# Studies on Aldosterone Biosynthesis *in Vitro**

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ABSTRACT: Biosynthesis of 18-hydroxycorticosterone and aldosterone from corticosterone by bovine, guinea pig, and particularly sheep adrenal tissue has been studied. Both "18-hydroxylase" and "18-ol-dehydrogenase" were located mainly in the mitochondrial fraction.

Reduced triphosphopyridine nucleotide (TPNH) and not reduced diphosphopyridine nucleotide (DPNH) was the cofactor for C-18 hydroxylation; presence of oxidized triphosphopyridine ($TPN^+$) or diphosphopyridine nucleotide ($DPN^+$) in the incubation media resulted in the conversion of 18-hydroxycorticosterone into 18-hydroxy-11-dehydrocorticosterone and not into aldosterone. $Ca^{2+}$ stimulated the conversion of corticosterone into 18-hydroxycorticosterone. SU 4885 [1,2-bis(3-pyridyl)-2-methyl-1-propanone] and SU 9055 [3-(1,2,3,4-tetrahydr-1-oxo-2-naphthyl)pyridine] had inhibitory effect on C-18-hydroxylation while SU 8000 [3-(6-chloro-3-methyl-2-indenyl)pyridine] inhibited "18-ol dehydrogenase." *N*-Ethylmaleimide at lower concentrations inhibited the conversion of 18-hydroxycorticosterone into 18-hydroxy-11-dehydrocorticosterone without affecting the biosynthesis of 18-hydroxycorticosterone and aldosterone; other sulfhydryl inhibitors affected both "18-hydroxylase" and "18-ol dehydrogenase" by varying degrees. Although aldosterone had no effect on the conversion of corticosterone into 18-hydroxycorticosterone or of the latter into 18-hydroxy-11-dehydrocorticosterone, "18-hydroxylase" was inhibited by the intermediate 18-hydroxycorticosterone. The formation of 18-hydroxy-11-dehydrocorticosterone and the inhibiting effect of 18-hydrocorticosterone appears to be of significance in the control of aldosterone biosynthesis.

Progesterone, 11-deoxycorticosterone,[1] and corticosterone have been shown to be precursors of aldosterone biosynthesis (Wettstein *et al.*, 1954; Kahnt *et al.*, 1956; Travis and Farrell, 1958; Ulick and Solomon, 1960; Sandor and Lanthier, 1963; Stachenko and Giroud, 1959). Among these steroids corticosterone appears to be the more immediate precursor (Ayres *et al.*, 1960). Although the steps in the conversion of corticosterone to aldosterone are not clear, it has been proposed that the most likely intermediate is 18-OH-corticosterone (Ulick and Kusch, 1960; Neher, 1964; Stachenko and Giroud, 1964). *In vitro* experiments with radioactive 18-OH-corticosterone (Pasqualini, 1964; Nicolis and Ulick, 1965) seem to support this view.

In humans 18-OH-corticosterone is a normal secretory product and its secretion is proportional to aldosterone (Ulick and Vetter, 1962). Visser and Cost (1964) suggested that an inborn aldosterone biosynthetic defect in children is most likely a defect in the 18-oxidation process from corticosterone to aldosterone. In patients with a salt-losing syndrome, the steroid pattern could be best explained by a defect in the dehydrogenation of 18-OH-corticosterone to aldosterone (Ulick *et al.*, 1964a,b). In the adrenocortical tumor from a patient of primary aldosteronism (Conn and Conn, 1961) also, the preferred pathway for aldosterone biosynthesis was found to be: progesterone → DOC → corticosterone → 18-OH-corticosterone → aldosterone (Raman *et al.*, 1965). A detailed investigation of the conversion of corticosterone to aldosterone by way of 18-OH-corticosterone is reported here.

## Experimental Procedure

*Substrates.* Progesterone-4-$^{14}$C (sp act. 29 mc/mmole), DOC-4-$^{14}$C (sp act. 35 mc/mmole), and corticosterone-1,2-$^3$H (sp act. 1.04 c/mmole) were obtained from New England Nuclear Corp. These radioactive substrates

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[1] Abbreviations and trivial names used: DEA, 3β-hydroxyandrost-5-en-17-one, dehydroepiandrosterone; DOC or 11-deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione; 18-OH-DOC or 18-hydroxy-11-deoxycorticosterone, 18,21-dihydroxy-4-pregnene-3,20-dione; 18-OH-corticosterone or 18-hydroxycorticosterone, 11β,18,21-trihydroxy-4-pregnene-3,20-dione; 18-hydroxy-11-dehydrocorticosterone, 18,21-dihydroxy-4-pregnene-3,11,20-trione; aldosterone diacetate, 18,21-diacetoxy-11β-hydroxy-3,20-dioxo-4-pregnen-18-al-18→11 hemiacetal; 18-hydroxy-11-dehydrocorticosterone monoacetate, 21-acetoxy-18-hydroxy-4-pregnene-3,11,20-trione; DETC, diethyldithiocarbamate; PCMB, *p*-mercuribenzoic acid as sodium salt; NEM, *N*-ethylmaleimide; FAA, formamidine acetate; IAA, iodoacetic acid as sodium salt; SU-4885 or metopirone, 1,2-bis-(3-pyridyl)-2-methyl-1-propanone; SU 8000, 3-(6-chloro-3-methyl-2-indenyl)pyridine; SU 9055, 3-(1,2,3,4-tetrahydro-1-oxo-2-naphthyl)pyridine; and SU-10603, 3-(1,2,3,4-tetrahydro-1-oxo-7-chloro-2-naphthyl)pyridine; TPNH and $TPN^+$, reduced and oxidized triphosphopyridine nucleotides; DPNH and $DPN^+$, reduced and oxidized diphosphopyridine nucleotides; FAD, flavin-adenine dinucleotide; FMN, flavin mononucleotide.

