. Table VI summarizes the results of these studies. At concentrations used in these experiments, puromycin (1 mmole/liter) blocked the stimulation of aldosterone, corticosterone, and cortisol by ACTH. Aldosterone biosynthesis was inhibited in slices from sodium-replete and from sodium-depleted dogs. However, puromycin did not inhibit the incorporation of progesterone-$^{3}$H or corticosterone-$^{3}$H into aldosterone by slices from sodium-depleted dogs even though it did inhibit over-all aldosterone synthesis. In the presence of saturating quantities of progesterone, puromycin was also without effect on the biosynthesis of aldosterone. Therefore, it appears that in the dog, puromycin inhibits aldosterone biosynthesis at a step before the production of progesterone in the biosynthetic pathway. These results differ from those of Burrow, Mulrow, and Bondy (26) in the rat. Their studies showed that puromycin inhibits the conversion of progesterone-$^{14}$C into aldosterone but not into corticosterone. Experiment 7 of Table VI does confirm their results with rat adrenal quarters incubated with the same concentration of puromycin used for the dog slices. Therefore, it appears that in the rat,

TABLE VI

*Effect of Puromycin on Biosynthesis of Aldosterone and on Incorporation of Progesterone-$^3H$ and Corticosterone-$^3H$ into Aldosterone in Vitro*

| Expt. no. | Additive | $^3H$ in aldosterone | $^3H$ in corticosterone | $^3H$ in cortisol | Aldosterone | Corticosterone | Cortisol |
|---|---|---|---|---|---|---|---|
| | | *dpm* × $10^{-4}$ | *dpm* × $10^{-5}$ | *dpm* × $10^{-5}$ | *μg/g per hr* | *μg/g per hr* | *μg/g per hr* |
| 1* | 0 (4)‡ | | | | 1.4 ± 0.1 | 3.0 ± 0.5 | 3.3 ± 0.5 |
| | ACTH§ (4) | | | | 1.9 ± 0.2 | 9.5 ± 0.6 | 14 ± 1 |
| | ACTH (4) | | | | 2.7 ± 0.2 | 6.1 ± 0.47 | 14.7 ± 0.9 |
| | ACTH plus puromycin‖ | | | | 0.3 ± 0.03¶ | 1.2 ± 0.5¶ | 5 ± 0.4¶ |
| 2** | 0 (4) | | | | 8.0 ± 1.2 | 2.3 ± 0.4 | 0.8 ± 0.1 |
| | Puromycin (4) | | | | 4.0 ± 0.6¶ | 1.2 ± 0.3‡‡ | 0.8 ± 0.1 |
| 3** | Progesterone-$^3H$, *1.3 μc* (4) | 5.1 ± 0.7 | 4.3 ± 0.2 | 1.5 ± 0.3 | 7.0 | 2.2 | 4.2 |
| | Progesterone-$^3H$ plus puromycin | 4.3 ± 0.5 | 3.5 ± 0.9 | 1.5 ± 0.1 | 1.7 | 0.4 | 1.5 |
| 4** | Progesterone-$^3H$, *1.3 μc* (4) | 3.0 ± 0.3 | | | 3.7 × $10^4$§§ | | |
| | Progesterone-$^3H$ plus puromycin (4) | 2.9 ± 0.3 | | | 7.4 × $10^4$‖‖ | | |
| 5** | Corticosterone-$^3H$, *1.2 μc* (4) | 5.0 ± 0.3 | | | | | |
| | Corticosterone-$^3H$, *1.2 μc*, plus puromycin (4) | 4.9 ± 0.4 | | | | | |
| 6** | Progesterone, *25 μg* (4) | | | | 5.0 ± 1.0 | 16 ± 1 | 22 ± 1 |
| | Progesterone plus puromycin (4) | | | | 4.3 ± 0.7 | 23 ± 2 | 21 ± 1.5 |
| 7** | Progesterone-$^3H$, *1.3 μc*, (4) (rat adrenal quarters) | 6.2 ± 1 | 8.8 ± 0.8 | 3.9 ± 0.3¶¶ | | | |
| | Progesterone-$^3H$ plus puromycin (4) | 1.5 ± 0.7§ | 14.7 ± 1.8§ | 6.3 ± 0.7¶¶ | | | |

