Biosynthesis of C-18-oxygenated Steroids by an Aldosterone-secreting Human Adrenal Tumor.* Metabolism of [4-^{14}C]Progesterone, [1,2-^{3}H]11-Deoxycorticosterone, and [4-^{14}C]Pregnenolone

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ABSTRACT: Homogenate as well as subcellular fractions of an adrenal tumor from a female patient with primary aldosteronism were incubated with [4-^{14}C]progesterone, [1,2-^{3}H]11-deoxycorticosterone, and [4-^{14}C]pregnenolone. The incubation mixtures were extracted, partitioned, and chromatographed along with steroid carriers on a Celite column with gradient elution. Radioactive metabolites corresponding to carrier steroids were further characterized by several paper and thin-layer chromatography systems and finally by conversion into suitable derivatives. Besides 11-deoxycorticosterone and corticosterone, aldosterone and 18-hydroxycorticosterone were among the incubation products. As compared to the whole homogenate, subcellular fraction (6500 × *g* sediment) more actively converted 11-deoxycorticosterone into 18-hydroxycorticosterone. Subcellular fraction (105,000 × *g* sediment) alone failed to convert 11-deoxycorticosterone into aldosterone. 18-Hydroxy-11-deoxycorticosterone could not be detected in incubation experiments with these substrates. The results suggest that the preferred pathway for aldosterone biosynthesis in the tumor tissue was progesterone → 11-deoxycorticosterone → corticosterone → 18-hydroxycorticosterone → aldosterone.

Comparative studies (Bailey *et al.*, 1960; Biglieri *et al.*, 1963) of various adrenal disorders have shown that in "Primary Aldosteronism" (Conn and Conn, 1961; Luetscher, 1964) there is an increased production of corticosterone and aldosterone. Increased secretion rates and high content of aldosterone in tumors from such patients have also been reported (Laragh *et al.*, 1960; Louis and Conn, 1961). However, no attempt has been made to isolate possible intermediates in the biosynthesis of aldosterone, viz., 18-hydroxy-11-deoxycorticosterone (18-OH-DOC)[1] and/or 18-hydroxycorticosterone (18-OH-corticosterone) in these aldosterone-producing tumors. In this investigation we have studied the metabolism of [4-^{14}C]progesterone, [4-^{14}C]-pregnenolone, and [1,2-^{3}H]11-deoxycorticosterone (DOC) in a tumor from such a patient. Radioactive metabolites corresponding to carrier aldosterone, as well as these intermediates, were isolated to determine the pathway favored for the biosynthesis of aldosterone in the tumor.

Experimental Procedure

Tumor Tissue. The patient (L. B.), a 34-year-old female, had an 8 year history of hypertension. The urinary excretion of the neutral 17-ketosteroids was 7.4 mg/24 hours and of the 17-hydroxycorticoids (Porter-Silber) 7.5 mg/24 hours. The urinary aldosterone titer, 69 μg/24 hours, as determined by the double isotope method of Kliman and Peterson (1960), was three times the normal level, 5–19 μg/24 hours. At surgery a benign adrenocortical tumor weighing 20 g was removed with the left adrenal. Postoperatively the blood pressure fell from 240/140 to 130/100 mm. The tumor tissue was used for the incubation studies reported here.

Incubation Procedure. Immediately after removal, the tissue was placed on crushed ice. Within 15 minutes, 750 mg of the tumor tissue was homogenized in a glass homogenizer with 4 ml of 0.05 M Tris-maleate buffer, *p*H 7.4 (Gomori, 1955). One ml of this homogenate preparation from the fresh tissue was added to each of the incubation flasks.

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[1] Abbreviations and trivial names used in this paper: 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, 11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al-18→11 hemiacetal; 21-acetoxy-18-OH-corticosterone, 21-acetoxy-11β,18-dihydroxy-4-pregnene-3,20-dione; aldosterone diacetate, 18,21-diacetoxy-11β-hydroxy-3,20-dioxo-4-pregnen-18-al-18→11 hemiacetal; aldolactone, 21-acetoxy,11β-hydroxy-3,20-dioxo-4-pregnene-(18→11) lactone; TPN, triphosphopyridine nucleotide.

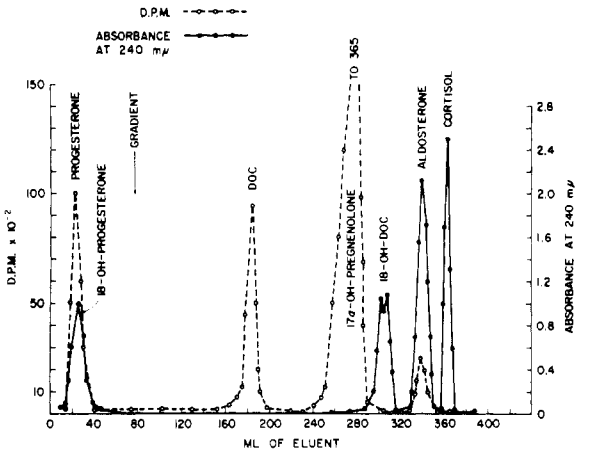

FIGURE 1: Partition column chromatography of a mixture of [4-^{14}C]progesterone, 18-OH-progesterone, [7α-^{3}H]17α-OH-pregnenolone, [1,2-^{3}H]DOC, 18-OH-DOC, [1,2-^{3}H]aldosterone, nonradioactive aldosterone, and cortisol on a Celite (25 g) column. The column was 1 cm in diameter, 15 ml of 80% aqueous methanol was the stationary phase, and 2,2,4-trimethylpentane was the eluent. Volume of each fraction was 1 ml. Exponential gradient of 1,2-dichloroethane was started after 60.0 ml of effluent was collected from the column. The radioactivity in d.p.m. (--O--O--) is plotted on the left ordinate and absorbance at240 mμ (–O–O–) is plotted on the right.

