Studies on Aldosterone Biosynthesis *in Vitro*. II*

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ABSTRACT: Effect of heparin, Ca^{2+}, Na^+, and K^+ on the *in vitro* biosynthesis of 18-hydroxycorticosterone and aldosterone by bullfrog adrenal mitochondria has been studied, using radioactive corticosterone as the substrate. Calcium (3.5 mM) had a significant stimulatory effect, while heparin (0.13 μM and higher) had a very marked inhibitory effect on the conversion of the substrate into 18-hydroxycorticosterone and aldosterone. This inhibitory effect of heparin has been studied in detail, and it appears that the aldosterone-suppressive effect of heparin is, at least partially, related to its effectiveness as a Ca^{2+} binder. Presence of Na^+ higher than 100 mM had an inhibitory effect, while K^+ higher than 65 mM had a less pronounced stimulatory effect on the *in vitro* conversion of corticosterone into 18-hydroxycorticosterone and aldosterone. These effects can be seen even in the presence of Ca^{2+}, and can be of significance in controlling aldosterone biosynthesis.

Increased secretion of aldosterone[1] in several clinical disorders and its role in their pathogenesis (Conn, 1963; Laragh *et al.*, 1964; Hudson *et al.*, 1957; Davis 1964) have made it attractive to search for means of blocking its production, secretion, and/or action. There have been two approaches to this problem: (1) to inhibit the aldosterone effect on sodium reabsorption by the renal tubule; and (2) to inhibit the biosynthesis or secretion of aldosterone by the adrenal cortex.

Under the first approach 17-spirolactone steroids (Figure 1) (Kagawa *et al.*, 1957; Kagawa, 1960a; Liddle, 1957; Beyer, 1960), besides progesterone (Landau *et al.*, 1955; Landau and Lugibihil, 1958), were found to block the effect of aldosterone on the tubular transport of electrolytes (Kagawa *et al.*, 1959; Coppage and Liddle, 1960; Bartter, 1960). The spirolactones do not inhibit the secretion of aldosterone or other corticosteroids (Coppage and Liddle, 1960). In fact, their antagonizing effect can be blocked by deoxycorticosterone acetate (Kagawa, 1960b); a compensatory increase in aldosterone secretion rate, following administration of spirolactone, SC-8109 (3-(3-keto-17β-hydroxy-19-nor-4-androsten-17α-yl)propionic acid-γ-lactone), has also been reported (Singer, 1959).

Under the second approach, various inhibitors of steroid biosynthesis (Hertz *et al.*, 1955; Renold *et al.*, 1957; Mach and Muller, 1957; Chart and Sheppard, 1959, 1964), particularly pyridine derivatives, have attracted special attention (Neher and Kahnt, 1963; Gaunt *et al.*, 1960; Raman *et al.*, 1966) owing to the remarkable success of metopirone (SU-4885). However, these compounds have not been used for aldosterone suppression in humans due to their effect not only on the biosynthesis of aldosterone, but on other corticosteroids also. The 4 isomer of metopirone (SU-5482) (1,2-bis(4-pyridyl)-2-methyl-1-propanone) has been reported to block 18-hydroxylation preferentially (Neher and Kahnt, 1963), but it could not be used in humans owing to toxicity[2] (Figure 1).

On the other hand, heparin and some related polysulfated polysaccharides have been used successfully by several workers for the suppression of aldosterone (Godlowski, 1933; Beiglbock *et al.*, 1952; Raynaud *et al.*, 1952; Engelberg *et al.*, 1952; Schlatmann *et al.*, 1960, 1964; Cejka *et al.*, 1960; Wilson and Goetz, 1964). It has been reported that heparin inhibits aldosterone production and lowers plasma aldosterone (Bailey and Ford, 1965; Gláz and Sugar, 1964). The mechanism by which this is mediated has been of concern.

Earlier we have reported the biosynthesis of 18-hydroxycorticosterone and aldosterone from progesterone, 11-deoxycorticosterone, and corticosterone by the adrenals of cow, guinea pig, sheep, and human with primary aldosteronism (Raman *et al.*, 1965, 1966). In the present study the effects of heparin, calcium, sodium, and potassium on the conversion of corticosterone to 18-OH-corticosterone and aldosterone have been investigated in detail in the frog adrenal.

