Journal of Steroid Biochemistry, 1977, Vol. 8, pp. 643–646. Pergamon Press. Printed in Great Britain

THE SYNTHESIS OF C-19 STEROIDS BY GUINEA-PIG ADRENAL HOMOGENATES

EZRA S. LWOWSKI AND D. W. KILLINGER*
Departments of Physiology and Medicine and the Wellesley Hospital, University of Toronto, Toronto, Ontario, Canada

(*Received* 4 *October* 1976)

SUMMARY

Guinea-pig adrenal homogenates were incubated with [^{3}H]-pregnenolone as substrate. There was conversion to progesterone, cortisol, cortisone, 11β-hydroxyandrostenedione (11βOHA), androstenedione, adrenosterone and testosterone. Dehydroepiandrosterone could not be isolated. Of the total amount of substrate, 3.26% was converted to 11βOHA, and 4.08% was converted to cortisol. In an incubation using [^{14}C]-cortisol as substrate, 2.34% conversion to 11βOHA was obtained. When adrenal homogenates were incubated with both [^{3}H]-pregnenolone and [^{14}C]-cortisol, the ^{3}H/^{14}C ratio in isolated 11βOHA was 3–4 fold higher than the ^{3}H/^{14}C ratio in isolated cortisol. These results indicate that cortisol is not the major precursor of 11βOHA in guinea-pig adrenal. It is likely that most of 11βOHA is formed from androstenedione.

INTRODUCTION

There has been some controversy regarding the biosynthesis of specific C-19 steroids by the guinea-pig adrenal. Dehydroepiandrosterone (DHA) was found in adrenal incubates when 17-hydroxypregnenolone was used as a substrate [1–3]; however, when pregnenolone or progesterone was used as substrate [4, 5], no DHA was found. Both androstenedione and 11β-hydroxyandrostenedione (11βOHA) have been found in guinea-pig adrenal incubations using either pregnenolone or progesterone as substrates [6–9]. Grossman and Block, however, observed the conversion of pregnenolone to androstenedione in homogenates of fetal guinea-pig adrenals, but were unable to demonstrate this in adults [4]. Deshpande *et al.*[5] were also unable to find any androstenedione, after perfusing guinea-pig adrenals with [^{3}H]-pregnenolone. They did report significant conversion to 11βOHA. This suggested that in the guinea-pig, 11βOHA is formed by the side-chain cleavage of cortisol. We investigated the production of 11βOHA and other C–19 steroids by guinea-pig adrenal homogenates using both pregnenolone and cortisol as substrates.

MATERIALS AND METHODS

Animals. Coloured virgin female guinea-pigs were obtained from Connaught Medical Research Labs, Toronto, and were caged in pairs with unlimited food and water, in a photoperiod of 12L:12D. The animals were 6 months old and weighed approximately 600 g.

Chemicals. All radioactive steroids were obtained from New England Nuclear Corp., Boston, and were purified by paper chromatography prior to use. Purity of substrates was confirmed by the recrystallization of an aliquot with authentic unlabeled standard. Non-radioactive andrenosterone was obtained from Mann Research Laboratories, New York. All other steroids were from Ikapharm, Ramat-Gan, Israel. NADPH and ATP were purchased from Sigma Chemicals, St. Louis, and Mann Research Laboratories respectively.

Experimental design. The following 3 incubations were carried out. A. Tissue was incubated with 27.2 μCi [7-^{3}H]-pregnenolone (S.A. = 15.8 Ci/mmol). Appropriate amounts of [^{14}C]-labeled progesterone, androstenedione, DHA, DHA sulphate, testosterone, 11βOHA, cortisone, and cortisol were added after termination of the incubation to determine the recovery. B. Tissue was incubated with 0.3 μCi [4-^{14}C]-cortisol (S.A. = 54.9 mCi/mmol) as substrate. Tritiated 11βOHA was added after the incubation was terminated to check for recovery. C. Tissue was incubated with both [^{3}H]-pregnenolone (19.5 μCi) and [^{14}C]-cortisol (0.3 μCi) as substrates.

