EVIDENCE FOR THE PRESENCE OF 17β-HYDROXYSTEROID OXIDO-REDUCTASE AND 19-HYDROXYLASE SYSTEMS IN DOMESTIC DUCK (ANAS PLATYRHYNCHOS) ADRENAL MITOCHONDRIA*.

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ABSTRACT

Adrenal "mitochondrial" preparations of domestic duck (Anas platyrhynchos) were incubated with ^{14}C labelled androstenedione (Δ^4A)***, testosterone (T) and 11-deoxycorticosterone (DOC) as model substrates in the presence of NADPH generating system. Δ^4A was converted to 19-OH-Δ^4A (≈1%), 11β-OH-Δ^4A (38%) and testosterone (32%). 11β-OH-Δ^4A (3%), 11β-OH-T (60%) and 18-OH-T (2%) were the characterizable metabolites of T. Among the many unidentified metabolites, using T as a precursor, a compound presumed to be 19-OH-T was also isolated. The C_{21} labelled substrate (DOC) gave rise to 19-OH-DOC (<1%) in addition to many other conversion products. All the above mentioned metabolites were identified by isotopic dilution techniques and radiochemical purity was obtained. It appears that duck adrenal "mitochondria" possesses: 1) 17β-hydroxysteroid dehydrogenase and 17β-reductase, though adrenal tissue lacks 17α-hydroxylase activity; 2) 19-hydroxylase activity, the physiological significance of which in the adrenal gland is unknown.

INTRODUCTION

In previous years considerable insight has been gained on the intimate nature of enzymatic reactions involved in the mammalian adrenal gland (1,2). Thus the localization of some of the enzyma-

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tic reactions at the subcellular level and their cofactor(s) requirements have been fairly elucidated (3). The avian adrenal gland has been shown to possess cholesterol desmolase, Δ^5-3β-hydroxysteroid dehydrogenase and 11β, 18 and 21-hydroxylase activities but it lacks 17α-hydroxylase activity (4,5). Most of these enzymes are mitochondrial bound (6,7). Since there is a lack of 17α-hydroxylase in the avian adrenal, reactions of steroids of the C_{19} series have not been investigated. However, the rat adrenal gland which also lacks 17α-hydroxylase activity can metabolize exogenous C_{19} steroids (8). Thus the present investigation was undertaken in an attempt to explore whether the avian adrenal gland could accept exogenous C_{19} steroids as substrates. The possible biotransformation of androstenedione-4-^{14}C and testosterone-4-^{14}C by duck adrenal mitochondria was investigated. In addition, the possible 19-hydroxylation of 11-deoxycorticosterone-4-^{14}C was also studied.

MATERIALS AND METHODS

Tissue preparation. Male ducks of the Pekin white variety, weighing between 1.5 - 2 Kg, were used. The animals were killed by exsanguination and adrenals were removed through an abdominal incision. Adrenals were carefully dissected free of renal parenchyma. Adrenal "mitochondria" (6000 x g) were prepared by the 0.25 M sucrose method (9) in an L2 -65B preparative ultracentrifuge (Spinco Division, Beckman Instrument, Palo Alto, Calif. Rotors used: No. 30 and No. 50-Ti).
Substrates. Androstenedione-4-^{14}C (SA: 40 μCi/μmole), 11-deoxycorticosterone-4-^{14}C (SA: 25 μCi/μmole) and testosterone-4-^{14}C (SA: 40 μCi/μmole) were obtained from New England Nuclear Corporation, Boston, Mass. Their chromatographic homogeneity was verified before use. Two μCi of the above mentioned steroids were employed as substrates. These precursors were further diluted with 20 μg of the corresponding radio-inert substances. After evaporating the original solvent mixtures in which they were contained, the substances were dissolved in 0.2 ml of propylene glycol.
Medium. In all experiments, Krebs-Ringer bicarbonate solution (pH 7.4), supplemented with 11 mM glucose was used. The incubation medium was further supplemented with NADPH generating system, consisting of NADP (2 mg), glucose-6-phosphate (2 mg) and glucose-6-phosphate dehydrogenase. (The additives were obtained from Boehringer Mannheim Corp., New York, N.Y.)
Incubations. Each incubation flask contained "mitochondria" obtained from 100 mg of the adrenal tissue (about 2 mg of protein). Final incubation volume was 5 ml. Incubations were carried out in a metabolic shaking incubator for 30 minutes at 41°C in an O_2 (95%) + CO_2 (5%) atmosphere. Most of these incubations were done in duplicate.