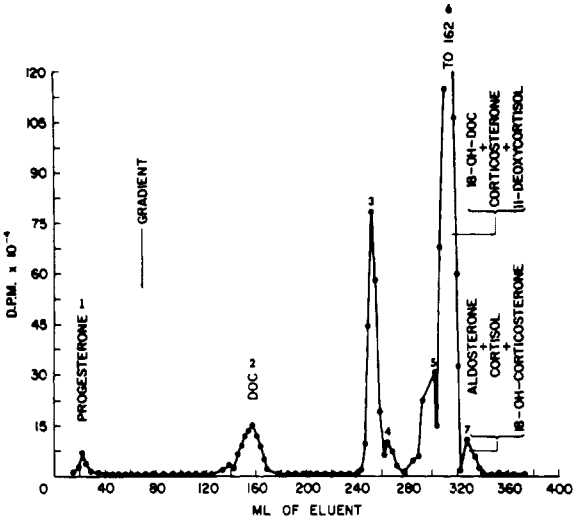

FIGURE 2: Separation of radioactive metabolites obtained from the incubation of [4-^{14}C]progesterone with homogenate of the tumor tissue, on a Celite (25 g) column. Solvent system was 2,2,4-trimethylpentane–80% methanol; exponential gradient of 1,2-dichloroethane was applied after 70 ml of the effluent from the column was collected. Volume of each fraction was 1 ml. Radioactivity in d.p.m. is plotted as ordinate. The peaks have been numbered.

The remaining portion of the tumor was frozen in dry ice and stored in a deepfreeze for 10 weeks. A part of it (5.4 g) was then freed from adipose tissue (1.0 g), homogenized with 9 ml of 0.25 M sucrose solution, and fractionated (Sharma and Dorfman, 1964) into "mitochondrial" (6500 × *g*), "microsomal" (105,000 × *g*), and "supernatant" fractions in an ultracentrifuge (Spinco Model L II). The "mitochondrial" and the "microsomal" fractions were washed twice by suspending in 0.25 M sucrose solution and recentrifuging for 15 minutes. The cell fractions thus isolated were then added to the appropriate incubation flasks.

Besides the substrate each incubation flask contained TPN (5 μmoles), glucose 6-phosphate (20 μmoles), magnesium chloride (5 μmoles), phosphate buffer, *p*H 7.2 (10 μmoles), Tris-maleate buffer, *p*H 7.4 (100 μmoles), and glucose 6-phosphate dehydrogenase (2 Kornberg units) in a total volume of 4.3 ml. Incubation with fresh tissue was for 90 minutes and with cell particulates of the frozen tissue was for 120 minutes in air at 37° in a Dubnoff metabolic shaker.

TPN "98% pure" as the sodium salt, glucose 6-phosphate "99% pure" also as the sodium salt, Tris, and maleic anhydride were obtained from Sigma Chemical Co. Glucose 6-phosphate dehydrogenase was obtained from Calbiochem, Inc.

Extraction and Preliminary Purification Procedure to Obtain "Polar Lipids." The extraction of radioactive metabolites and other lipids from the incubation mixture and their partition into "polar" and "nonpolar" fractions were accomplished by a procedure similar to that described earlier (Sharma *et al.*, 1965).

Partition Column Chromatography. Partition column chromatography of the "polar lipids" was carried out on a Celite column by a procedure similar to that described by Engel *et al.* (1961). Since the relative chromatographic characteristics of progesterone, 18-OH-progesterone, 17α-hydroxypregnenolone, DOC, 18-OH-DOC, corticosterone, aldosterone, 18-OH-corticosterone, and cortisol were not known, a mixture of authentic samples of these steroids was subjected to partition column chromatography on a Celite column (25 g). Figure 1 shows the elution pattern of the compounds recovered from the mixture.

The fractions collected on partition column chromatography were evaporated under vacuum, the residues were dissolved in 95% aqueous ethanol, and aliquots were removed for analyses. Fractions corresponding to various radioactive peaks were pooled and an appropriate aliquot was taken for radioactivity determination; the remaining portion was further purified by paper and thin-layer chromatography procedures (Sharma *et al.*, 1965).

Paper Chromatography. Descending chromatography on Whatman No. 1 paper was carried out at 26–28° with the solvent systems described:

B_3 (Bush, 1952): benzene-heptane (67:33) and methanol-water (80:20)

B_5 (Bush, 1952): benzene-methanol-water (100:50:50)

E_2B (Eberlein and Bongiovanni, 1955): *t*-butyl alcohol–isooctane–water (50:100:90)

EEG (Ulick and Vetter, 1962a): ethylene dichloride–ethylene glycol (100:100)

In system EEG, the paper was impregnated with a 25% solution of ethylene glycol in acetone as described by Reich *et al.* (1950). The steroids were extracted from paper chromatograms with absolute ethanol.

Thin-Layer Chromatography. Glass plates coated with a 0.25 mm-thick layer of silica gel GF_{254} (Brinkmann, Great Neck, N.Y.) were used in the following chromatography systems:

TLC-A (Quesenberry and Ungar, 1964): methylene chloride–methanol–water (150:10:0.4)

TLC-B (Quesenberry and Ungar, 1964): methylene chloride–methanol–water–glycerine (150:10:1:0.4)

TLC-C (Raman *et al.*, 1964): ethyl acetate (100%)

TLC-D (Raman *et al.*, 1964): methanol–ethyl acetate (1:20)

The steroids were extracted from silica gel with ethanol in 90% yield by using a zone extractor as decribed by Matthews *et al.* (1962).