TABLE I: Biosynthesis of 18-OH-Corticosterone and Aldosterone by Cow, Sheep, and Guinea Pig Adrenals.[a]

| Substrate | μc | mμmoles | Species | % Conversion | |
|---|---|---|---|---|---|
| | | | | 18-OH-Corti-costerone | Aldosterone |
| Progesterone-4-$^{14}$C | 1.22 | 42.1 | Cow | 1.50 | 0.96 |
| | 1.36 | 46.9 | Sheep | 3.10 | 1.60 |
| | 1.10 | 37.9 | Guinea pig | 0.34 | 0.16 |
| DOC-4-$^{14}$C | 1.10 | 31.4 | Cow | 2.40 | 1.20 |
| | 1.05 | 30.0 | Sheep | 7.00 | 1.80 |
| | 1.05 | 30.0 | Guinea pig | 0.40 | 0.19 |
| Corticosterone-1,2-$^{3}$H | 4.0 | 43.2 | Sheep | 14.00 | 1.50 |

[a] In addition to the radioactive substrates, each incubation flask contained a homogenate of adrenal cortex (1 g), $TPN^+$ (3 μmoles), glucose-6-P (20 μmoles), glucose-6-P dehydrogenase (2 Kornberg units), and Krebs–Ringer bicarbonate buffer (2 ml) (pH 7.3) in a total volume of 3 ml. Incubation was for 90 min at 37° in air.

were checked for homogeneity in at least two paper chromatography systems.

Radioactive 18-OH-corticosterone was prepared biosynthetically from corticosterone-1,2,-$^{3}$H and was purified by paper chromatography in systems $B_5$ (Bush, 1952) and $E_2B$ (Eberlein and Bongiovanni, 1955). Its identity was further established by oxidizing a portion of it into lactone of 11β,18-dihydroxy-3-keto-4-etienic acid by periodic acid (Péron, 1961; Raman *et al.*, 1964).

18-Hydroxy-11-dehydrocorticosterone was prepared biosynthetically from 18-OH-corticosterone. It was also purified by paper chromatography in systems $B_5$ and $E_2B$ and its identity was confirmed by converting a portion to the lactone of 18-OH-11,3-diketo-4-etienic acid with periodic acid.

*Cofactors and Other Chemicals.* $TPN^+$ "98% pure," glucose-6-P "98% pure," and TPNH enzymatically reduced "90–95%," as sodium salts, α-$DPN^+$, β-$DPN^+$, Tris, formamidine acetate, *N*-ethylmaleimide, and sodium salt of iodoacetic acid were obtained from Sigma Chemical Co. L-Ascorbic acid, FAD, and FMN as their sodium salts were purchased from Nutritional Biochemical Corp. Sodium salts of diethyldithiocarbamate and *p*-mercuribenzoic acid were obtained from Matheson Coleman and Bell Co., Cincinnati, Ohio, and Mann Research Laboratory, N. Y., respectively.

SU 4885, SU 8000, SU 9055, and SU 10603 were generous gifts of Dr. J. J. Chart, Ciba Pharmaceutical Products, Inc., N. J. Other chemicals were obtained commercially.

*Adrenal Tissue.* The adrenals from cow and sheep were obtained as soon as possible, usually within 20 min, after slaughter and were transported from the slaughter house to the laboratory in ice. Guinea pig adrenals were removed immediately after sacrificing the animal. Subsequent operations mentioned below were carried out at 4°. Adhering fat and medulla were removed from the adrenals and the cortex tissue was homogenized with two times its weight of ice-cold 0.25 M sucrose solution in an all-glass homogenizer. The homogenate was centrifuged at 700*g* for 20 min to remove the cell debris and nuclear fraction. The supernatant was centrifuged at 6500*g* for 40 min to obtain the "mitochondrial fraction." After removing the "mitochondrial fraction," the supernatant was centrifuged for 60 min at 105,000*g* to obtain the "microsomal fraction" and the "supernatant fraction." The mitochondrial and the microsomal fractions were washed once in ice-cold 0.25 M sucrose and recentrifuged.

*Incubation and Extraction Procedures.* Incubations were done in air at 37° in a Dubnoff metabolic shaker. Krebs–Ringer bicarbonate buffer (pH 7.3) containing 200 mg of glucose/100 ml was used in these incubations. Unless otherwise mentioned, TPNH-generating system consisted of $TPN^+$ (3μmoles), glucose-6-P (20 μmoles), glucose-6-P dehydrogenase (2 Kornberg units), and buffer (2 ml)/fresh adrenal cortex tissue (g). The total volume of the incubation mixture was 3 ml. The substrates were added to the incubation flasks in 0.05 ml of propylene glycol (propane-1,2-diol) before other additions.

After incubation, the contents of the incubation flasks were transferred to a separatory funnel with 5 ml of water followed by 20 ml of acetone in five portions. The contents of the separatory funnel were then extracted three times with 20-ml portions of methylene chloride. The recovery of the radioactivity was between 90 and 95%.

*Procedures for Purification and Characterization of Steroids.* Methods for the purification and identification of aldosterone and 18-OH-corticosterone were similar to those described in detail earlier (Raman *et al.*, 1965). In the experiments summarized in Table I, radioactive 18-OH-corticosterone and aldosterone were characterized by converting them to suitable derivatives, *viz.*, etiolactone of 11β,18-dihydroxy-3-keto-4-etienic acid (Péron, 1961; Raman *et al.*, 1964) and diacetate of aldosterone. In other experiments, unless otherwise mentioned, the products were purified in $B_5$ and $E_2B$ systems (Bush, 1952). Authentic standards to check the

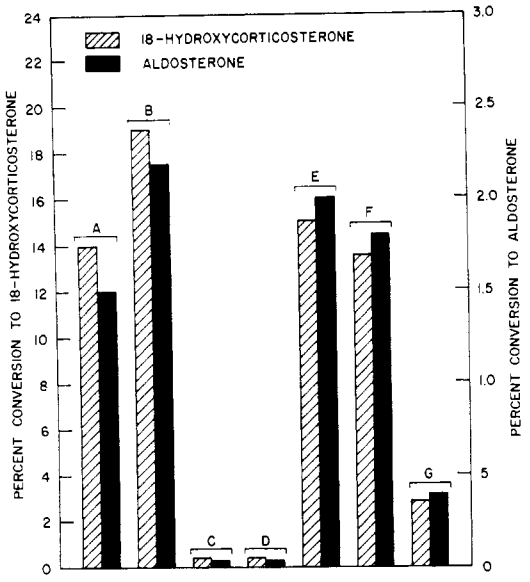


FIGURE 1: Conversion of corticosterone-1,2-$^3$H into 18-OH-corticosterone and aldosterone by different cell fractions and the effect of ultrasonic treatment on enzyme activity. (A) Whole homogenate; (B) "mitochondrial fraction;" (C) "microsomal fraction;" (D) "supernatant fraction;" (E) "mitochondrial fraction" after ultrasonic treatment for 90 sec; (F) 105,000*g* sediment of ultrasonic-treated mitochondria; and (G) supernatant fluid from (F) above. Each incubation flask contained corticosterone-1,2-$^3$H (2 $\mu$c) as substrate and the respective tissue fraction corresponding to 1 g of adrenal cortex in a total volume of 3 ml. Incubation was for 90 min at 37° in presence of the TPNH-generating system.