Extraction and isolation of steroids. All incubation media were extracted with chloroform and ethyl acetate as reported alsewhere (10). The extracts were evaporated to dryness under reduced pressure.

Extracts were fractionated by serial paper chromatography as shown in Figure 1. The following chromatographic systems, both paper (PPC) and thin-layer (TLC), were used for the isolation and identification of biosynthetic products and their derivatives: toluene/ethylene glycol (T.E.G.), ligrion/propylene glycol (L.P.G.), toluene/propylene glycol (T.P.G.), E_2B, E_4, Bush C and Bush A (11); TLC of Randerath (12); TLC-1, chloroform:acetone (95:5); TLC-2, cyclohexane: ethyl acetate (50:50) and TLC-3, benzene:ethyl acetate (1:10).

Detection and assay of radioactive substances. Radioactive zones were detected on paper chromatograms and thin-layer plates with radio-autography and/or a radio-chromatogram scanner (Model 7200, Packard Instrument Company, Downers Grove, Ill.). Quantitative measurement of radioactivity was done with a 3-channel liquid scintillation spectrometer, equipped with automatic external standardization for control of quenching (Tri-Carb, Model 3375, Packard Instrument Company). Counting error was kept at + 1% (10^4 net cpm accumulated) and isotope contents were expressed as disintegrations per minute (dpm).

Results have been expressed as percentage conversion of radioactivity (^{14}C dpm) added as a substrate. The data have not been corrected for experimental losses.

Characterization of metabolites. Individual metabolites isolated by the chromatography scheme shown in Figure 1 were further run in 2-3 suitable PPC and/or TLC systems in which their isopolarity with the corresponding radio-inert carrier was established. Subsequently these metabolites were mixed with authentic tritiated marker steroids (always recently purified whenever possible).

Acetylation and hydrolysis of the acetate were done as previously described (11). Oxidations at carbon 11 and 17 were carried out with chromium trioxide (CrO_3) and sodium bismuthate ($NaBiO_3$) respectively, as described by Bush (13). Some of the metabolites were mixed with 25 mg of authentic radio-inert carriers (previously recrystallized 3 times from pentane-ethanol) and successive crystallizations were performed using appropriate solvent mixtures.

A conversion product was considered characterized when one or more of the following criteria could be established: constancy of $^{3}H/^{14}C$ ratios after 1) formation of derivative(s), 2) constant specific activity after successive crystallization of the metabolite or its derivative.

RESULTS

1. Incubation of androstenedione-4-^{14}C with duck adrenal "mitochondria."

From this incubation the following ^{14}C-labelled metabolites were isolated: 11β-OH-Δ^4A, 19-OH-Δ^4A and testosterone.

11β-OH-Δ^4A. A radioactive substance having chromatographic mobility parallel to 11β-OH-Δ^4A appeared in E_2B system (Fig. 1). After elution

of the ^{14}C-metabolite, 100 µg of authentic radio-inert 11β-OH-Δ^4A and tritiated 11β-OH-Δ^4A, were added and the mixture chromatographed in TLC-1. After the first chromatogram the ^3H and ^{14}C mixture was oxidized with CrO_3 and the adrenosterone thus formed was chromatographed in TLC-1, TLC-2 and TLC-3. Subsequently, 25 mg of adrenosterone was added to the mixture and it was crystallized three times from petroleum ether containing small amounts of benzene. The counting data obtained after TLC and on crystallizations are shown in Tables 1 and 2 respectively. The amount of radioactivity in 11β-OH-Δ^4A showed a 38% conversion of the substrate.