Experimental Section

Substrates. Corticosterone-1,2-^{3}H (sp act. 15 c/

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[1] Abbreviations used: aldosterone, 11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al-(18→11)-hemiacetal; aldactone, spironolactone (SC-9420), 3-(3-keto-7α-acetylthio-17β-hydroxy-4-androsten-17γ-yl)propionic acid-γ-lactone; metopirone (SU-4885), 1,2-bis(3-pyridyl)-2-methyl-1-propanone; 18-OH-corticosterone or 18-hydroxycorticosterone, 11β,18,21-trihydroxypregn-4-ene-3,20-dione; aldosterone diacetate, 18,21-diacetoxy-11β-hydroxy-3,20-dioxo-4-pregnen-18-al-(18→11)-hemiacetal; lactone of 18-OH-corticosterone-11β,18-dihydroxy-3-keto-4-etienic acid lactone; TPN^+ and TPNH, oxidized and reduced triphosphopyridine nucleotides; glucose-6-P, glucose 6-phosphate.

[2] J. J. Chart, personal communication.

mmole), D-aldosterone-4-^{14}C (sp act. 40 mc/mmole), and corticosterone-4-^{14}C (sp act. 40 mc/mmole) were obtained from New England Nuclear Corp. and checked for homogeneity by paper chromatography in the Bush B_5 system (Bush, 1952) before use. Authentic radioactive 18-OH-corticosterone prepared from corticosterone-1,2-^{3}H was kindly supplied by Drs. S. Ulick and G. L. Nicolis, and also prepared biosynthetically in this laboratory (Raman *et al.*, 1966); the identity of these compounds was further established by oxidizing a portion of them into the lactone of 11β,18-dihydroxy-3-keto-4-etienic acid by periodic acid (Péron, 1961). Nonradioactive corticosterone and D-aldosterone were obtained from Mann Research Laboratory, N. Y.

Cofactors and Other Chemicals. TPN$^+$, "98% pure," glucose-6-P, "98% pure," and TPNH enzymatically reduced "90–95%" as sodium salts were obtained from Sigma Chemical Co. Heparin (B grade, lot 52832, 141 U.S.P. units/mg) was obtained from Calbiochem, Los Angeles; for various calculations the molecular weight was taken to be 12,000 and the formula weight of the repeating disaccharide unit was taken as 583 (Helbert and Marini, 1963). Silica gel (activated, grade 923, mesh size 100–200) was obtained from Grace-Davison Chemical Co., Baltimore. Periodic acid (H_5IO_6) crystalline and other chemicals were obtained from Matheson Coleman and Bell, Cincinnati, Ohio.

Adrenal Tissue and Incubation Procedure. Bullfrogs obtained from various sources were kept in fresh water until the adrenal tissue was needed for experiments (usually 2–4 days). The adrenal glands were obtained by careful dissection after pithing the animal; in general, about 1 g of fresh tissue was available from 20 to 24 animals. Immediately on removal, the tissue was placed on ice and then homogenized with five times its weight of 0.25 M sucrose in a motor-driven Potter-Elvehjem glass homogenizer, and the mitochondrial fraction was obtained by differential centrifugation (Sharma *et al.*, 1962). The mitochondria were resuspended in Tris-HCl buffer (0.05 M, pH 7.3) and a portion of this preparation, equivalent to about 100 mg of the fresh adrenal tissue, was added to each of the incubation flasks.

Besides the enzyme preparation and other additions mentioned in individual experiments, each incubation flask contained corticosterone-1,2-^{3}H (1.5 μc), nonradioactive corticosterone (15 mμmoles), TPN$^+$ (3 μmoles), glucose-6-P (16 μmoles), glucose-6-P dehydrogenase (2 Kornberg units), and Tris-HCl buffer (pH 7.3, 150 μmoles) in a total volume of 3 ml. The substrates were added to the incubation flasks in 0.05 ml of propylene glycol (propane-1,2-diol) before other additions. Incubations were done in air at 37° for 60 min in a Dubnoff metabolic shaker.

Extraction, Characterization, and Estimation of Products. After incubation the steroids were extracted from the incubation media with 60 ml (three 20-ml portions) of methylene chloride and the pooled extract was evaporated to dryness in a flash evaporator. The residue was dissolved in 10 ml of methylene chloride, a suitable aliquot was taken for radioactivity, and the remainder was chromatographed on a 5-g silica gel column (1 × 24 cm). The column was first eluted with 20–25 ml of methylene chloride–methanol (97:3) and then with 25 ml of methylene chloride–methanol (85:15). The steroids were eluted in the latter fraction, the recovery of radioactivity was 96–100%. The eluate, after determining the radioactivity in an aliquot, was chromatographed in the Bush B_5 system (Bush, 1952).