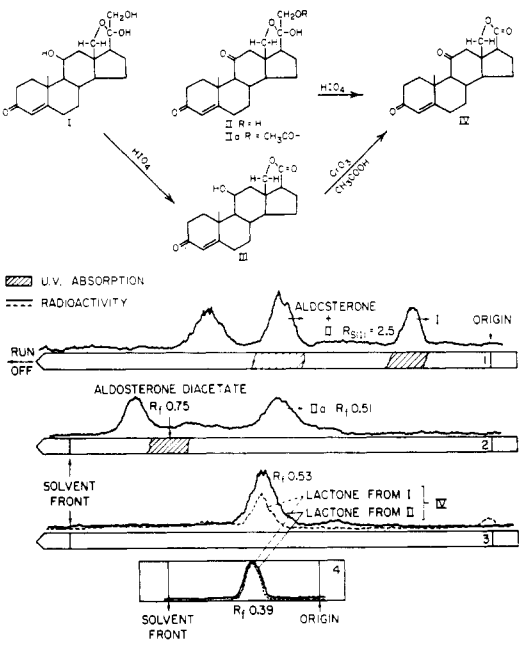


FIGURE 2: Characterization of 18-OH-11-dehydrocorticosterone (II), obtained on incubation of sheep adrenal mitochondria with 18-OH-corticosterone in the presence of TPN$^+$ or DPN$^+$. (I) 18-OH-Corticosterone; (II) 18-OH-11-dehydrocorticosterone; (IIa) 21-acetoxy-18-OH-11-dehydrocorticosterone; (III) lactone of 11$\beta$,-18-dihydroxy-3-keto-4-etienic acid; and (IV) lactone of 18-OH-11,3-diketo-4-etienic acid. Chromatogram 1. Separation of authentic aldosterone and incubation product II from the substrate I in Bush $B_5$ system. Chromatogram 2. Resolution of aldosterone diacetate from the acetate of II (IIa) in Bush $B_3$ system. Chromatograms 3 and 4 indicate that the lactone IV obtained from II has the chromatographic characteristics of the lactone IV obtained from 18-OH-corticosterone (I) by way of III in the Bush $B_3$ system and in the thin layer chromatography system, benzene–ethyl acetate, 1:2, respectively. Similar identity of chromatographic characteristics was observed in a toluene–propylene glycol system (impregnation with propylene glycol–methanol, 20:80), also.

chromatographic mobilities and recoveries were used in all experiments. Details of chromatographic systems, mobilities of standard compounds, methods for preparing derivatives, procedures for locating steroids, and measurement of radioactivity have been reported earlier (Raman *et al.*, 1965; Sharma *et al.*, 1965).

## Results

*Bovine, Sheep, and Guinea Pig Adrenals.* Homogenates of cow, sheep, and guinea pig adrenals were incubated separately with progesterone-4-$^{14}$C and DOC-4-$^{14}$C in the presence of a TPNH-generating system. Corticosterone-1,2-$^3$H was the substrate for sheep adrenals only. Results summarized in Table I indicate that sheep adrenals showed maximum conversion of the substrates into 18-OH-corticosterone and aldosterone followed by bovine and guinea pig adrenals. Since sheep adrenals showed maximum activity of "C-18-hydroxylase" and "18-ol-dehydrogenase," further studies were done with this tissue.

*Distribution of Enzyme Activities.* Whole homogenate and "mitochondrial," "microsomal," and "supernatant" fractions corresponding to 1 g of sheep adrenal cortex were incubated with corticosterone-1,2-$^3$H (4 $\mu$c) for 90 min. Figure 1 shows the per cent conversion of the substrate into 18-OH-corticosterone and aldosterone. It is apparent that "C-18 hydroxylase" and "18-ol dehydrogenase" are principally located in the "mitochondrial fraction." The per cent conversion of corticosterone-1,2-$^3$H into 18-OH corticosterone (19%) and into aldosterone (2.2%) by the "mitochondrial fraction" was comparable to the biosynthesis of these products by the whole homogenate.

Attempts were made to obtain "C-18 hydroxylase" activity in a soluble form by subjecting the mitochondria to ultrasonic vibrations.[2] However, on ultrasonic treat-

[2] "Sonifier S-125" with a tip of 0.5-in. diameter from Branson Instruments, Inc., Danbury, Conn., was used.

TABLE II: Cofactor Requirement for "C-18 Hydroxylase" and "18-ol-Dehydrogenase."[a]

| Substrate | Cofactors | Product | | |
|---|---|---|---|---|
| | | 18-OH-Corticosterone | Aldosterone | 18-OH-11-Dehydrocorticosterone |
| Corticosterone-1,2-$^3$H (1.8 $\mu$c) | None | 2.2 | 0.05 | 0 |
| Corticosterone-1,2-$^3$H (1.8 $\mu$c) | TPNH (generating system)[b] | 28.7 | 4.1 | 0 |
| Corticosterone-1,2-$^3$H (1.8 $\mu$c) | TPNH[c] | 10.6 | 1.5 | 1.9 |
| Corticosterone-1,2-$^3$H (1.8 $\mu$c) | DPNH[c] | 1.9 | 0.00 | 0 |
| 18-OH-Corticosterone-1,2-$^3$H (0.8 $\mu$c) | None | ... | 3.3 | 0 |
| 18-OH-Corticosterone-1,2-$^3$H (0.8 $\mu$c) | TPNH (generating system)[b] | ... | 2.1 | 0 |
| 18-OH-Corticosterone-1,2-$^3$H (0.8 $\mu$c) | TPNH[c] | ... | 1.7 | 1.1 |
| 18-OH-Corticosterone-1,2-$^3$H (0.8 $\mu$c) | TPN$^+$[c] | ... | 0 | 33.8 |
| 18-OH-Corticosterone-1,2-$^3$H (0.8 $\mu$c) | DPN$^+$[c] | ... | 0 | 35.0 |

[a] Mitochondria from 1 g of sheep adrenal cortex were incubated at 37° for 150 min with the substrate and the cofactor in a total volume of 3 ml. Krebs–Ringer bicarbonate buffer (2 ml) (pH 7.3) was used in each incubation flask. [b] Described under Experimental Procedure. [c] 3 $\mu$moles.