19-OH-Δ^4A. The ^{14}C material, having similar mobility to 19-OH-Δ^4A in E_2B system (Fig. 1: fraction C) was eluted. It was combined with 100 µg of authentic 19-OH-Δ^4A and run in T.E.G. system for 4 hours. The radioactive material and the carrier remained homogeneous. Finally the radioactive material was mixed with 25 mg of 19-OH-Δ^4A and crystallized four times from petroleum ether containing a trace of benzene. Table 2, shows the counting data of crystalline samples. The amount of radioactivity in 19-OH-^{4}A, in terms of percentage conversion was <1%.

Testosterone. The radioactive material, appearing in the overflow (Fig. 1: fraction D) had similar polarity as authentic testosterone in L.P.G. system for 24 hrs. When combined with 100 µg of authentic testosterone and rechromatographed in TLC-1, the radioactivity remained associated with the carrier. At this stage testosterone-^3H was added to the ^{14}C metabolite and the mixture chromatographed successively on TLC-1, TLC-2 and TLC-3. The mixture was acetylated and again subjected to TLC on the above mentioned systems. Finally the ^3H and ^{14}C labelled acetate mixture was diluted with 25 mg of testosterone acetate and crystallized three times with petroleum ether and benzene. The $^3H/^{14}C$ data of free and acetylated compound and of crystallized samples are shown in Tables 1 and 2. The transformation of the substrate to testosterone was 32%.

2. Incubation of testosterone-4-^{14}C with duck adrenal "mitochondria."

Testosterone-4-^{14}C was incubated with duck adrenal mitochondria and following identifiable metabolites were obtained: 11β-OH-Δ^4A

11β-OH-T and 18-OH-T.

11β-OH-Δ^4A. A radioactive zone having chromatographic mobility similar to 11β-OH-Δ^4A appeared in E_2B, system (Fig. 1: fraction C). The amount of radioactivity in this zone was 3% of the substrate added. Further steps for the identification of the metabolite were similar to those used in the earlier experiment when androstenedione-4-^{14}C was employed as a substrate (see Tables 1 and 2).

11β-OH-T. A radioactive substance present in the third overflow (Fig. 1: fraction B) was rechromatographed in the T.E.G. system for 17 hrs. In this system it was isopolar with authentic 11β-OH-T. After elution, the radioactive material was combined with 100 µg of authentic carrier and chromatographed in Bush C system where the radioactivity remained associated with the carrier. The mixture was oxidized with $NaBio_3$ and the resultant substance was chromatographed in TLC-1. Tritiated 11β-OH-Δ^4A was added to the oxidized ^{14}C material and constant $^3H/^{14}C$ ratios were established in TLC systems as shown in Table 1. The $^3H/^{14}C$ mixture of 11β-OH-Δ^4A was further oxidized with CrO_3 to adrenosterone and chromatographed in TLC-1 and TLC-2 systems. Conversion of the substrate to 11β-OH-T was 60%.

18-OH-T. This radioactive material was isopolar with 18-OH-T in the E_2B system (Fig. 1). Subsequently it was combined with 100 µg of radio-inert 18-OH-T and acetylated. The mixture of the acetylated biosynthetic material and the carrier remained homogeneous in Bush A and TLC-1 systems. After saponification to free 18-OH-T, the carrier and the ^{14}C material did not separate in Bush C and TLC-1 systems. Further identification by crystallization could not be carried out because of lack of authentic material in mg amounts. The conversion of the substrate to 18-OH-T was 2%.