Incubation procedure. Guinea-pigs were lightly anaesthetized with CO_2 and decapitated. The

The following trivial names and abbreviations are used: adrenosterone: 4-androsten-3,11,17-trione; androstenedione: 4-androsten-3,17-dione; cortisol: 11β,17,21-trihydroxy-4-pregnen-3,20-dione; cortisone: 17,21-dihydroxy-4-pregnen-3,11,20-trione; dehydroepiandrosterone (DHA): 3β- hydroxy-5-androsten-17-one; dehydroepiandrosterone sulphate: 17-oxo-5-androsten-3β-yl sulphate; 11β-hydroxyandrostenedione (11βOHA): 11β-hydroxy-4-androsten-3,17-dione; 17-hydroxypregnenolone: 3β,17-dihydroxy-5-pregnen-20-one; 17-hydroxyprogesterone: 17-hydroxy-4-pregnen-3,20-dione; pregnenolone: 3β-hydroxy-5-pregnen-20-one; progesterone: 4-pregnen-3,20-dione; testosterone: 17β-hydroxy-4-androsten-3-one.

* Address reprint requests to Dr. D. W. Killinger, Medical Sciences Building, Room 7366, University of Toronto, Toronto, Ontario, Canada M5S 1A8.

Table 1. Paper chromatographic systems used for the purification of substrates and isolated products

System	Compounds
Heptane:Benzene:MeOH:H_2O (2:1:4:1)	pregnenolone, progesterone, androstenedione, DHA, testosterone, adrenosterone, 11βOHA
Hexane:MeOH:H_2O (10:9:1)	pregnenolone, progesterone, androstenedione, DHA, testosterone
Isooctane:Toluene:MeOH:H_2O (3:2:3:1)	DHA, 11βOHA
Isooctane: *t*-butanol:MeOH:H_2O (10:6:10:3)	testosterone, adrenosterone, 11βOHA
Isooctane: *t*-butanol: H_2O (10:5:9)	cortisol, cortisone
Benzene:MeOH:H_2O (2:1:1)	cortisol, cortisone

adrenals were removed, freed of adhering fat, and finely minced. They were then homogenized by hand in a Ten Broeck all-glass hand homogenizer in phosphate buffer (pH = 7.4). The homogenate was added to 25 ml incubation flasks containing the substrate dissolved in 4 drops propylene glycol, and incubated for 60 min at 37°C in phosphate buffer (pH = 7.4) with 0.2 mM NADPH and 2 mM ATP. The ratio of tissue to buffer was 100 mg per ml. All reactions were terminated by the addition of sufficient ice-cold ethanol to make an 80% solution. After addition of the steroids to monitor losses, the aqueous ethanol solution was evaporated *in vacuo*.

Product isolation and quantification. The residue was dissolved in water, and extracted 5 times with dichloromethane. The organic fractions were pooled, dried with Na_2SO_4 and evaporated *in vacuo*. An aliquot of the aqueous phase was removed for determination of radioactivity. The organic extract was chromatographed on a 25 g celite column using the solvent system isooctane:methanol:water (10:9:1 v/v), as described by Engel *et al.*[10]. Gradient elution was performed with increasing concentrations of dichloroethane in isooctane. One-hundred and fifty 5 ml fractions were collected, and pooled on the basis of their content of radioactivity.

Isolated compounds were further purified by descending paper chromatography on Whatman Number 1 filter paper. The systems used for each steroid are shown in Table 1. Chromatograms were scanned with a Packard Radiochromatogram Scanner, and the appropriate peak of radioactivity was eluted. The final eluate was added to approximately 40 mg of authentic standard, and recrystallized to constant S.A.

Radioactivity was determined in a Philips Liquid Scintillation Analyser in vials containing 10 ml toluene with 4% Permafluor (Packard Instrument Co.) and 2% methanol. Efficiency of counting, determined by use of an external standard, was approximately 25% for tritium and 35% for ^{14}C.

RESULTS

Recrystallization data for the purification of substrates and all isolated products are shown in Table 2.

Incubation A. The products of conversion following incubation with [^{3}H]-pregnenolone are listed in Table 3. The C-21 products isolated were progesterone (9.58%), cortisol (4.08%) and cortisone (2.06%). The C-19 steroids isolated were androstenedione (1.67%), testosterone (0.10%), and adrenosterone (0.13% uncorrected), with the major C-19 compound being 11βOHA (3.26%). The presence of DHA could not be established, as the S.A. of the crystals was still not constant after six crystallizations (Table 2). A second incubation under similar conditions confirmed the presence of androstenedione (0.38%), testosterone (0.14%), adrenosterone (0.07% uncorrected), and 11βOHA (3.39%); but once again DHA could not be detected. The aqueous phase contained 5.47% of the radioactivity. Celite partition chromatography revealed 4 radioactive peaks, which were not further analysed.