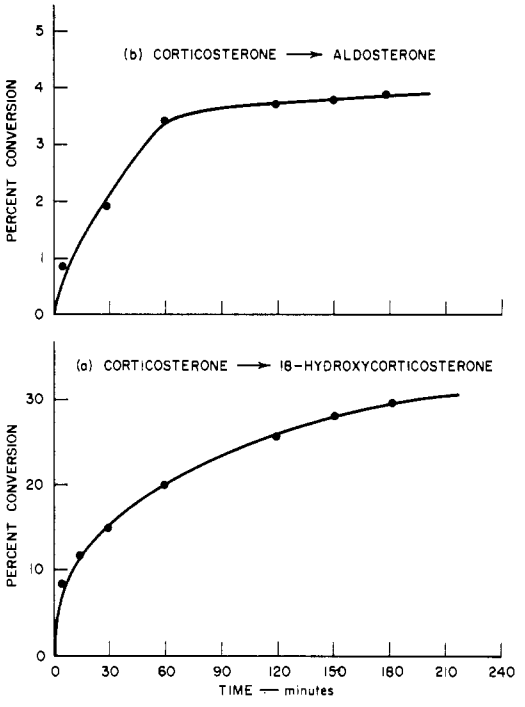


FIGURE 3: Time course of C-18 hydroxylation of corticosterone-1,2-$^3$H (a) and for the formation of aldosterone from corticosterone-1,2-$^3$H (b). Incubation conditions are given in the text.

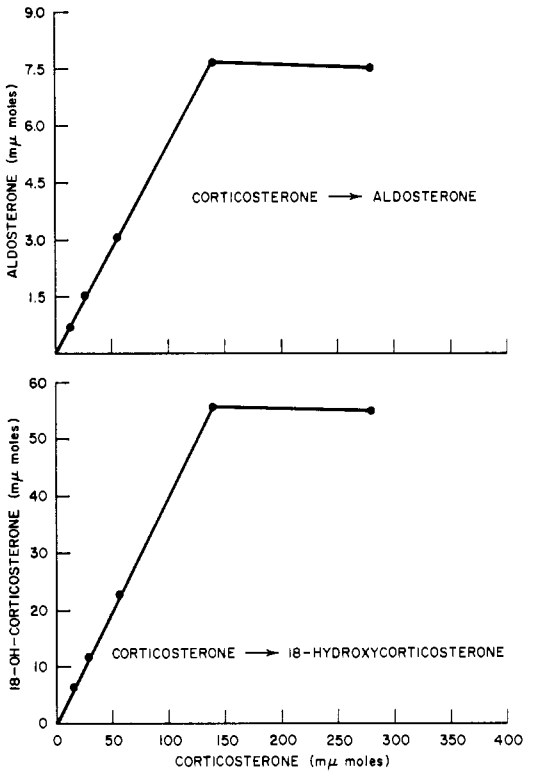


FIGURE 4: Effect of substrate (corticosterone) concentration on the formation of 18-OH-corticosterone and aldosterone. Mitochondria equivalent to 1 g of sheep adrenal cortex were incubated with corticosterone-1,2-$^3$H (2 $\mu$c) and different amounts of nonradioactive corticosterone for 150 min in air. Other conditions are given in the text.

ment of the mitochondria for 1, 5, 10, 15, and 20 min most of the C-18-hydroxylase activity remained with the sediment obtained on centrifugation at 105,000$g$ for 60 min. On prolonged exposure (30 min) to ultrasonic vibrations the "C-18-hydroxylase" activity was lost in the sediment also.

TABLE III: Effect of SU 4885, SU 8000, SU 9055, and SU 10603 on the Biosynthesis of 18-Hydroxycorticosterone and Aldosterone from Corticosterone.[a]

| Expt | Addn | Amount Added (mμmoles) | Product Formed (% of the Substrate) | | Inhibn (% of the Control) | |
|---|---|---|---|---|---|---|
| | | | 18-OH-B | Aldosterone | 18-OH-B | Aldosterone |
| | None | .. | 15.0 | 2.5 | .. | .. |
| | SU 4885 | 18 | 3.8 | 0.7 | 75 | 73 |
| | | 36 | 2.4 | 0.6 | 84 | 76 |
| | | 45 | 2.1 | 0.4 | 86 | 84 |
| | | 72 | 1.5 | 0.5 | 90 | 80 |
| I | SU 9055 | 18 | 5.6 | 1.0 | 63 | 62 |
| | | 36 | 4.2 | 0.6 | 72 | 76 |
| | | 45 | 3.7 | 0.3 | 75 | 88 |
| | | 72 | 2.5 | 0.33 | 84 | 87 |
| | SU 10603 | 45 | 14.3 | 2.2 | 12 | 5 |
| II | None | .. | 12.5 | 5.5 | .. | .. |
| | SU 8000 | 72 | 9.1 | 2.3 | 27 | 58 |

[a] In addition to the inhibitor each incubation flask contained: substrate corticosterone-1,2-$^3$H (2 μc) (72 mμmoles); $TPN^+$ (3 μmoles); glucose-6-P (5 μmoles); glucose-6-P dehydrogenase, 2 Kornberg units; Krebs–Ringer bicarbonate buffer (pH 7.3) (2 ml); washed mitochondria obtained from 1 g of sheep adrenal cortex, in a total volume of 3 ml. Incubation was for 135 min at 37° in air.

*Cofactor Requirements.* Data summarized in Table II show that C-18 hydroxylation of corticosterone is greatly increased in the presence of TPNH. Although the amount of 18-OH-corticosterone formed in the presence of exogenous TPNH was five times the control value, the per cent conversion was less than half as compared to when a TPNH-generating system was used; the ratio of aldosterone:18-OH-corticosterone (1:7) was the same in both instances. Addition of DPNH as a cofactor did not increase C-18 hydroxylation of corticosterone.