3. Incubation of 11-deoxycorticosterone-4-^{14}C with duck adrenal "mitochondria".

The presence of 19-hydroxylase activity has been already reported in various species of mammalian adrenal gland (8, 14-22) and fish interrenals (8). As shown in this study, that duck adrenal mitochondria have the enzymatic capacity to form 19-OH-Δ^4A using a C_{19} steroid as a precursor. In consequence, we attempted to explore whether

a C_{21} steroid substrate could also be 19-hydroxylated by these "mitochondrial" preparations. 11-Deoxycorticosterone-4-^{14}C was chosen as a model precursor. The isolation and identification of ^{14}C labelled 19-OH-DOC is described as following:

19-OH-DOC. A radioactive conversion product present in the third overflow (Fig. 1: fraction B) was rechromatographed in T.E.G. system for 17 hrs. From previous knowledge, it had a mobility similar to 19-OH-DOC (20). The labelled material was acetylated and the chromatographic behaviour of the diacetate in T.P.G. system was found to be similar to that reported by Neher (16) for 19-OH-DOC, 19,21-diacetate.

Tritiated 19-OH-DOC was prepared by incubation of beef adrenals with 11-deoxycorticosterone-1,2-^{3}H. This compound was acetylated and its infrared spectrum was taken. Carbon 14 labelled diacetate from the duck adrenal and the ^{3}H diacetate from beef adrenals were mixed and purified in several chromatographic systems. The mixture of $^{3}H/^{14}C$ 19-OH-DOC, 19,21-diacetate was hydrolyzed and chromatographed to constant isotope ratios (Table 1). These data were taken as proof that the biosynthetic material was indeed 19-OH-DOC-^{14}C. The conversion of this ^{14}C metabolite was <1% of the substrate used.

DISCUSSION

Results described in this paper indicate that duck adrenal tissue metabolized exogenous Δ^4A and T in a manner similar to mammalian adrenals (8,21). Hydroxylations of C_{19} precursors by rat adrenals at 11β,18 and 19 positions have been reported in the literature (8). It has also been shown that the interrenals of the Atlantic Salmon (Salmo salar) transform exogenous testosterone to 19-OH-T and 19-OH-Δ^4A (8).

Hydroxylation at 11β-position of C_{21} steroid is known to occur in the adrenals of all the vertebrate classes studied (22). However, there are few in vitro studies where the steroids of C_{19} series have been employed as precursors (8,19). The present investigation has demonstrated that duck adrenal "mitochondria" will actively hydrosylate exogenous Δ^4A and T at 11β-position ($\Delta^4A \longrightarrow$ 11β-OH-Δ^4A: 38% conversion; T$\longrightarrow$11β-OH-T: 60% conversion).

Carbon-18 hydroxylation of C_{21} steroids is known to occur in the "mitochondrial" fraction of mammalian (23), avian (6), amphibian

(25) and fish (26) adrenocortical tissue. On the other hand 18-hydroxylation of C_{19} steroids has been shown with mammalian adrenal gland only (8,21). Previously Sandor et al (6) using steroids of C_{21} series have demonstrated 18-hydroxylase activity in the duck adrenal "mitochondria". In the present study, testosterone was converted to 18-OH-T (2%). However, the degree of conversion was very low as compared to the conversion of corticosterone to 18-OH-B (15%) by a similar tissue preparation (24). This could be attributed to the fact that there is a competition between 11β and 18-hydroxylations. This speculation was based upon the fact that there was a high degree of conversion of testosterone to 11β-OH-T (60%). Also the metabolism of testosterone and corticosterone cannot be compared due to the fact that corticosterone is already hydroxylated at C-11.

The metabolic reaction $\Delta^4A \rightleftharpoons T$ is known to occur in cortisol producing mammals (8,21). However, Lucis et al. (8) have demonstrated that the rat adrenal, which similarly to avian adrenal does not possess 17α-hydroxylase, converted T to Δ^4A. In the duck adrenal tissue, both 17β-hydroxysteroid dehydrogenase and 17β-reductase activities were shown to be present.