Detection of Steroid Carriers and Radioactive Metabolites. Methods for locating radioactivity on paper chromatograms and thin-layer plates, and also for precise estimation of radioactivity by a liquid scintillation spectrometer, have been described earlier (Sharma *et al.*, 1965).

To detect steroids having an α-ketol side chain the paper chromatograms were sprayed with a mixture of 1 ml of 0.2% blue tetrazolium in ethanol and 40 ml of 10% sodium hydroxide in water; the compounds showed blue color immediately. In addition, when the paper chromatogram following spraying of the blue tetrazolium reagent was heated for 40 minutes at 60°, the compounds having a Δ^4-3-keto group gave a yellow fluorescence under ultraviolet (360 mμ) (Bush, 1961). Aldosterone and 18-OH-DOC gave soda fluorescence as well as positive blue tetrazolium reaction. The latter compound, however, gave the blue tetrazolium reaction only on heating. 18-OH-Corticosterone gave only soda fluorescence (Péron, 1961). Procedures for periodic acid oxidation and acetylation have been described earlier (Raman *et al.*, 1964; Sharma *et al.*, 1965).

Chromic Acid Oxidation. In a 40-ml glass-stoppered centrifuge tube 0.2 ml of the oxidizing reagent (0.5% chromic acid in glacial acetic acid) was added to aldosterone diacetate and kept at room temperature for 20 min. One ml of 20% aqueous ethanol was added to arrest the reaction. The reaction mixture was extracted with 10 ml of methylene chloride; the methylene chloride layer was then washed with 2 ml of water, dried over anhydrous sodium sulfate, and evaporated under nitrogen.

Results

[4-^{14}C]*Progesterone as Substrate*

[4-^{14}C]Progesterone (7.20 μc, specific activity 166 mc/mmole) was incubated with homogenate corresponding to 190 mg of the fresh tumor tissue. After the initial extraction procedure, 90% of the added total radioactivity was recovered from the incubation medium. The radioactive residue together with nonradioactive carriers 18-OH-DOC (200 μg) and aldosterone (500 μg) were chromatographed on a Celite column. The elution pattern of radioactivity and the carrier steroids are given in Figure 2.

Radioactive Metabolites in Peak 6. The pooled radioactive material from peak 6 along with nonradioactive 18-OH-DOC added as carrier before column chromatography was chromatographed on paper in the B_5 system (Figure 3). Four radioactive zones, viz., a, b, c, and d in order of decreasing polarity, and a nonradioactive, ultraviolet light absorbing zone, e, corresponding to the carrier 18-OH-DOC (R_F 0.59) were obtained. Most of the radioactivity (1.18×10^6 dpm) was lodged in zone c, corresponding with standard corticosterone. The radioactive metabolite eluted from zone c migrated as a single radioactive zone in Bush B_3 system also and corresponded with authentic corticosterone (R_F 0.25) (Figure 3).

No radioactive peak was apparent in the position occupied by the nonradioactive 18-OH-DOC. To determine whether the radioactivity in zone c had conceivably obscured a small amount of radioactivity in 18-OH-DOC zone, zone e was eluted (2000 dpm) and rechromatographed in B_5 system. The radioactivity attributable to 18-OH-DOC was reduced to 60 dpm without any definite peak, indicating that [4-^{14}C]-progesterone was not converted into 18-OH-DOC in appreciable amounts (Figure 3).

Radioactive Metabolites in Peak 7. Pooled radioactive material from peak 7 on chromatography in B_5 system gave four radioactive zones; zone 2 (R_F 0.11) corresponded with authentic 18-OH corticosterone, and zone 3 (R_F 0.32) with aldosterone (Figure 3). The radioactive metabolites from these zones were further purified and characterized as described below.

ZONE 2. The radioactive material eluted from zone 2 was further checked in two paper and three thin-layer chromatography systems (Table I). In each of these systems it migrated as a single substance and was similar to 18-OH-corticosterone. One-half of the radioactive material from zone 2 (1700 dpm) was oxidized with periodic acid; the oxidation product (Figure 4, V) had the mobility of authentic lactone of 18-OH-corticosterone (Ulick and Vetter, 1960) in B_3 and B_5 systems. The remaining portion (1700 dpm) of the radioactive material from zone 2 was acetylated. Chromatography of the product in the B_3 system indicated that the principal radioactive product (80%) was 21-acetoxy-18-OH-corticosterone (Figure 4, IV). Besides this peak, two other less polar minor radioactive zones were observed. Standard 18-OH-corticosterone behaved similarly on acetylation. The details are summarized in Table I.