Conversion of 18-OH-corticosterone into aldosterone in the presence of a TPNH-generating system or exogenous TPNH was comparable to the control value (no cofactors added). Addition of $TPN^+$ or $DPN^+$ to the incubation system converted 34–35% of the substrate to a radioactive metabolite, which behaved similar to aldosterone in chromatography systems $B_5$ and $E_2B$. However, the acetate of this twice chromatographically purified product did not correspond with aldosterone diacetate (Raman *et al.*, 1965) in the Bush $B_3$ system (Figure 2). The etiolactone of this compound, obtained on periodic acid oxidation (Péron, 1961; Raman *et al.*, 1964), was compared with the etiolactone (IV) obtained from 18-OH-corticosterone by periodic acid oxidation and then chromic acid oxidation (Raman *et al*, 1965; Péron, 1961; Poos *et al.*, 1953). The two etiolactones had identical mobilities in paper chromatography systems $B_3$ (Bush, 1952) and toluene–propylene glycol (Burton *et al.*, 1951), and in the thin layer chromatography system benzene–ethyl acetate, 1:2 (Figure 2). Hence, it appears that $TPN^+$ and $DPN^+$ favor the conversion of 18-OH-corticosterone into 18-OH-11-dehydrocorticosterone and not into aldosterone.

It may be noted that 18-OH-11-dehydrocorticosterone was among the incubation products of corticosterone in the presence of exogenous TPNH and not in the presence of a TPNH-generating system (Table II). In a time study, using exogenous TPNH as the cofactor, 18-OH-11-dehydrocorticosterone was not detected after 15 min of incubation but a significant amount of it was formed in 150 min (1.9% of the substrate corticosterone-1,2-$^3$H).

Other hydrogen acceptors, *viz.*, FAD, FMN, cytochrome *c*, glutathione, and dehydroascorbate, were tried separately as possible cofactors for the dehydrogenation of 18-OH-corticosterone into aldosterone, but the results were negative. Also 18-OH-11-dehydrocorticosterone was not detected when these hydrogen acceptors were added to the incubation mixture.

*Time Course of the Enzyme Reactions.* Figures 3a and b show the conversion of corticosterone into 18-OH-corticosterone and aldosterone, respectively, with varying time intervals, the substrate and cofactor concentrations being constant. Although the conversion of corticosterone into 18-OH-corticosterone did not reach a steady state until about 150 min, the conversion into aldosterone (3.5%) reached a steady state within 60 min. This is most likely due to the fact that enough 18-OH-corticosterone was formed during the first 60 min and a further increase in the substrate for "18-ol-dehydrogenase" during the later period had no effect.

*Effect of Substrate Concentration.* The effect of the concentration of corticosterone on the formation of 18-OH-corticosterone and aldosterone is shown in Figure 4. First-order kinetics pertain up to a concen-

TABLE IV: Effect of Enzyme Inhibitors on the Conversion of Corticosterone into 18-Hydroxycorticosterone and Aldosterone.[a]

| Inhibitor Added | Moles | Product (% of Substrate) | | | Inhibn (% of Control) | | |
|---|---|---|---|---|---|---|---|
| | | 18-OH-B | Aldosterone | 18-OH-A | 18-OH-B | Aldosterone | 18-OH-A |
| | | Period of Incubation (15 min) | | | | | |
| Control | ... | 9.2 | 1.3 | 0 | ... | ... | ... |
| DETC | $2 \times 10^{-4}$ | 4.1 | 1.0 | ... | 56 | 16 | ... |
| | $2 \times 10^{-2}$ | 0.6 | 0 | ... | 83 | 100 | ... |
| PCMB | $2 \times 10^{-4}$ | 7.6 | 0.8 | ... | 18 | 33 | ... |
| | $2 \times 10^{-2}$ | 0.4 | 0 | ... | 89 | 100 | ... |
| IAA | $2 \times 10^{-4}$ | 7.2 | 1.2 | ... | 22 | 0 | ... |
| | $2 \times 10^{-2}$ | 1.8 | 0.5 | ... | 48 | 54 | ... |
| NEM | $3 \times 10^{-4}$ | 10.5 | 1.7 | ... | 0 | 0 | ... |
| | $3 \times 10^{-2}$ | 4.5 | 0.9 | ... | 51 | 31 | ... |
| FAA | $2 \times 10^{-4}$ | 6.1 | 1.2 | ... | 33 | 0 | ... |
| | $2 \times 10^{-2}$ | 4.1 | 0.7 | ... | 56 | 46 | ... |
| | | Period of Incubation (150 min) | | | | | |
| Control | ... | 10.5 | 1.6 | 1.83 | ... | ... | ... |
| DETC | $2 \times 10^{-4}$ | 5.7 | 1.0 | 0.9 | 46 | 36 | 51 |
| | $2 \times 10^{-2}$ | 1.4 | 0 | 0 | 83 | 100 | 100 |
| PCMB | $2 \times 10^{-4}$ | 9.1 | 1.2 | 0 | 14 | 23 | 100 |
| | $2 \times 10^{-2}$ | 1.2 | 0.1 | 0 | 86 | 94 | 100 |
| IAA | $2 \times 10^{-4}$ | 2.6 | 1.1 | 1.8 | 75 | 29 | 0 |
| | $2 \times 10^{-2}$ | 3.8 | 0.8 | 1.1 | 64 | 50 | 40 |
| NEM | $3 \times 10^{-4}$ | 12.8 | 1.4 | 0 | −22 | 10 | 100 |
| | $3 \times 10^{-2}$ | 3.0 | 1.7 | 0 | 63 | −10 | 100 |
| FAA | $2 \times 10^{-4}$ | 7.7 | 1.4 | 2.0 | 26 | 17 | −9 |
| | $2 \times 10^{-2}$ | 4.0 | 0.8 | 2.1 | 62 | 50 | −10 |

[a] In addition to the inhibitor each incubation flask contained: substrate, corticosterone-1,2-$^3$H (2 $\mu$c) (72 m$\mu$moles); TPNH (2 $\mu$moles); Krebs–Ringer bicarbonate buffer (pH 7.3) (2 ml); washed mitochondria obtained from 1 g of sheep adrenal cortex, in a total volume of 3 ml. Incubation was at 37° in air.

tration of 110 m$\mu$moles/3 ml. Because of the limitation of the amount at our disposal, 18-OH-corticosterone could not be used as the substrate for the "18-ol-dehydrogenase" reaction.