Incubations B and C: In the incubation with cortisol as the substrate, 2.36% was converted to 11βOHA. It has previously been shown that the spontaneous conversion of cortisol to 11βOHA is undetectable under similar conditions[11]. The results of the incubation with both [^{3}H]-pregnenolone and [^{14}C]-cortisol as substrates are shown in Table 4. The ratio of ^3H d.p.m. to ^{14}C d.p.m. after recrystallization of cortisol was 7.34. It was slightly lower for cortisone (6.88), and considerably higher for 11βOHA (26.61). The significance of these isotope ratios is discussed below.

DISCUSSION

The conversion of pregnenolone to cortisol and its subsequent oxidation to cortisone (Tables 3 and 4), serve to validate the adrenal homogenate system used here, as these steroids are known to be synthesized by the guinea-pig adrenal [12–14]. The substantial production of progesterone has not previously been demonstrated in the guinea-pig adrenal, but has been shown in the adrenal of other species [15–18].

The biosynthesis of specific C-19 steroids by the guinea-pig adrenal has been a controversial matter. Lipsett and Hokfelt[1], and Trout and co-workers [2, 3] detected DHA in adrenal tissue incubated with 17-hydroxypregnenolone. When pregnenolone was

Table 2. Recrystallization data showing S.A. of crystals and mother liquors of substrates and products of incubations with guinea-pig adrenal homogenates

		Specific Activity (d.p.m./mg)					
		^3H			^{14}C		
	Compound	C_{n-1}	C_n	ML_n	C_{n-1}	C_n	ML_n
Substrates	pregnenolone	2883	2737	2943	—	—	—
	cortisol	—	—	—	181	176	174
Products of Incubation							
A.	progesterone	5160	5217	5286	126	134	128
	androstenedione	8093	8126	8391	425	421	436
	DHA*	103	76	124	49	48	51
	testosterone	583	618	670	154	146	156
	adrenosterone	1984	1938	2090	—	—	—
	11βOHA	3177	3163	3339	113	102	105
	cortisone	2436	2377	2468	61	59	60
	cortisol	4038	4308	4351	147	152	156
B.	11βOHA	342	356	349	140	137	129
C.	cortisol	3628	3956	3962	490	542	541
	cortisone	220	213	221	33	31	31
	1βOHA	3175	3162	3243	122	115	122

n = number of crystallizations.
C = crystals.
ML = mother liquor.
* —specific activities of crystals and mother liquor were not constant after 6 crystallizations.

the substrate, however, no DHA was found [4, 5]. In similar incubations with pregnenolone as substrate we were also unable to establish the presence of DHA (Table 2). We did find a substantial conversion of pregnenolone to progesterone, suggesting the presence of an active 3β-hydroxysteroid dehydrogenase. This would mean that 17-hydroxypregnenolone is not a major intermediate in guinea-pig adrenal biosynthesis. The conversion of exogenous 17-hydroxypregnenolone to DHA therefore may not represent the physiological situation.

The conversion of pregnenolone to androstenedione (Table 3) confirms previous reports [6–9] that this androgen is produced by the guinea-pig adrenal. The production of testosterone (Table 3) lends further support for the presence of androstenedione, since this steroid is thought to be the major precursor of testosterone.

Despande *et al.*[5] observed that when guinea-pig adrenals were perfused with [^{3}H]-pregnenolone,

Table 3. Steroids isolated after incubation of guinea-pig adrenal homogenates with radioactive precursors

Substrate	Product	% conversion
pregnenolone	progesterone	9.58
	androstenedione	1.67
	testosterone	0.10
	andrenosterone	0.13*
	11βOHA	3.26
	cortisone	2.06
	cortisol	4.08
cortisol	11βOHA	2.34

Incubations were carried out with either 27.2 μCi [^{3}H]-pregnenolone or 0.3 μCi [^{14}C]-cortisol.
* uncorrected for recovery.