ZONE 3. The radioactive material from this zone had chromatographic mobility similar to the carrier aldosterone in B_5 and EEG systems (Table I). However, it separated into two radioactive peaks [$R_{S\ (cortisol)}$ 0.93 and 1.10] with an equal distribution of radioactivity in E_2B system. The less polar radioactive metab-

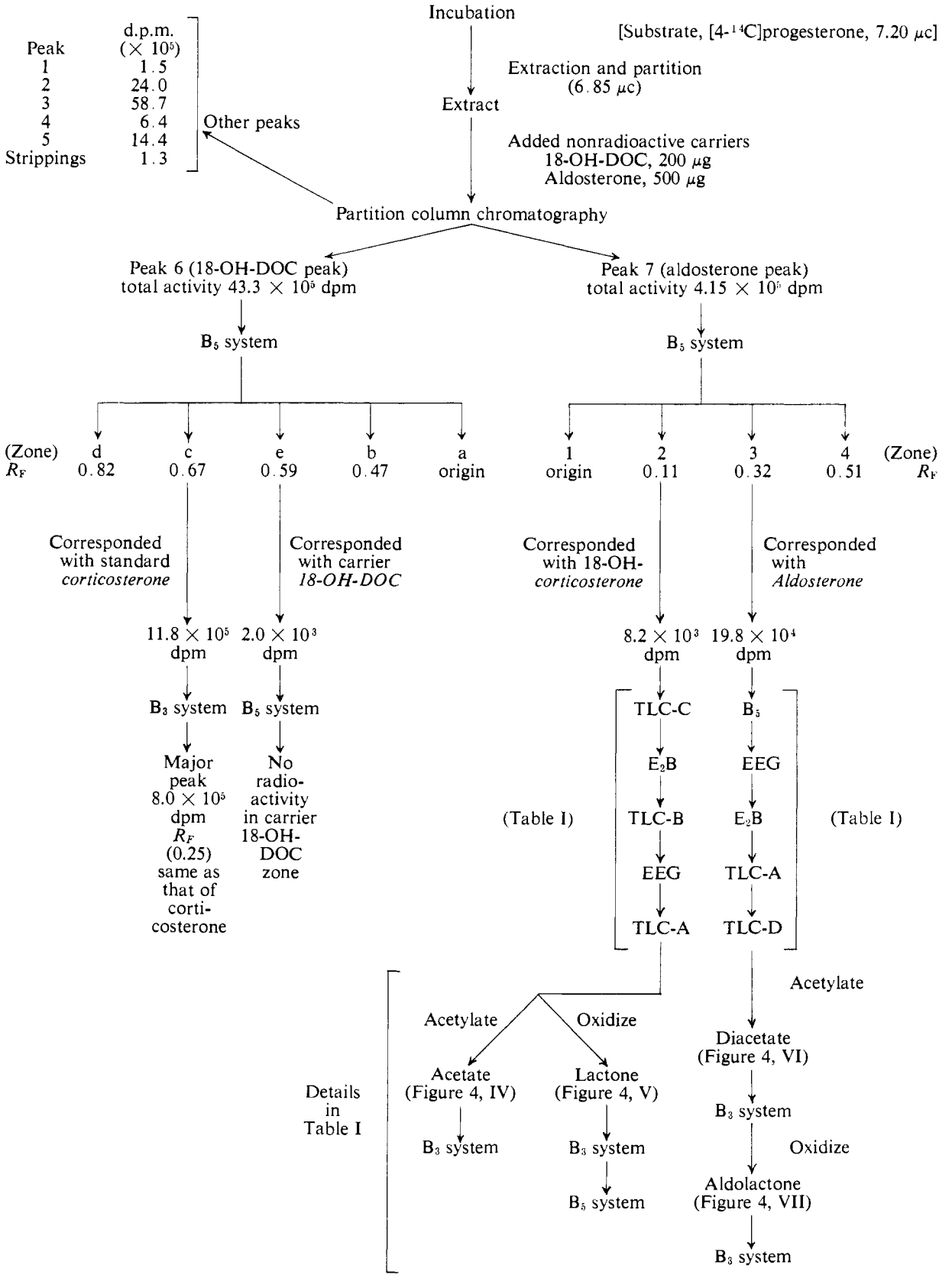

FIGURE 3: Schematic representation of the preliminary steps in the characterization of radioactive metabolites.

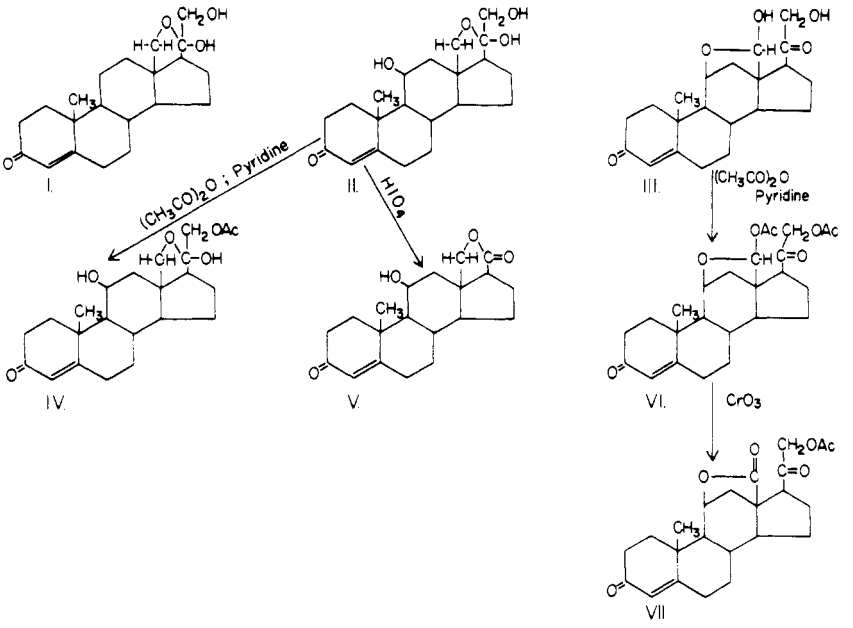

FIGURE 4: 18-OH-DOC (I) and derivatives of 18-OH-corticosterone (II) and aldosterone (III) prepared for their identification. 21-Acetoxy-18-OH-corticosterone (IV); lactone of 11β,18-dihydroxy-3 keto-4-etienic acid (V); aldosterone diacetate (VI), aldolactone (VII).