*Effect of Pharmacologic Inhibitors.* Table III summarizes the effect of SU 4885, SU 8000, SU 9055, and SU 10603 on the conversion of corticosterone into 18-OH-corticosterone and aldosterone. SU 4885 and SU 9055 significantly inhibited C-18 hydroxylation and hence aldosterone biosynthesis; SU 10603 and SU 8000 at the same concentration were less effective. The latter compound (SU 8000) seems to inhibit the "18-ol dehydrogenase" more than "C-18 hydroxylase."

*Effect of Enzyme Inhibitors.* Table IV summarizes the data on the effect of various sulfhydryl-binding agents on the formation of 18-OH-corticosterone, aldosterone, and 18-OH-11-dehydrocorticosterone from corticosterone-1,2-$^3$H. During the 15-min incubation period, formation of 18-OH-corticosterone was inhibited by varying degrees by all of the inhibitors except *N*-ethylmaleimide (NEM) at a concentration of $3 \times 10^{-4}$ M. The latter, however, inhibited the C-18 hydroxylation reaction *ca.* 51% at a concentration of $3 \times 10^{-2}$ M. Aldosterone biosynthesis seems to be affected only at the higher concentration of $2 \times 10^{-2}$ M by iodoacetic acid (IAA), NEM, and formamidine acetate (FAA). No 18-OH-11-dehydrocorticosterone was formed during this period of incubation.

During the 150-min incubation period, presence of *N*-ethylmaleimide (NEM, $3 \times 10^{-4}$ M) in the incubation media inhibited the conversion of 18-OH-corticosterone into 18-OH-11-dehydrocorticosterone; aldosterone formation did not change significantly. In contrast, presence of iodoacetic acid ($2 \times 10^{-4}$ M) or formamidine acetate ($2 \times 10^{-4}$ M) did not adversely affect the formation of 18-OH-11-dehydrocorticosterone. PCMB and DETC inhibited all of the three enzyme systems even at the lower concentration.

*Effect of Products and Other Steroids.* The data on the effect of products, *viz.*, 18-OH-corticosterone, aldosterone, and of some androgens and estrogens, on the *in vitro* biosynthesis of aldosterone and 18-OH-corticosterone from corticosterone are summarized in Table V. While aldosterone had no effect on its biosynthesis or

TABLE V: Effect of Added Steroids on the Conversion of Corticosterone into 18-Hydroxycorticosterone and Aldosterone.[a]

| Expt | Substrate Corticosterone-1,2-$^3$H | | Addn | mμmoles | Product (% of the Substrate) | |
|---|---|---|---|---|---|---|
| | mμmoles | μc | | | 18-OH-B | Aldosterone |
| I | 72 | 2 | Control | None | 13.0 | 5.0 |
| | 72 | 2 | 18-OH-DOC | 72 | 13.3 | 4.8 |
| II | 43 | 2 | Control | None | 13.3 | 2.3 |
| | 43 | 2 | 18-OH-Corticosterone | 14 | 8.1 | 2.4 |
| | 43 | 2 | 18-OH-Corticosterone | 28 | 8.4 | 1.9 |
| | 43 | 2 | Aldosterone | 14 | 14.5 | 2.4 |
| | 43 | 2 | Aldosterone | 28 | 13.2 | 2.6 |
| III | 72 | 2 | Control | None | 22.5 | 3.7 |
| | 72 | 2 | Estrone | 52 | 20.0 | 3.1 |
| | 72 | 2 | 17β-Estradiol | 55 | 19.0 | 3.9 |
| | 72 | 2 | Testosterone | 51 | 19.0 | 3.4 |
| | 72 | 2 | DEA | 54 | 22.0 | 3.9 |
| IV | 72 | 2 | Control | None | 11.0 | 3.4 |
| | 72 | 2 | Androst-4-ene-3,17-dione | 51 | 9.5 | 2.8 |
| | 72 | 2 | Estriol | 54 | 9.3 | 3.1 |

[a] In addition to the steroids, each incubation flask contained: $TPN^+$, 3 μmoles; glucose-6-P, 5 μmoles; glucose-6-P dehydrogenase, 2 Kornberg units; Krebs–Ringer bicarbonate buffer (pH 7.3), 2 ml; washed mitochondria obtained from 1 g of sheep adrenal cortex, in a total volume of 3 ml. Incubation was for 135 min at 37° in air.

that of the intermediate, 18-OH-corticosterone, the latter compound when present in the incubation mixture inhibited the 18-hydroxylation of corticosterone; 18-OH-DOC had no effect.

In contrast to the inhibitory action of testosterone, androst-4-ene-3,17-dione, and DEA on 11β and C-21 hydroxylation (Sharma *et al.*, 1963; Sharma and Dorfman, 1964), these androgens had no effect on C-18 hydroxylation of corticosterone as well as on the conversion of 18-OH-corticosterone into 18-OH-11-dehydrocorticosterone. Neither estrone nor 17β-estradiol exerted any inhibitory effect on aldosterone biosynthesis (Table V).

*Effect of Metal Ions.* C-18 hydroxylation of corticosterone and the conversion of 18-OH-corticosterone into 18-OH-11-dehydrocorticosterone in the presence of Krebs–Ringer bicarbonate buffer (pH 7.3) as compared to Tris–HCl buffer (pH 7.3, 0.05 M) was *ca.* five- and ninefold, respectively. This suggested the possibility of a metal ion requirement for these enzyme systems. Results summarized in Table VI indicate that $Ca^{2+}$ is required for C-18 hydroxylation, and that $Na^+$ or $K^+$ either alone or together are ineffective. Conversion of 18-OH-11-dehydrocorticosterone was influenced by $Ca^{2+}$, $Na^+$, $K^+$, and to a much lesser degree $Li^+$ ions also seem to stimulate the biosynthesis of 18-OH-11-dehydrocorticosterone.

Since *N*-ethylmaleimide (NEM) was effective in inhibiting the conversion of 18-OH-corticosterone into 18-OH-11-dehydrocorticosterone and not into aldosterone the effect of $Na^+$ and $K^+$ was studied in the presence of NEM. The results indicate that although the biosynthesis of 18-OH-11-dehydrocorticosterone was inhibited, the formation of aldosterone could not be increased.