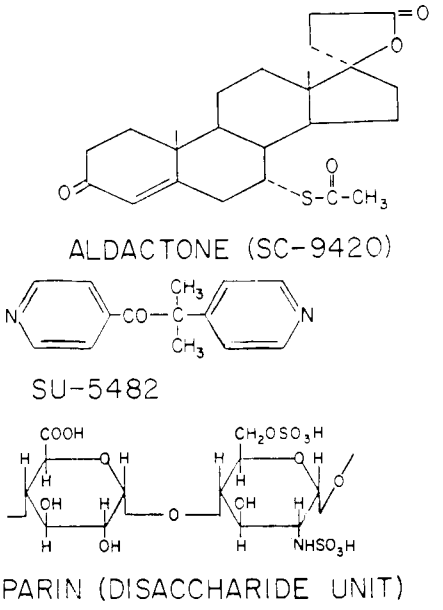

FIGURE 1: Inhibitors of aldosterone.

Both 18-OH-corticosterone and aldosterone were characterized by oxidizing a portion with periodic acid into lactones (Péron, 1961; Nicolis and Ulick, 1965; Bush, 1961) and the remainder was acetylated. Authentic standards to check the chromatographic mobilities and recoveries were used in all experiments. Details of chromatographic systems, mobilities of standard compounds, and determination of radioactivity have been reported earlier (Raman *et al.*, 1965; Sharma *et al.*, 1965).

Acetylation Procedure. An aliquot of the product corresponding to 18-OH-corticosterone or aldosterone was placed in 0.2 ml of pyridine and treated with 0.5 ml of acetic anhydride at 60° for 90 min. The reaction products along with authentic standards were chromatographed in the Bush B_3 system (Bush, 1952) (Figure 2).

Periodic Acid Oxidation Procedure. Dried steroid fractions were dissolved in 0.25 ml of ethanol in a 13-ml, glass-stoppered centrifuge tube and an equal volume of 0.1 M periodic acid solution in 2% pyridine was added. The reaction mixture was allowed to stand overnight in the dark. The products were extracted with methylene chloride (three 5-ml portions). The combined methylene chloride extracts were washed with 2% sodium bicarbonate solution (two 1-ml portions) and then with water (two 1-ml portions). The bicarbonate and aqueous washings were combined and extracted once with 5 ml of methylene chloride.

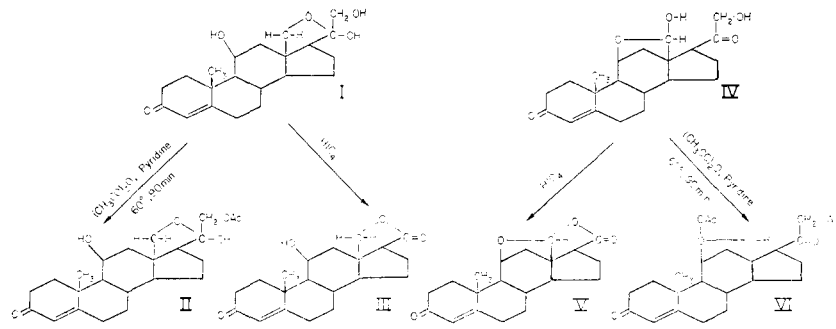

FIGURE 2: Characterization of 18-OH-corticosterone (I) and aldosterone (IV), (II) 21-acetoxy-18-OH-corticosterone (III) lactone of 11β,18-dihydroxy-3-keto-4-etienic acid, (V) lactone of etienic acid from aldosterone, and (VI) aldosterone diacetate.

The pooled methylene chloride extract (20 ml) was evaporated to dryness under reduced pressure and the residue was chromatographed in the Bush B_3 system (Figure 2).

Results

Effect of Calcium. Mitochondria from bullfrog adrenals were incubated with corticosterone-1,2-^{3}H in the presence of a TPNH-generating system as described under Experimental Section. The effect of added Ca^{2+} up to a concentration of 33.3 mM is shown in Figure 3. Biosynthesis of both 18-OH-corticosterone and aldosterone was increased by the presence of Ca^{2+} up to a concentration of 7 mM; at 3 mM concentration the increase was about sixfold, while at concentrations above 12 mM the stimulatory effect was small. Results of another experiment are summarized in Table I; maximum stimulation (sevenfold) was again at about 3.3 mM concentration.