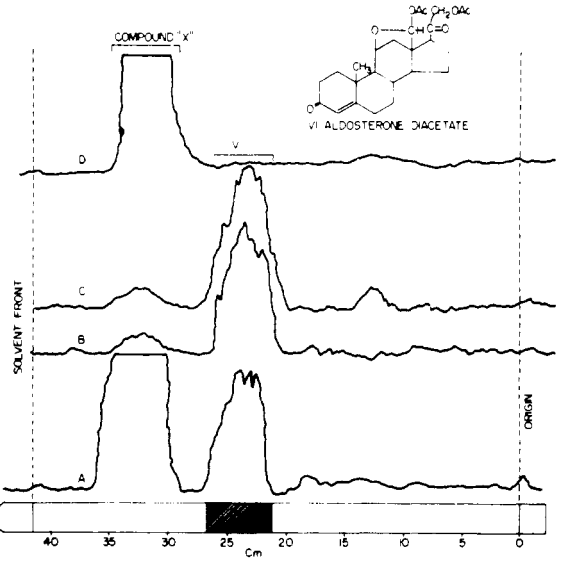

FIGURE 5: Radioscanograms of aldosterone diacetate (VI) and unknown "Compound X." The acetates were chromatographed on paper in B_3 system for 3.5 hours. A, B, C, and D represent tracings of radioactivity distribution in the following incubations: A = incubations of [4-^{14}C]progesterone with tissue homogenate; B = incubation of [1,2-^{3}H]DOC with tissue homogenate; C = incubation of [1,2-^{3}H]DOC with "mitochondrial" fraction; D = incubation of [1,2-^{3}H]DOC with "microsomal" fraction. The lower chromatogram indicates ultraviolet-absorbing area of authentical dosterone diacetate.

olite, corresponding to aldosterone [$R_{s(\text{cortisol})}$ = 0.93], was further checked for its homogenity in TLC-A and TLC-D systems. In both of these systems the radioactivity corresponded in mobility with the carrier aldosterone (Table I). The radioactive metabolite was then acetylated and chromatographed in the B_3 system (Figure 5). Nine per cent of the radioactivity coincided with aldosterone diacetate (Figure 4, VI), R_F 0.54, and the remaining activity was lodged in a less polar material, R_F 0.65. Chromic acid oxidation of the diacetate yielded a lactone (Figure 6) chromatographically identical with authentic aldolactone (Figure 4, VII) (Kliman and Peterson, 1960). Table I summarizes the evidence for the presence of aldosterone in zone 3.

11-[1,2-^{3}H]Deoxycorticosterone as Substrate

Whole homogenate corresponding to 900 mg of the frozen tissue and the "mitochondrial" and "microsomal" fractions obtained from 3.50 g of the tumor tissue were incubated separately, each with 3.78 μc of [1,2-^{3}H]DOC (specific activity 1.05 mc/μmole). Nonradioactive carriers, 18-OH-DOC (200 μg) and aldosterone (500 μg), were added to the radioactive materials extracted from each of the three incubation mixtures. Preliminary fractionation of the radioactive metabolites was done on Celite partition columns; the elution patterns of the radioactive compounds and the carriers are shown in Figure 7.

Incubation Experiments with Homogenate and "Mitochondria." RADIOACTIVE METABOLITES IN PEAK 5. Partition column chromatography of radioactive materials obtained from incubations with the tissue homogenate

and the "mitochondrial" fraction yielded a total of 31.40×10^5 and 20.45×10^5 dpm, respectively, in peak 5 (Figure 7B). The radioactive materials from these peaks were chromatographed in the B_5 system. Both chromatograms indicated the presence of four radioactive zones, a, b, c, and d, and one nonradioactive ultraviolet-absorbing zone, e, as described earlier when [4-^{14}C]progesterone was the substrate (Figure 3, peak 6). Zone c (R_F identical with authentic corticosterone) from polarity. Of these four zones, from the two chromatograms, radioactive zone 2 corresponded with standard 18-OH-corticosterone (R_F 0.11) and zone 3 corresponded with the carrier aldosterone (R_F 0.32). Further characterization of these radioactive metabolites was done by the procedures outlined for zones 2 and 3 obtained from peak 7 when [4-^{14}C]progesterone was the substrate (Figure 3). The data are summarized in Table II.

TABLE I: Characterization of Radioactive Metabolites in Peak 7.[a]

Zones Studied	Reaction	Chromatographic Mobility Corresponding to	Chromatography[b] System	Mobilities (R_F)	Running Time (min)	Total Radioactivity (dpm $\times 10^{-3}$)
Zone 2		18-OH-Corticosterone	TLC-C	0.19	75	6.55
		18-OH-Corticosterone	E_2B	0.91[c]	420	6.15
		18-OH-Corticosterone	TLC-B	0.23	60	
		18-OH-Corticosterone	EEG	0.95[c]	420	4.33
		18-OH-Corticosterone	TLC-A	0.35	60	3.42
	Acetylation (1700 dpm)	21-Acetoxy-18-OH-corticosterone	B_3	0.50	240	1.02
	Oxidation (periodic) (1700 dpm)	11β,18-Dihydroxyetienic acid lactone	B_3	0.25	240	1.42
		11β,18-Dihydroxyetienic acid lactone	B_5	0.85	210	1.22
Zone 3		Aldosterone	B_5	0.32	240	178.70
		Aldosterone	EEG	0.28	300	163.60
		Aldosterone	E_2B	0.93[c]	420	78.85
		Aldosterone	TLC-A	0.54	75	69.00
		Aldosterone	TLC-D	0.35	75	58.24
	Acetylation	18,21-Diacetoxyaldosterone + "X" acetate	B_3	0.57	240	5.23
				0.65	240	48.07
	Oxidation (chromic acid)	Aldolactone	B_3	0.25	240	4.08