Table 4. Steroids isolated after incubation of guinea-pig adrenal homogenate with [^{3}H]-pregnenolone (19.5 μCi) and [^{14}C]-cortisol (0.3 μCi) as substrates

Product	Isotope ratio (d.p.m. ^{3}H/d.p.m. ^{14}C)
cortisol	7.34
cortisone	6.88
11βOHA	26.61

11βOHA was found in the perfusate, even though androstenedione was not. This led them to suggest that 11βOHA was formed by the side-chain cleavage of cortisol. To test this hypothesis, we incubated adrenal homogenates with either pregnenolone or cortisol. Table 3 shows that 4.08% of [^{3}H]-pregnenolone was converted to cortisol, while 3.26% was converted to 11βOHA. When the substrate was cortisol only 2.34% was converted to 11βOHA. This strongly suggests that most of 11βOHA derived from pregnenolone did not come through cortisol. To further clarify this matter, both [^{3}H]-pregnenolone and [^{14}C]-cortisol were used as substrates in the same incubation. The $^3H/^{14}C$ ratio in 11βOHA and its putative precursor (cortisol) can then be compared. A $^3H/^{14}C$ ratio in 11βOHA less than or equal to that in cortisol indicates that all of the 11βOHA which was formed from pregnenolone came through cortisol; since any 11βOHA produced through another intermediate would be labeled with ^{3}H but not with ^{14}C, thereby increasing the $^3H/^{14}C$ ratio in the final product. Table 4 shows that the ratio in 11βOHA was 3–4 times higher than that in cortisol,

indicating that under the conditions studied, cortisol was not the major precursor of 11βOHA.

In addition to 11βOHA, another 11-oxygenated C-19 compound, adrenosterone, was also detected in small amounts (Table 3). This steroid has previously been found in bovine adrenal [19]. At present, there is no information concerning its biosynthetic pathways, but it can be speculated that it is formed by reduction of 11βOHA in a reaction similar to the formation of cortisone from cortisol.

In conclusion, guinea-pig adrenal homogenates produce 11βOHA from both pregnenolone and cortisol, but cortisol is not the major precursor of 11βOHA. It is more likely that 11βOHA is formed by the hydroxylation of androstenedione, as has been demonstrated in other species [20].

Acknowledgements—This work was supported by grant MA 2613 from the Medical Research Council of Canada. ESL was the recipient of a Medical Research Council Studentship. The authors wish to thank Mrs. E. Perel for helpful discussions and for reviewing the manuscript, and Miss A. Pritchard for her help in its preparation.

REFERENCES

1. Lipsett M. B. and Hokfelt B.: *Experientia* **17** (1961) 449–450.
2. Trout E. C., Kao T. Y. and McGavack T. H.: *Steroids* **9** (1967) 57–70.
3. Trout E. C.: *Steroids* **18** (1971) 505–510.
4. Grossman S. B. and Block E.: *Steroids* **21** (1973) 813–832.
5. Deshpande N., Carson P. and Harley S.: *J. Endocr.* **50** (1971) 467–484.
6. Spatz L. and Hofman F. G.: *Endocrinology* **79** (1966) 971–990.
7. Bloch E.: *Steroids* **13** (1969) 589–603.
8. Weiss M. and Ford V. L.: *Int. J. Biochem.* **6** (1975) 683–687.
9. Billiar R. B. and Eik-Nes K. B.: *Biochim. biophys. Acta* **104** (1965) 503–514.
10. Engel L. L., Cameron C. B., Stoffyn A., Alexander J. A., Klein O. and Trofimow N. D.: Analyt. Biochem. **2** (1961) 114–125.
11. Hudson R. W. and Killinger D. W.: *J. clin. Endocr. Metab.* **34** (1972) 215–224.
12. Seth P.: *Gen. comp. Endocr. Suppl.* **2** (1969) 317–324.
13. Sandor T.: *Gen. comp. Endocr. Suppl.* **2** (1969) 284–298.
14. Kolanowski J. and Crabbé J.: *J. steroid Biochem.* **5** (1974) 310.
15. Highfill D. R. and Mead R. A.: *Gen. comp. Endocr.* **27** (1975) 389–400.
16. Callard I. P. and Leathem J. H.: *Proc. Soc. exp. Biol. Med.* **115** (1964) 567–569.
17. Resko J. A.: *Science* **164** (1969) 70–71.
18. Wasserman G. F. and Eik-Nes K. B.: *Acta endocr., Copenh.* **61** (1969) 33–47.
19. Meyer A. S., Hayano M., Lindberg M. C., Gut M. and Rodgers O. G.: *Acta endocr., Copenh.* **18** (1955) 148–168.
20. Hudson R. W., Schachter H. and Killinger D. W.: *J. steroid Biochem.* **7** (1976) 255–262.