Effect of Heparin. Heparin had a very marked inhibitory effect on the *in vitro* biosynthesis of 18-OH-corticosterone and aldosterone from corticosterone (Figure 4); the approximate first-order plot indicates that for the range of heparin concentration studied, the inhibition of the formation of 18-OH-corticosterone and aldosterone from corticosterone is directly proportional to the concentration of heparin. In another experiment a concentration of about 0.7 μM was sufficient to reduce the aldosterone biosynthesis to one-third and the 18-OH-corticosterone levels to about one-half of the control levels (Table II).

Effect of Heparin and Calcium. Heparin and other mucopolysaccharides have affinity for cations. This affinity is dependent on the structure of the polysaccharide (Mathews, 1960, 1964) and the cation (Dunstone, 1962); among the cations of constant ionic strength a marked selectivity is also shown (Mathews, 1964). Since conversion of corticosterone into 18-OH-corticosterone and aldosterone is stimulated by Ca^{2+} and since heparin has greater affinity for Ca^{2+} (Dunstone, 1962) experiments were done to investigate whether the inhibitory effect of heparin can be compensated by adding a calculated amount of additional Ca^{2+}.

These calculations were based on the linear form

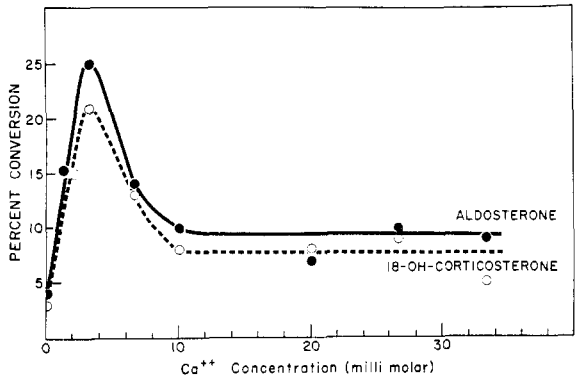

FIGURE 3: Effect of Ca^{2+}. Mitochondria from 100 mg of bullfrog adrenals were incubated with corticosterone-1,2-^{3}H (1.1 μc, 5 μg) for 60 min in air. Other conditions are given in the text.

TABLE I: Effect of Added Calcium.[a]

Addition Ca^{2+} (mM)	Products (% conversion)	
	18-OH-Corticosterone	Aldosterone
None	3.4	2.7
1.7	23.0	10.1
3.3	30.5	20.0
10.0	11.3	8.9

[a] Mitochondria from 58 mg of frog adrenals were incubated with corticosterone-1,2-^{3}H (1.14 μc) and 5 μg of nonradioactive corticosterone in the presence of a TPNH-generating system in a total volume of 3 ml. Details under Experimental Section.

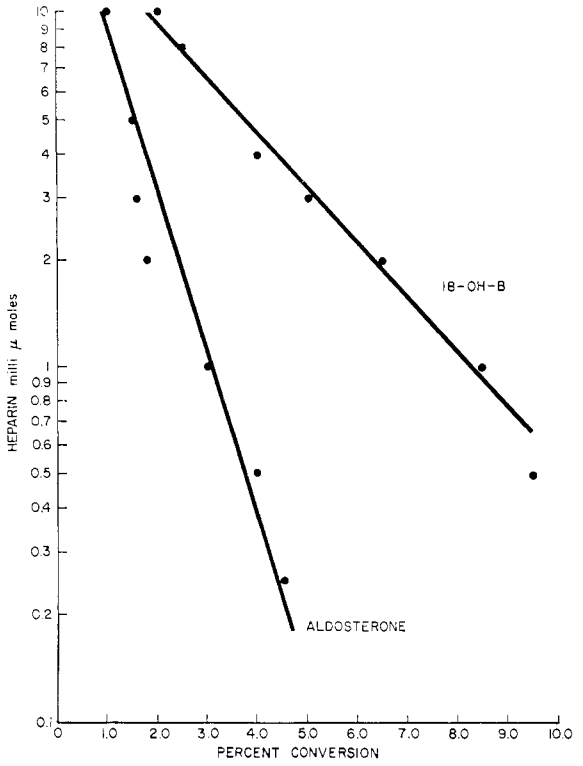

FIGURE 4: Effect of heparin. Mitochondria from 100 mg of bullfrog adrenals were incubated with corticosterone-1,2-^{3}H (1.6 μc, 5 μg) for 60 min in air. Other conditions are given in the text.