In experiments 1–6 dog adrenal was used.
* Animals in this experiment were on normal sodium intake.
‡ Number of slices incubated.
§ 1 U of ACTH was added to flasks where indicated.
‖ Concentration of puromycin was 1 mmole/liter for all experiments.
¶ $P < 0.001$, puromycine vs. appropriate control.
** Animals in this experiment were sodium-depleted.
‡‡ $P < 0.05$, puromycin vs. appropriate control.
§§ These numbers represent specific activity of aldosterone (in dpm/μg).
‖‖ $P < 0.05$, puromycin vs. appropriate control. These numbers represent specific activity of aldosterone (in dpm/μg).
¶¶ For experiment 7, this column represents $^3H$ in DOC.

puromycin inhibits aldosterone synthesis by inhibiting the conversion of corticosterone to aldosterone, but that in the dog puromycin does not inhibit this conversion, but acts only at an earlier step.

## DISCUSSION

The present experiments indicate that sodium depletion increases synthesis of aldosterone at at least two sites in the biosynthetic pathway which is summarized in Fig. 4. The first step that is stimulated by sodium depletion is above the position of DOC, as indicated by the higher production of DOC by slices from sodium-depleted animals in the presence of metyrapone. The increase in activity at this early locus accounts for the increase in production of all steroids measured in vivo during sodium depletion. The activity at this locus is probably regulated, at least in part, by the renin-angiotensin system. Similar results with metyrapone inhibition of steroidogenesis during sodium depletion have been reported in man (27). The precise location of this early step could not be obtained in the dog slices because of several difficulties: there was, inevitably, contamination of zona glomerulosa cells with cells from the zona fasiculata, the response of dog slices to angiotensin added in vitro is variable, and finally, the activity of this early step "decays" in vitro. A decline in activity of steroid production similar to the decay of this early stimulus has also been observed during in vitro superfusion of rat adrenal capsules (28). This has been attributed to the decay in

vitro of an in vivo stimulus. From previous studies of aldosterone production in beef adrenal slices, it is likely that the earlier site is at the conversion of cholesterol to pregnenolone (2).

The present experiments indicate that sodium depletion increases the conversion of corticosterone to aldosterone by adrenal slices. The evidence can be summarized as follows: (1) Sodium depletion accelerates biosynthesis of aldosterone in vitro, whereas the release of its precursor, corticosterone, is decreased. (2) Addition of corticosterone in saturating quantities increases production of aldosterone by slices from sodium-depleted animals more than it does in slices from sodium-replete ones. (3) More corticosterone-$^3$H is converted into aldosterone by slices from sodium-depleted dogs. Despite this, the aldosterone has a lower specific activity, because of augmented incorporation of nonradioactive (endogenous) precursors as well. Evidence for increased activity of this conversion by sodium depletion has also been obtained in studies with rats in vivo (29) and with isolated rat adrenal mitochondria (30, 31).

Sodium depletion, therefore, appears to increase aldosterone secretion by stimulation of at least two sites in the biosynthetic pathway. It is possible that more than two enzymatic steps are affected by sodium depletion. The in vitro studies did not reveal any potentiation of 11- or 21-hydroxylation in slices from sodium-depleted animals. Thus, saturating quantities of progesterone produced the same quantity of corticosterone in both types of slices. However, these enzymatic steps might be increased with more long-term sodium depletion, when cellular hyperplasia of the zona glomerulosa has occurred.

It is difficult to determine which step stimulated by acute sodium depletion is primarily responsible for the control of the rate of aldosterone synthesis; however, the increase of the conversion of corticosterone into aldosterone during sodium depletion does offer an explanation for the finding that ACTH, which acts early in the pathway of aldosterone formation (2), stimulates secretion of aldosterone more effectively in a sodium-depleted state (32).

It is clear that in the dog adrenal slices puromycin inhibits the biosynthesis of aldosterone at a site above that of progesterone. If it is acting, as is generally accepted, by prevention of protein synthesis, this indicates that protein synthesis is necessary for the normal regulation of aldosterone production. It is interesting that the major site of inhibition of aldosterone biosynthesis by puromycin is different in the dog and in the rat. The reason is not clear at present; it may indicate that the sites of control of aldosterone biosynthesis are different in the rat and in the dog. The lack of influence of the renin-angiotensin system in regulation of aldosterone secretion in the rat (29, 33, 34) may be related to this difference.

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