Hydroxylation at position 19 of the steroid molecule has been shown to occur in mammalian (8,21) and fish (9) adrenocortical parenchyma. With regard to the C_{19} steroids, Meyer (15) has reported 19-hydroxylation of androstenedione and dehydroepiandrosterone by bovine adrenals and Griffiths (19) that of androstenedione and testosterone by the adrenals of golden hamster. Also Lucis et al. (8) demonstrated the presence of this enzyme in the adrenocortical tissue of beef, rat, human and Atlantic salmon using labelled steroids of C_{19} and C_{21} series. Using 11-deoxycorticosterone as substrate, several groups of investigators have shown the in vitro conversion to this steroid by bovine (16,27,28), hog (21), rat (20) and human (8) adrenals. Also 19-OH-DOC has been isolated as a metabolite of radio-progesterone using Atlantic salmon interrenal incubation (8). The isolation of 19-OH-Δ^4A and 19-OH-DOC, as conversion products of androstenedione and 11-deoxycorticosterone respectively, by duck adrenal "mitochondria", shows that 19-hydroxylation also occurs in the avian adrenals. The physiological significance of 19-hydroxy C_{21} steroids is not known.

19-OH-DOC shows only weak mineralocorticoid activity (14).

In conclucion, though there is no evidence of C_{19} steroid production by duck adrenals, these compounds are utilized *in vitro* studies as substrates by duck adrenal mitochondria.

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*** The following trivial names and abbreviations are used in the text:

1. 19-Hydroxy-11deoxycorticosterone (19-OH-DOC)- 19, 21-dihydroxy-pregn-4-ene-3,20-dione.
2. 11-Deoxycorticosterone (DOC)- 21-hydroxy-pregn-4-ene-3,20-dione.
3. 19-Hydroxy-testosterone (19-OH-T)- 17β,19-dihydroxy-androst-4-ene-3-one.
4. 18-Hydroxy-testosterone (18-OH-T)- 17β,18-dihydroxy-androst-4-ene-3-one.
5. 11β-Hydroxy-testosterone (11β-OH-T)- 11β, 17β-dihydroxy-androst-4-ene-3-one.
6. Testosterone (T)- 17β-hydroxy-androst-4-ene-3-one.
7. Adrenosteron-Androst-4-ene-3,11,17-trione.
8. 19-Hydroxy-androstenedione (19-OH-Δ^4A)- 19-hydroxy-androst-4-ene-3,17-dione.
9. 11β-Hydroxy-androstenedione (11β-OH-Δ^4A)- 11β-hydroxy-androst-4-ene-3,17-dione.
10. Androstenedione (Δ^4A)- androst-4-ene-3,17-dione.
11. 18-Hydroxycorticosterone (18-OH-B)- 11β,18,21-trihydroxy-pregn-4-ene-3,20-dione.
12. Corticosterone (B)- 11β,21-dihydroxy-pregn-4-ene-3,20-dione.

TABLE 1

IDENTIFICATION OF ^{14}C-METABOLITES: $^{3}H/^{14}C$ RATIOS OF ISOLATED STEROIDS AND THEIR DERIVATIVES IN VARIOUS CHROMATOGRAPHIC SYSTEMS

^{14}C Substrate	Metabolite and Derivative	Chemical Treatment	Chromatographic systems	$^{3}H/^{14}C$
Δ^4A	11β-OH-Δ^4A			
	a) Parent compound	None	TLC-1	3.99
	b) Adrenosterone	CrO_3 oxidation	TLC-1	3.92
			TLC-2	3.91
			TLC-3	3.90
	Testosterone			
	a) Parent compound	None	TLC-1	8.98
			TLC-2	9.69
			TLC-3	9.92
	b) Testosterone-acetate	Acetylation	TLC-1	9.87
			TLC-2	9.97
			TLC-3	10.05
T	11β-OH-Δ^4A			
	a) Parent compound	None	TLC-1	3.61
	b) Adrenosterone	CrO_3 oxidation	TLC-1	4.12
			TLC-2	4.28
			TLC-3	4.32
	11β-OH-T			
	a) 11β-OH-Δ^4A	$NaBio_3$ oxidation	TLC-1	9.69
			TLC-2	9.92
	b) Adrenosterone	CrO_3 oxidation	TLC-1	9.84
			TLC-2	10.12
DOC	19-OH-DOC			
	a) 19-OH-DOC, 19,21-diacetate	Acetylation	E_4	1.68
			T.P.G.	1.36
			TLC-1	1.27
	b) 19-OH-DOC	Saponification	T.P.G.	1.40
			TLC-1	1.43