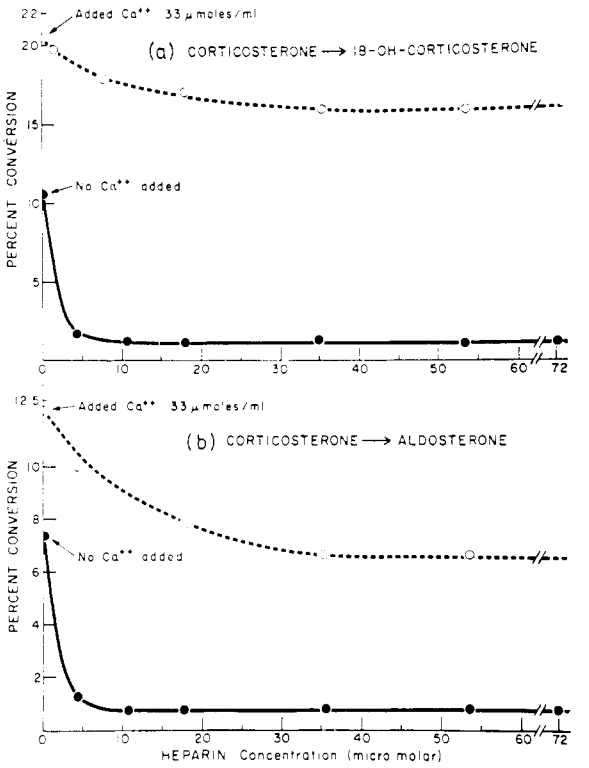

FIGURE 5: Effect of heparin and Ca^{2+}. Mitochondria from 100 mg of bullfrog adrenals were incubated with corticosterone-1,2-^{3}H (1.6 μc, 5 μg) in presence of a TPNH-generating system in air. Other conditions are given in the text.

of the Langmuir adsorption isotherm (Langmuir, 1918)

$$\frac{1}{r} = \frac{1}{nk}\frac{1}{[\text{Me}]} + \frac{1}{n}$$

where r = moles of bound cation per total concentration of the repeating disaccharide units; n = binding sites per repeating period = 1; k = stability constant of the complex = 147; and [Me] = Ca^{2+} concentration.

It has been used earlier in studies on the binding of cations by heparin and other mucopolysaccharides (Mathews, 1960, 1964; Dunstone, 1962; Buddecke and Drzenick, 1962).

The results of the experiments with heparin and Ca^{2+} are shown in Figure 5. Heparin alone (5 μM) inhibited the biosynthesis of 18-OH-corticosterone and aldosterone by more than 80%. On addition of Ca^{2+} (33 mmoles), even at a much higher concentration of heparin (72 μM), the conversion of corticosterone into 18-OH-corticosterone and aldosterone could be maintained comparable to the controls which contained no heparin or Ca^{2+} (Figure 5).

Effect of Sodium. The effect of the presence of increasing concentrations of Na^+ in the incubation media on the conversion of corticosterone into 18-OH-corticosterone and aldosterone is shown in Figure 6. It is apparent that Na^+ concentrations above 300 mM have an inhibitory effect on the formation of aldosterone, whereas the effect on the formation of 18-OH-corticosterone is less pronounced.

The effect of increasing Na^+ concentration in the

TABLE II: Effect of Heparin.[a]

Heparin (μM)	Products (% conversion)	
	18-OH-Corticosterone	Aldosterone
None	12.0	6.0
0.13	10.0	3.0
0.43	12.0	3.0
0.67	7.0	2.0
1.00	5.0	2.0
1.67	3.0	2.0
2.83	3.0	1.0

[a] Mitochondria from 100 mg of frog adrenals were incubated with corticosterone-1,2-^{3}H (1.52 μc, 5.01 μg) in a total volume of 3 ml.