[a] Radioactive metabolites (0.19 μc) from peak 7 obtained on partition column chromatography of polar lipids extracted from the incubation mixture (substrate [4-^{14}C]progesterone, 7.2 μc) along with nonradioactive carrier aldosterone (500 μg) were separated on paper chromatography (B_5) into four radioactive zones (1–4) in the decreasing order of polarity. Zones 2 and 3 corresponded in mobility with authentic samples of 18-OH-corticosterone and aldosterone, chromatographed on parallel strips. The table summarizes the chromatographic behavior of the radioactive metabolites in zones 2 and 3 and their derivatives in various chromatography systems along with those of the authentic samples of 18-OH-corticosterone and aldosterone. [b] Chromatographic systems are described in detail under Experimental Procedure. [c] Mobilities represent R_s values when $R_{s(\text{cortisol})} = 1$.

the homogenate and "mitochondrial" incubations contained 14.13×10^5 and 11.25×10^5 dpm, respectively. Zone e corresponding with the carrier 18-OH-DOC showed no radioactivity, indicating the lack of conversion of the substrate into this intermediate.

RADIOACTIVE METABOLITES IN PEAK 6. Individual chromatography of the material eluted in peak 6 (Figure 7B) from both the incubations in the B_5 system revealed four zones, viz., 1, 2, 3, and 4, in decreasing order of

Incubation Experiment with "Microsomes." Radioactive metabolite along with the nonradioactive carrier 18-OH-DOC, from peak 6 (total activity 5.67×10^5 dpm) of the partition column (Figure 7A), was chromatographed in the B_5 system. Most of the radioactivity was located in the zone with R_F 0.88. No radioactivity corresponded with the carrier 18-OH-DOC.

The pooled material from peak 7 (Figure 7A; total activity 6.7×10^5 dpm) along with the carrier aldos-

TABLE II: Characterization of Radioactive Metabolites in Peak 6 of Incubation Experiments with Whole Homogenate and "Mitochondrial" Fraction.[a]

Step	Chromatographic[b] Mobility Corresponding to	Total Radioactivity Whole Homogenate (dpm × 10^{-4})	Total Radioactivity "Mitochondria" (dpm × 10^{-4})
Substrate: [1,2-^{3}H]DOC		831.60	831.60
Partition column chromatography			
Whole homogenate experiment peak 6	Aldosterone and 18-OH-corticosterone	113.51	
"Mitochondrial" fraction experiment peak 6			129.45
Paper chromatography in B_5 system			
Zone 2	18-OH-Corticosterone	11.88	68.92
Zone 3	Aldosterone	22.13	31.78
Identification of radioactive metabolite in zone 2			
TLC-C	18-OH-Corticosterone	8.67	60.49
E_2B	18-OH-Corticosterone	7.81	52.63
TLC-B	18-OH-Corticosterone	6.60	42.10
EEG	18-OH-Corticosterone	5.94	36.21
TLC-A	18-OH-Corticosterone	4.78	28.60
Acetylation after TLC-A		1.43	2.62
Chromatography of acetate in B_3 system	21-Acetoxy-18-OH-corticosterone	0.96	1.95
Periodic acid oxidation after TLC-A		1.43	2.62
Chromatography of the oxidation product in	11β,18-Dihydroxy-etienic acid lactone		
B_3		1.35	2.48
B_5		1.08	2.37
Identification of radioactive metabolite in zone 3			
B_5	Aldosterone	18.14	2.89
EEG	Aldosterone	14.30	23.68
E_2B	Aldosterone	11.92	18.85
TLC-A	Aldosterone	9.54	15.08
TLC-D	Aldosterone	7.33	11 40
Acetylation after TLC-D		7.33	11 40
Chromatography of acetate in B_3	18,21-Diacetoxy aldosterone	5.71	7.35
Chromic acid oxidation		5.71	7.35
Chromatography of the oxidation product in B_3	Aldolactone	4.74	4.84

[a] Details of chromatographic procedures are given under Experimental Procedure. [b] The mobilities (R_F) of 18-hydroxycorticosterone, aldosterone, and their derivatives in various chromatography systems are listed in column 5 of Table I.

terone was chromatographed in system B_5. The chromatogram indicated two radioactive zones, zone 1 at the origin and zone 2 corresponding with the carrier aldosterone (R_F 0.32). No radioactivity was apparent in the 18-OH-corticosterone region.

The radioactive metabolite from zone 2 (3.9×10^5 dpm) had the mobility of aldosterone in systems B_5, EEG, E_2B, TLC-A, and TLC-D. However, on acetylation and subsequent chromatography in B_3 this radioactive metabolite showed a peak (R_F 0.65) different from that of aldosterone diacetate (Figure 5D). This indicated that the initial radioactive metabolite in zone 2, which had the mobility pattern similar to that of aldosterone in all chromatography systems, was not aldosterone. Attempts were not made to characterize this compound (compound X) further owing to the lack of authentic standards of the 18-hydroxy series.