The following metal ions, $Cu^{2+}$, $Zn^{2+}$, $Co^{2+}$, $Hg^+$, $Fe^{3+}$, and $Mn^{2+}$, when present at concentrations of $1 \times 10^{-2}$ M and $1 \times 10^{-3}$ M in the incubation media (Krebs–Ringer bicarbonate buffer and TPNH) inhibited the conversion of corticosterone to 18-OH-corticosterone and aldosterone.

## Discussion

The results from this investigation support earlier observations that 18-OH-corticosterone is a likely intermediate in the biosynthesis of aldosterone from corticosterone. Both "18-hydroxylase" and "18-ol dehydrogenase" activities seem to be located in the "mitochondrial fraction." In two recent studies with rat adrenals the "18-hydroxylase" activity was found to be mainly located in the "mitochondrial fraction," although the "microsomal fraction" also showed some activity (Nakamura and Tamaoki, 1964; Nakamura *et al.*, 1965). There was no significant loss in the "18-hydroxylase" and "18-ol dehydrogenase" activities in the mitochondria following ultrasonic treatment and these activities were principally located in the sediment after centrifugation at 105,000*g* for 60 min. This would indicate that intact mitochondria are not needed for

TABLE VI: Effect of Buffer Metal Ions and *N*-Ethylmaleimide on the *in Vitro* Biosynthesis of 18-OH-Corticosterone, Aldosterone, and 18-OH-11-Dehydrocorticosterone.[a]

| Tissue | Substrate | Buffer (pH 7.3) | Addn | Product (% of Substrate) 18-OH-Corticosterone | Aldosterone | 18-OH-11-Dehydrocorticosterone |
|---|---|---|---|---|---|---|
| | | Cofactor–TPNH (Generating System) | | | | |
| Sheep adrenal cortex slices | Corticosterone-1,2-$^3$H (2 $\mu$c, 72 $\mu$moles) | Krebs–Ringer bicarbonate | None | 19.7 | 5.5 | 0 |
| | Corticosterone-1,2-$^3$H (2 $\mu$c, 72 $\mu$moles) | Tris | None | 3.8 | 1.4 | 0 |
| | Corticosterone-1,2-$^3$H (2 $\mu$c, 72 $\mu$moles) | Tris | $Na^+$ | 3.6 | 1.9 | 0 |
| | Corticosterone-1,2-$^3$H (2 $\mu$c, 72 $\mu$moles) | Tris | $K^+$ | 3.9 | 1.7 | 0 |
| | Corticosterone-1,2-$^3$H (2 $\mu$c, 72 $\mu$moles) | Tris | $Ca^{2+}$ | 20.1 | 1.1 | 0 |
| Washed mitochondria | Corticosterone-1,2-$^3$H (2 $\mu$c, 72 $\mu$moles) | Krebs–Ringer bicarbonate | None | 18.2 | 4.0 | 0 |
| | Corticosterone-1,2-$^3$H (2 $\mu$c, 72 $\mu$moles) | Tris | None | 2.5 | 1.1 | 0 |
| | Corticosterone-1,2-$^3$H (2 $\mu$c, 72 $\mu$moles) | Tris | $Ca^{2+}$ | 19.8 | 1.5 | 0 |
| | | Cofactor–$DPN^+$ (3 $\mu$moles) | | | | |
| Sheep adrenal cortex slices | 18-OH-Corticosterone-1,2-$^3$H (0.1 $\mu$c) | Krebs–Ringer bicarbonate | None | ... | 0 | 54.0 |
| | 18-OH-Corticosterone-1,2-$^3$H (0.1 $\mu$c) | Tris | None | ... | 1.8 | 6.1 |
| | 18-OH-Corticosterone-1,2-$^3$H (0.1 $\mu$c) | Tris | $Na^+$ | ... | 0.5 | 34.5 |
| | 18-OH-Corticosterone-1,2-$^3$H (0.1 $\mu$c) | Tris | $K^+$ | ... | 0.3 | 31.6 |
| | 18-OH-Corticosterone-1,2-$^3$H (0.1 $\mu$c) | Tris | $Ca^{2+}$ | ... | 0.1 | 51.0 |
| | 18-OH-Corticosterone-1,2-$^3$H (0.1 $\mu$c) | Tris | $Li^+$ | ... | 1.1 | 18.0 |
| | 18-OH-Corticosterone-1,2-$^3$H (0.1 $\mu$c) | Tris | NEM | ... | 1.7 | 3.1 |
| | 18-OH-Corticosterone-1,2-$^3$H (0.1 $\mu$c) | Tris | NEM + $Na^+$ | ... | 2.1 | 5.1 |
| | 18-OH-Corticosterone-1,2-$^3$H (0.1 $\mu$c) | Tris | NEM + $K^+$ | ... | 1.9 | 7.0 |
| | | Cofactor–$TPN^+$ (3 $\mu$moles) | | | | |
| Sheep adrenal cortex slices | 18-OH-Corticosterone-1,2-$^3$H (0.1 $\mu$c) | Krebs–Ringer bicarbonate | None | ... | 0 | 51.0 |
| | 18-OH-Corticosterone-1,2-$^3$H (0.1 $\mu$c) | Tris | | | 2.0 | 5.7 |
| | 18-OH-Corticosterone-1,2-$^3$H (0.1 $\mu$c) | Tris | $Na^+$ | | 0.7 | 33.0 |
| | 18-OH-Corticosterone-1,2-$^3$H (0.1 $\mu$c) | Tris | $K^+$ | | 0.5 | 30.2 |
| | 18-OH-Corticosterone-1,2-$^3$H (0.1 $\mu$c) | Tris | $Ca^{2+}$ | | 0.1 | 43.8 |
| | 18-OH-Corticosterone-1,2-$^3$H (0.1 $\mu$c) | Tris | $Li^+$ | | 0.9 | 15.7 |

TABLE VI (*Continued*)

| Tissue | Substrate | Buffer (pH 7.3) | Addn | Product (% of Substrate) 18-OH-Corticosterone | Aldosterone | 18-OH-11-Dehydrocorticosterone |
|---|---|---|---|---|---|---|
| | 18-OH-Corticosterone-1,2-$^3$H (0.1 $\mu$c) | Tris | NEM | | 1.4 | 2.0 |
| | 18-OH-Corticosterone-1,2-$^3$H (0.1 $\mu$c) | Tris | NEM + $Na^+$ | | 1.9 | 6.7 |
| | 18-OH-Corticosterone-1,2-$^3$H (0.1 $\mu$c) | Tris | NEM + $K^+$ | | 2.2 | 8.1 |

[a] In addition to the substrate and cofactors indicated the incubation mixture contained tissue equivalent to 1 g of adrenal cortex, 2 ml of Krebs–Ringer bicarbonate or Tris–HCl (0.05 M) buffer, in a total volume of 3 ml. When metal ions were added to the incubation mixtures containing Tris–HCl buffer, the concentration of the additions was the same as in the Krebs–Ringer bicarbonate buffer. Amount of *N*-ethylmaleamide, when added, was 0.9 $\mu$mole. Incubation was for 150 min.