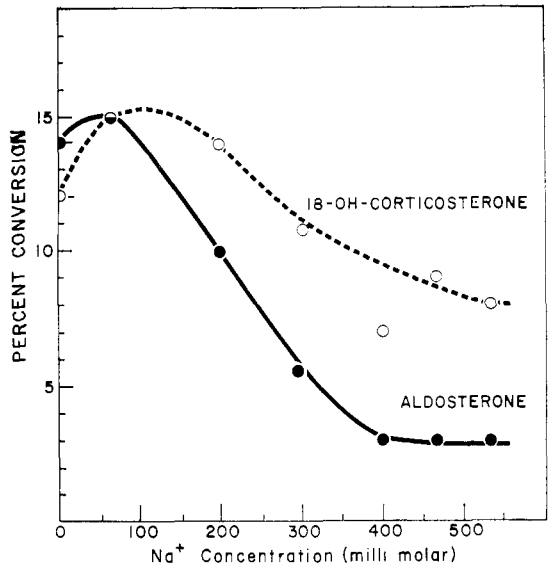

FIGURE 6: Effect of Na^+. Mitochondria from 62 mg of the adrenal tissue were incubated with corticosterone-1,2-^3H (1.5 μc, 5 μg) in presence of a TPNH-generating system. Other conditions are given in the text.

presence of 3.3 mM Ca^{2+} on *in vitro* aldosterone biosynthesis was also studied (Table III); again there was a decline with increasing Na^+ concentration in the incubation media.

Effect of Potassium. Table IV summarizes the effect of the presence of K^+ on the conversion of corticosterone in 18-OH-corticosterone and aldosterone. The data indicate that increased levels of K^+ have slight stimulatory effects on aldosterone biosynthesis although it is not as pronounced as the effect of Ca^{2+}. It may be pointed out that due to the nonavailability of potassium salts, sodium salts of the cofactors were used, thus each of the incubation flasks contained some Na^+ (30 μmoles) also.

TABLE III: Effects of Added Na^+ and Ca^{2+}.[a]

Addition (mM)		Products (% conversion)	
Ca^{2+}	Na^+	18-OH-Corticosterone	Aldosterone
3.0	None	15.8	4.9
3.0	200	11.8	2.4
3.0	400	7.8	1.3
3.0	530	10.0	1.3
3.0	600	10.5	1.3

[a] Mitochondria from 49 mg of frog adrenals were incubated with corticosterone-1,2-^3H and 5 μg of nonradioactive corticosterone in presence of a TPNH-generating system and 150 μmoles of Tris-HCl buffer (pH 7.3) in a total volume of 3 ml.

TABLE IV: Effect of Added K^+.

Expt	Addition (mM) K^+	Products (% conversion) 18-OH-Corticosterone	Aldosterone
27[a]	None	1.2	1.4
	10	2.3	0.9
	20	2.2	0.9
	60	3.0	0.7
	120	2.6	1.8
	160	3.2	2.8
30[b]	None	3.4	3.0
	20	3.2	3.9
	100	4.4	4.2
	200	5.8	10.6
	300	3.3	3.3
	400	4.9	3.4
	500	4.2	2.8
33[c]	None	4.5	4.2
	80	5.5	4.3
	120	4.9	2.5
	160	6.0	3.5
	200	9.7	5.5
	260	7.8	7.3

[a] Experiment 27: Mitochondria from 70 mg of frog adrenals were incubated with 1.6 μc (5.01 μg) of corticosterone-1,2-^3H. [b] Experiment 30: Mitochondria from 77 mg of frog adrenals were incubated with 1.8 μc (5.01 μg) of corticosterone-1,2-^3H. [c] Experiment 33: Mitochondria from 100 mg of frog adrenals were incubated with 1.6 μc (5.01 μg) of corticosterone-1,2-^3H. Other conditions were as described under Experimental section.

The effect of increasing K^+ concentration in presence of Ca^{2+} on the conversion of corticosterone into 18-OH-corticosterone and aldosterone is summarized in Table V.

Discussion

The conversion of corticosterone to 18-OH-corticosterone and aldosterone by the mitochondria from bullfrog adrenals was much higher than by a similar preparation from the adrenal cortices of cow, sheep, and guinea pig reported earlier (Raman *et al.*, 1966). Recently Psychoyos *et al.* (1966) have also observed higher enzyme(s) activity in the mitochondria from bullfrog adrenals and suggested that the lower activity of the bovine and rat adrenal gland is due to the presence of a heat-labile inhibitor.