[4-^{14}C]Pregnenolone as Substrate

The whole homogenate corresponding to 190 mg of the tumor tissue was incubated with 0.56 μc of [4-^{14}C]-pregnenolone, specific activity 39 μc/μmole. The radio-

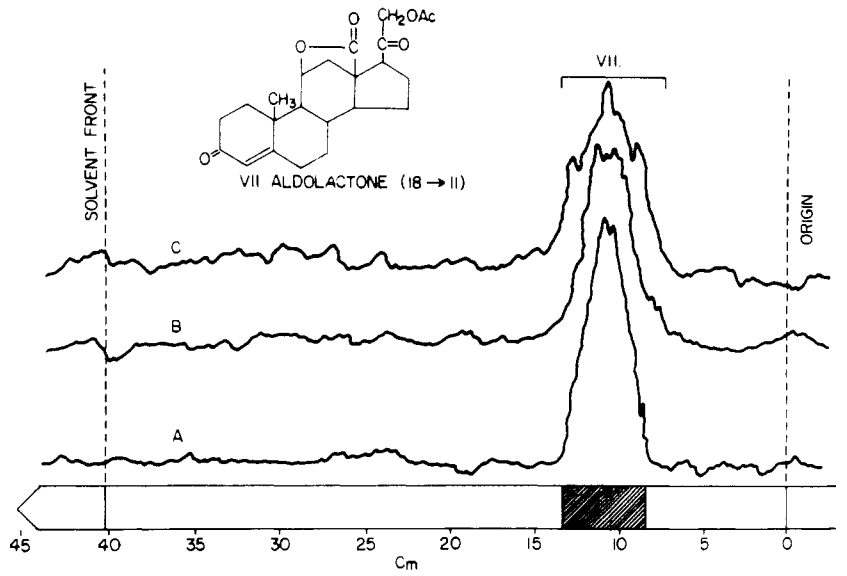

FIGURE 6: Radioscanograms of aldolactone (VII) chromatographed in B_3 system for 3.5 hours. The aldolactone samples chromatographed were obtained on chromic acid oxidation of aldosterone diacetate from the following incubation experiments: A = incubation of [4-^{14}C]progesterone with tissue homogenate; B = incubation of [1,2-^{3}H]DOC with tissue homogenate; C = incubation of [1,2-^{3}H]DOC with "mitochondrial" fraction.

TABLE III: *In Vitro* Conversion[a] of [4-^{14}C]Progesterone and [1,2-^{3}H]DOC into 18-OH-Corticosterone and Aldosterone.

Substrate (Radioactivity)	[4-^{14}C]Progesterone (7.20 μc)		[1,2-^{3}H]DOC (3.78 μc)		(3.78 μc) Frozen Tumor Tissue		(3.78 μc)	
Tissue	Fresh Tumor Whole Homogenate		Whole Homogenate		Mitochondrial Fraction		Microsomal Fraction	
Products	(%)	(dpm × 10^{-3})	(%)	(dpm × 10^{-3})	(%)	(dpm × 10^{-3})	(%)	(dpm × 10^{-3})
18-OH-DOC	—	—	—	—	—	—	—	—
18-OH-Corticosterone[b]	0.16	22.60	1.10	66.00	2.04	103.40	—	—
Aldosterone[c]	0.21	27.40	1.06	63.40	0.46	21.00	—	—
"X" compound[c]	1.90	215.70	0.011	0.88	0.002	0.16	1.60	75.80

[a] Conversions are expressed per g of the tissue. Radioactivity recovered from the incubation mixture (90–92% of the total radioactivity used as substrate) was taken as 100% in computing the figures for the products. [b] The "polar lipid" fraction obtained from the extract of the incubation mixture was subjected to partition column chromatography along with nonradioactive carriers. The above figures represent per cent conversion of the substrate into 18-OH-corticosterone after purification of the pooled 18-OH-corticosterone fraction by three paper and two thin-layer chromatography systems. [c] The per cent conversion of the substrate into aldosterone and "X" compound has been calculated after the purification of the respective compounds in four paper and two thin-layer chromatography systems followed by acetylation and purification of the respective acetates by paper chromatography.

active metabolites were extracted and separated from the incubation media as described under Experimental Procedure. Aldosterone (990 dpm) and 18-OH-corticosterone (1150 dpm) were purified and investigated in detail. A description of purification and characterization of these compounds is omitted here since it was similar to that described for these radioactive metabolites when [4-^{14}C]progesterone and [1,2-^{3}H]DOC were the substrates.

Discussion

The results indicate that this adrenal tumor from a patient with primary aldosteronism readily forms, *in*