"C-18 hydroxylase" and "18-ol dehydrogenase" and that these enzymes are possibly closely bound to either the mitochondrial membrane or to cristae. In contrast 11$\beta$ hydroxylase could be obtained in a soluble form following ultrasonic treatment (Sharma *et al.*, 1962). Also, lyophilized sheep adrenal "mitochondria" had less than 10% of the activity of fresh mitochondria on a per milligram of protein basis.

As in the case of other steroid hydroxylations, TPNH was required for C-18 hydroxylation also. The formation of 18-OH-corticosterone and aldosterone was less in the presence of exogenous TPNH as compared to a TPNH-generating system (Table II). Addition of $TPN^+$ or $DPN^+$ resulted in the conversion of 18-OH-corticosterone into 18-OH-11-dehydrocorticosterone. A likely explanation for the formation of some 18-OH-11-dehydrocorticosterone in presence of exogenous TPNH in contrast to a TPNH-generating system is that a sufficient amount of $TPN^+$ is formed during the hydroxylation of corticosterone. It may also be noticed that corticosterone is hydroxylated at C-18 at a rate *ca.* seven times faster than the rate of conversion of 18-OH-corticosterone into aldosterone (Figure 3).

While SU 4885 and SU 9055 inhibited the biosynthesis of 18-OH-corticosterone as well as that of aldosterone SU 8000 seems to preferentially inhibit C-18 hydroxylation. Bledsoe *et al.* (1963, 1964) have earlier observed decreased aldosterone secretion in humans when SU 9055 was administered. The inhibition of C-18 hydroxylation by SU 4885 has also been reported (Kahnt and Neher, 1962). This compound is considered to be a specific inhibitor of 11$\beta$ hydroxylation (Kraulis and Birmingham, 1963; Chart and Sheppard, 1959) and SU 9055 that of 17$\alpha$ hydroxylation (Neher and Kahnt, 1965; Chart *et al.*, 1962). However, SU 4885 seems to inhibit hydroxylation at C-17 (Chart *et al.*, 1962) and at C-21 also (Sharma and Dorfman, 1964).

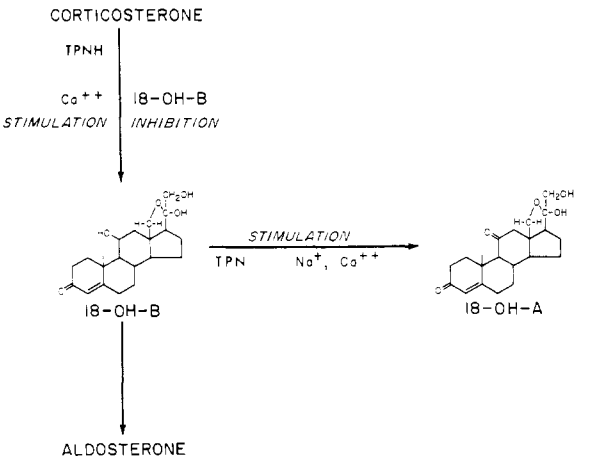


FIGURE 5: Effect of cofactors and some metal ions on the *in vitro* biosynthesis of aldosterone.

Among the inhibitors listed in Table VI, iodoacetic acid (IAA) and formamidine acetate, at a concentration of $2 \times 10^{-4}$ M, inhibited C-18 hydroxylation but not the aldosterone formation during the 15-min incubation period. Only at the higher concentration ($2 \times 10^{-2}$ M), when the formation of 18-OH-corticosterone was extensively inhibited, the aldosterone biosynthesis was affected. Whether this was due to an inhibitory effect of these compounds on "18-ol-dehydrogenase" or because the intermediate 18-OH-corticosterone was produced in a lesser quantity cannot be answered in the context of the present data. At the lower concentration *N*-ethylmaleimide (NEM, $3 \times 10^{-4}$ M) seems to specifically inhibit the formation of 18-hydroxy-11-dehydrocorticosterone; neither C-18 hydroxylation of corticosterone nor the conversion of 18-hydroxycorticosterone into

aldosterone was influenced by NEM (Tables IV and VI).

$Ca^{2+}$ significantly influenced the C-18 hydroxylation of corticosterone by sheep adrenal cortex slices as well as by the washed mitochondria from this tissue; conversion of 18-OH-corticosterone into aldosterone was not influenced. The requirement for $Ca^{2+}$ for C-18 hydroxylation of 11-deoxycorticosterone by rat adrenals has been indicated by Péron (1964). Increased *in vitro* formation of aldosterone from corticosterone in the presence of $Ca^{2+}$ has also been observed by Nicolis and Ulick (1965). Results from the present study indicate that neither $Na^+$ nor $K^+$ influences the *in vitro* conversion of 18-OH-corticosterone into aldosterone. However, these ions had a stimulating effect on the conversion of 18-OH-corticosterone into 18-OH-11-dehydrocorticosterone; $Li^+$ was much less effective.

It is interesting to note that among the wide variety of steroids studied for the effect on the conversion of corticosterone into 18-OH-corticosterone and aldosterone (Table V) only 18-OH-corticosterone had a significant inhibitory effect on the step corticosterone → 18-OH-corticosterone. Since aldosterone exerts the well-known salt-retaining effect *in vivo* we can attempt to summarize these effects in Figure 5.

18-Hydroxy-11-dehydrocorticosterone was not found to be a precursor of aldosterone in the *in vitro* system. Since $Ca^{2+}$ seems to influence the biosynthesis of 18-OH-corticosterone, it would be of interest to find whether the secretion of aldosterone and 18-OH-corticosterone are related to the $Ca^{2+}$ levels under certain clinical conditions.

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