As in the case of rat (Péron, 1962), guinea pig, sheep,

TABLE V: Effect of Added Ca^{2+} and K^+.[a]

Additions (mM)		Products (% conversion)	
Ca^{2+}	K^+	18-OH-Corticosterone	Aldosterone
None	None	11.6	14.1
3.3	None	22.2	34.0
3.3	33.3	24.2	36.5
3.3	66.6	30.1	42.0
3.3	100.0	33.6	39.0

[a] Mitochondria from 208 mg of frog adrenals were incubated with corticosterone-1,2-^{3}H (1.46 μc) and 5 μg of nonradioactive corticosterone in presence of a TPNH-generating system. Other conditions as described under Experimental Section.

cow (Raman *et al.*, 1966), and human adrenals (Raman *et al.*, 1965), TPNH fulfilled the cofactor requirement for the *in vitro* conversion of corticosterone to 18-OH-corticosterone and aldosterone by the bullfrog adrenal mitochondria. The need for fumarate reported by Psychoyos *et al.* (1966) appears to be concerned with the formation of TPNH, suggested by Grant (1956) in his studies on 11β-hydroxylation. The cofactor need for *in vitro* C-17 (Hofmann, 1960), C-21 (Ryan and Engel, 1957), and other steroid hydroxylations (Hayano, 1962) has also been found to be TPNH and molecular oxygen.

The experiments reported here, as well as several others not included, demonstrate a stimulatory effect of Ca^{2+} up to a certain concentration, maximum stimulation being approximately sixfold at about 3 mM concentration; at 14 mM and higher concentrations the stimulatory effect is much less. Thus the variation in the levels of Ca^{2+} stimulation reported in earlier studies (Péron, 1964; Nicolis and Ulick, 1965; Raman *et al.*, 1966; Psychoyos *et al.*, 1966) is probably due to the differences in the concentration of Ca^{2+} in the incubation media.

The efficacy of heparin in reducing blood pressure (Godlowski, 1933), renal hypertension in humans (Beiglbock *et al.*, 1952; Keller, 1957) and experimental animals (Keller and Wasser, 1954; Hardegg *et al.*, 1956), and alleviating edema in patients with nephrotic syndrome or cardiac failure (Raynaud *et al.*, 1952; Engelberg *et al.*, 1952) and nephrotic rats (Rosenman *et al.*, 1954; Rosen *et al.*, 1954) is related to its effect of increasing Na^+ and water excretion and K^+ retention (Majoor *et al.*, 1957; Hoppeler *et al.*, 1959; Schlatmann *et al.*, 1960, 1964).

That heparin exerts its natriuretic and diuretic effect by decreasing aldosterone levels in the body is shown by the fact that its administration leads to a fall in urinary aldosterone excretion (Schlatmann *et al.*, 1960, 1964; Cejka *et al.*, 1960; Bailey and Ford, 1965). Gláz and Sugar (1964) observed that heparin reduces the *in vivo* biosynthesis of aldosterone without decreasing the synthesis of other corticoids in rats. A possible direct action of heparinoids on adrenal cells has been suggested by Schlatmann *et al.* (1964) also.

In the present studies, conversion of corticosterone into 18-OH-corticosterone and aldosterone was inhibited by heparin. A similar inhibitory effect on aldosterone biosynthesis by the adrenocortical adenoma removed from a 29-year-old woman with primary aldosteronism was observed earlier (Sharma *et al.*, 1966). Detailed investigation of the inhibitory effect of heparin showed that the inhibition could be overcome by adding calculated amounts of Ca^{2+} to the *in vitro* system (Figure 5). Buddecke and Drzenick (1962) have determined the stability constants of calcium complexes of acid mucopolysaccharides under physiological conditions; these authors have suggested that the high Ca^{2+} binding ability of heparin probably plays an important regulatory function in biological systems by controlling certain enzymatic processes.

The use of Krebs–Ringer bicarbonate buffer which already contains 0.11 M Ca^{2+} would explain the *in vitro* results of Gláz and Sugar (1964), although they did suggest the possibility that heparin or one of its metabolites could be acting through the enzyme 18-hydroxlase. Wilson and Goetz (1964) also on the basis of their study of selective hypoaldosteronism after heparin administration and a review of the studies of Cejka *et al.* (1960, 1962) observed that heparin interferes with the addition of the aldehyde group during the synthesis of aldosterone. This is supported by the fact the inhibitory effect of heparin is limited to the production of aldosterone without reducing the biosynthesis of other corticosteroids (Veyrat, 1962).