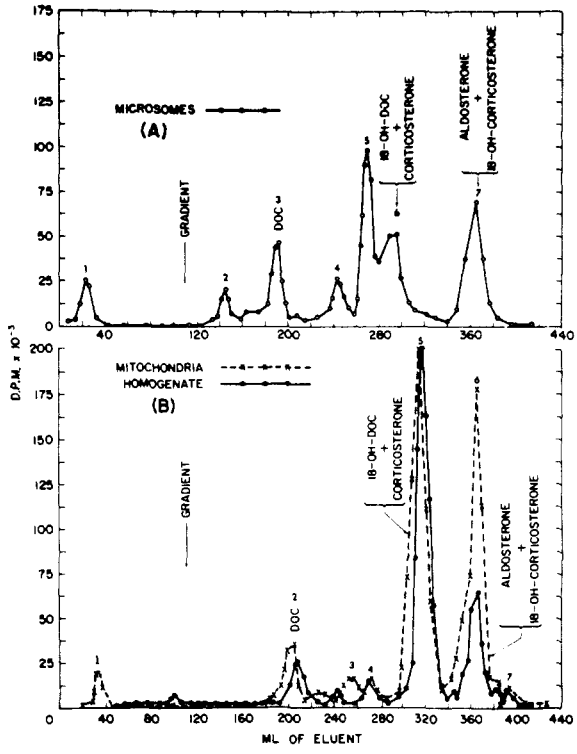

FIGURE 7: Partition column chromatography of radioactive metabolites from the incubation experiments with [1,2-^{3}H]DOC as the substrate. Celite (25 g) and 15 ml of 80% aqueous methanol were the stationary phase, 2,2,4-trimethylpentane was the mobile phase; exponential gradient of 1,2-dichloroethane was applied after 110 ml of the effluent was collected from the column. Total of 400 fractions, each 1 ml in volume, were collected. (A) Elution pattern of the radioactive metabolites obtained from the incubation of [1,2-^{3}H]-DOC with "microsomal" fraction (-O-O-). (B) Elution patterns of the radioactive metabolites from the incubations of [1,2-^{3}H]DOC with "mitochondrial" fraction (-×-×-) and tissue homogenate (-O-O-).

vitro, radioactive 18-OH-corticosterone and aldosterone from [4-^{14}C]progesterone, [1,2-^{3}H]DOC, and [4-^{14}C]-pregnenolone. The ratio of 18-OH-corticosterone to aldosterone formed from progesterone and DOC was comparable in the two incubation experiments with the tumor homogenate. However, the per cent conversion into these products was five- to sixfold greater for the substrate DOC as compared with progesterone, suggesting that C-21 hydroxylation facilitates further steps involved in aldosterone biosynthesis.

The conversion of DOC into 18-OH-corticosterone by the "mitochondria" alone was greater as compared with the tissue homogenate or "microsomes," indicating that "18-hydroxylase" is primarily located in the "mitochondria" (Nakamura and Tamaoki, 1964).

Although the "mitochondria" alone were capable of converting DOC into aldosterone, the formation of aldosterone was much greater with tissue homogenate. This would indicate that either the "enzyme system" responsible for conversion of C-18-OH into 18-CHO is present in other cellular fractions also or there is some other component in the whole homogenate responsible for carrying out this step more efficiently. Increased accumulation of 18-OH-corticosterone in the incubation experiment with "mitochondria" supports these observations.

A careful search for radioactive 18-OH-DOC among the incubation products was made in all four incubation experiments with [1,2-^{3}H]DOC and [4-^{14}C]progesterone as the substrate. The results indicated that the tumor tissue was capable of synthesizing DOC, corticosterone, 18-OH-corticosterone, and aldosterone but not 18-OH-DOC. Thus, the preferred pathway for aldosterone biosynthesis seems to be DOC → corticosterone → 18-OH-corticosterone → aldosterone. Existence of this pathway is further strengthened by the recent report of Pasqualini (1964), who showed the conversion of tritiated 18-OH-corticosterone to aldosterone by slices of an aldosterone-secreting tumor.

Ulick and Vetter (1962b) have shown the secretion rates of 18-OH-corticosterone in man and have noted that in all cases where aldosterone secretion was increased there was a parallel increase in 18-OH-corticosterone levels also. Similar findings have been reported in chicken and duck (Sandor *et al.*, 1963a), mice (Raman *et al.*, 1964; Triller and Birmingham, 1964), bullfrog (Ulick and Vetter, 1960), and turtle (Sandor *et al.*, 1964) adrenal glands also. However, rat (Péron, 1962; Ward and Birmingham, 1962) adrenal glands elaborate very little of 18-OH-corticosterone and aldosterone, but secrete significantly high levels of 18-OH-DOC. The concomitant increased production of 18-OH-corticosterone along with aldosterone in the former species signifies the importance of 18-OH-corticosterone as an intermediate in aldosterone biosynthesis. Lack of C-18 hydroxylation of DOC as compared with C-18 hydroxylation of corticosterone can be explained on the basis of substrate specificity of "C-18-hydroxylase."

From Table III it is apparent that in incubation experiments with tissue homogenate the formation of "compound X," which could be separated from aldosterone after acetylation, was significantly higher when progesterone was the substrate (1.9%). When DOC was the substrate the conversion was only 0.01%. Although the conversion of [1,2-^{3}H]DOC into "compound X" (0.01%) with "mitochondria" was comparable to that in the incubation with tissue homogenate, the biosynthesis was much higher (1.6%) when the "microsomal" fraction was used. In incubations of 18-OH-corticosterone with beef adrenal glomerulosa Sandor and Lanthier (1963b) obtained a similar metabolite which had mobilities similar to those of aldosterone in several chromatography systems and could only be separated following acetylation. It was characterized as 18-OH-11-dehydrocorticosterone. Although "compound X" from our studies seemed to behave similarly to 18-OH-11-dehydrocorticosterone, it is possibly a different metabolite, since neither "18-hydroxylase" nor "11β-hydroxylase" has been reported in "microsomes."

It would be of interest to determine the significance of this metabolite ("compound X") in aldosterone-secreting tumors.

The above mentioned results indicate that progesterone and DOC are precursors of 18-OH-corticosterone and aldosterone in this tumor. Comparing the yields of these compounds to those reported by Sandor and Lanthier (1963b) for normal human adrenal, it appears that the tumor tissue in this study had an increased activity of "C-18-hydroxylase."

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