Note: The coefficient of variation of the isotope ratios was always less than ± 3.6%.

TABLE 2

IDENTIFICATION OF ^{14}C-METABOLITES: SPECIFIC ACTIVITIES AND $^{3}H/^{14}C$ RATIOS AFTER SUCCESSIVE CRYSTALLIZATIONS

Steroid and Crystallization no.	Specific activity dpm/mg ^{3}H	Specific activity dpm/mg ^{14}C	$^{3}H/^{14}C$
Adrenosterone (Derivative of 11β-OH-Δ^4A*)			
1	8933	2316	3.85
2	9060	2366	3.82
3	8750	2296	3.81
19-OH-Δ^4A*			
1		357	
2		350	
3		362	
4		357	
Testosterone acetate (Derivative of testosterone*)			
1	1847	184	10.04
2	1914	190	10.07
3	1952	188	10.38
Adrenosterone (Derivative of 11β-OH-Δ^4A**)			
1	1634	370	4.41
2	1614	366	4.40
3	1647	357	4.51

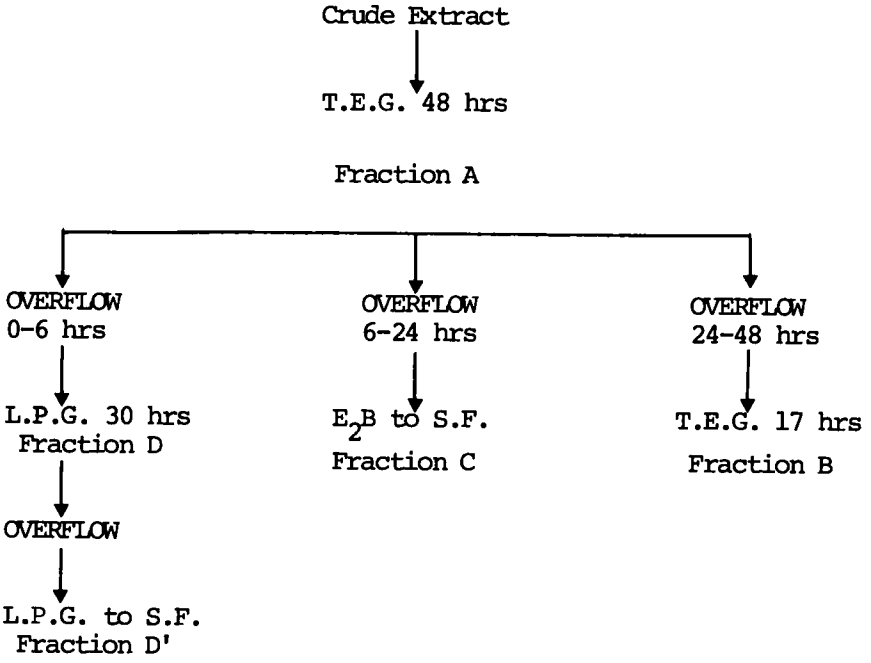
* From studies with androstenedione-4-^{14}C

** From studies with testosterone-4-^{14}C

Note: The coefficient of variation of the specific activities was always less than ± 2.9%.

FIGURE I

SERIAL FRACTIONATION



Serial paper-chromatographic scheme for the fractionation of steroids in crude extract from duck adrenal "mitochondria" incubations.

S.F.= Solvent Front.