In view of these studies and the data presented here heparin probably exerts its aldosterone suppressive action, at least in part, by inhibiting aldosterone biosynthesis, a possibility suggested by Gálz and Sugar (1964) and also Schlatmann *et al.* (1964). The latter workers have suggested that renin–angiotensin system may also be affected. Experimental evidences for this mechanism are conflicting. *In vitro* studies of Sealey *et al.* (1967) indicate that heparin can inhibit renin but Kloppenberg and his associates (1965) were unable to restore aldosterone secretion which was depressed by heparin administration, by angiotensin infusion. Wilson and Goetz (1964) were also unable to counteract the aldosterone suppressive action of heparin by intravenous infusion of angiotensin.

The *in vitro* effect of sodium and potassium ions on aldosterone biosynthesis appears to support the general concept of a regulatory system. In a regulatory system, there must be a stimulus to initiate operation of the system; if aldosterone controls the ratio of Na^+ to K^+ in the extracellular fluid, lowering or increasing of these ionic constituents can be expected to increase or decrease the rate of the hormone secretion by the adrenal cortex. *In vitro* experimental data seem to support this view; increasing Na^+ concentrations

had an inhibitory effect while increasing K^+ concentrations had a slight stimulatory effect, although not as pronounced. However, other mechanisms for the effect of Na^+ and K^+ on circulating aldosterone levels cannot be precluded.

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The Isolation and Characterization of Asperenone, a New Phenylpolyene from *Aspergillus niger**

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ABSTRACT: Asperenone, a new pigment from the vegetative mycelium of *Aspergillus niger*, has been isolated as a crystalline solid by solvent extraction and chromatographic procedures. Nuclear magnetic resonance, mass, and infrared spectroscopy of asperenone and its decahydro derivative shows asperenone to be a methyl derivative of 13-phenyltrideca-4,6,8,10,12-pentaen-3-one. The methyl group is located on one of the positions 5–11 and is tentatively suggested to be at position 8 on the basis of the abundance of hydrocarbon fragments in the mass spectra. The occurrence of two other substances with absorption spectra suggesting polyene chromophores in extracts of *A. niger* mycelium is also reported.

An accompanying paper (Jefferson, 1967) describes the accumulation of asperenone, a yellow pigment, when *Aspergillus niger* mycelium in replacement cultures is treated with small amounts of certain steroids. A spectroscopically identical pigment accumulates when the organism is grown or replaced on glycerol medium without added steroids. The present communication describes the isolation and characterization of this new metabolite of *A. niger* for which the name asperenone is proposed.

Materials and Methods

The organism used was *A. niger* NRRL-3. Cultures used for the isolation of the pigment were either grown, replaced, and treated with estradiol as described by Jefferson (1967) or were grown in glycerol medium consisting of the following per liter of tap water; glycerol, 20 ml; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; NH_4NO_3, 2.5 g; $ZnCl_2$, 0.52 mg; $FeSO_4 \cdot 7H_2O$, 6.5 mg; $CuCl_2 \cdot 2H_2O$, 0.32 mg; yeast extract, 30 mg; and HCl (37%), 0.5 ml. For small-scale production, 500 ml of the glycerol medium in 2-l. culture flasks was sterilized by autoclaving and inoculated with conidia. Incubation was at 26° on a reciprocating shaking machine. For larger scale production, 16 l. of unsterilized medium in carboys was inoculated with 300 ml of 48-hr cultures grown on sterile sucrose medium. The larger cultures were vigorously aerated by means of sintered-glass cylinders.

After 3–4 days, the pigmented mycelium was filtered through cheesecloth in a Buchner funnel, washed several times with water, and partially dried by covering the funnel with a rubber sheet (dental dam) while continuing the aspiration. Drying was completed at 50° in an oven equipped with a blower. The dried mycelium was stored in a refrigerator and ground immediately prior to extraction.

Extracts and samples were stored at refrigerator temperatures. Exposure to bright light was avoided.

Thin layer chromatographic plates were prepared by the dipping method of Peifer (1962) using silica gel G as adsorbent. They were developed with 25% ethyl ether in petroleum ether (bp 30–60°). Spots were observed under ultraviolet light and after spraying with concentrated sulfuric acid and heating.

Florisil and silicic acid columns were prepared from petroleum ether slurries of the adsorbents. Samples dissolved in petroleum ether were added to the columns. Development was with varying amounts of U.S.P. ethyl ether (usually 20% v/v) in petroleum ether.

Countercurrent distribution employed a stationary phase of 85%, v/v, aqueous methanol equilibrated with a mobile phase of petroleum ether.

Visible and ultraviolet spectra obtained with a

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