A Comparative Study of Steroid Biosyntheses *in Vitro*. in Clear Cell Adenoma and its Adjacent Tissue of Human Adrenal Gland *

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Synopsis

Cell-free homogenates of adrenal adenoma and its adjacent adrenal tissue obtained from a 28 yr-old female patient with Cushing's syndrome were incubated simultaneously with 4-¹⁴C-labelled steroid precursors, such as pregnenolone, progesterone, 17 α -hydroxyprogesterone, dehydroepiandrosterone and 11-desoxycortisol, at 37°C for 1 hr. under oxygenenriched atmosphere. After identification and quantitation of the steroids produced, *in vitro* biosynthetic activities of steroids were compared of both the above mentioned tissues. No significant difference was observed between the activities of steroid biosyntheses in these tissues, judging from the quantities of the corresponding steroids metabolized by the two tissues. Activities of 3 β -hydroxysteroid dehydrogenase with Δ^5 - Δ^4 isomerase, 17 α -hydroxylase and 21-hydroxylase were high, whereas the activity of 11 β -hydroxylase was found poor in both tissues. Pregnenolone was considered to be preferable to progesterone as precursor for cortisol biosynthesis in the human adrenal tissue, according to the quantitative determination of 11-desoxycortisol and cortisol produced from the two substances.

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The following trivial names were used in this text: pregnenolone; 3 β -hydroxypregn-5-en-20-one, progesterone; pregn-4-ene-3, 20-dione, 17 α -hydroxypregnenolone; 3 β , 17 α -dihydroxypregn-5-en-20-one, 17 α -hydroxyprogesterone; 17 α -hydroxypregn-4-ene-3,20-dione, 11-desoxycortisol; 17 α , 21-dihydroxypregn-4-ene-3, 20-dione, cortisol; 11 β , 17 α , 21-trihydroxypregn-4-ene-3, 20-dione, 11-desoxycorticosterone; 21-hydroxypregn-4-ene-3, 20-dione, corticosterone; 11 β , 21-dihydroxypregn-4-ene-3, 20-

dione, 16 α -hydroxyprogesterone; 16 α -hydroxypregn-4-ene-3, 20-dione, 11 β -hydroxyprogesterone; 11 β -hydroxypregn-4-ene-3, 20-dione, aldosterone; 11 β , 21-dihydroxy-18-oxo-pregn-4-ene-3, 20-dione, dehydroepiandrosterone; 3 β -hydroxyandrost-5-en-17-one, androstenedione; androst-4-ene-3, 17-dione, testosterone; 17 β -hydroxyandrost-4-en-3-one, 11 β -hydroxyandrostenedione; 11 β -hydroxyandrost-4-ene-3, 20-dione, adrenosterone; androst-4-ene-3, 11, 17-trione, dexamethasone; 9-fluolo-11 β , 17 α , 21-trihydroxy-16 α -methyl-pregna-1, 4-diene-3, 20-dione.

Following abbreviations were used: ACTH; adrenocorticotrophic hormone, 17-OHCS; 17 α -hydroxylated corticosteroids.

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It is well known that Cushing's syndrome is caused by the disturbance of adrenocortical functions. Although many reports are available on *in vitro* steroid metabolism in the adrenals obtained from patients with Cushing's syndrome (Bailey *et al.*, 1960; Villee *et al.*, 1962; Goldstein *et al.*, 1963; Adadevoh *et al.*, 1965), the comparison of steroid metabolism between adenoma and its adjoining adrenal tissue was reported only in 1 case of Conn's syndrome (Dufau *et al.*, 1968).

An adrenal gland bearing a clear cell adenoma was obtained from a patient with Cushing's syndrome. In this paper, patterns of steroid biosynthesis *in vitro* of the adenoma and adrenal tissue adjoining to it were investigated by incubating simultaneously several steroid precursors with these tissues under the same condition.

Materials and Methods

Clinical informations and histological findings of the adrenal gland

A 28 yr-old female patient with clinical signs and symptoms of Cushing's syndrome for the last decade was admitted to our clinic for the clinical evaluation. At the age of 24 yrs., partial resection of bilateral adrenals was performed at another hospital under the diagnosis of the left adrenal tumor, but the subsequent histological studies had revealed normal finding in the resected adrenal. Her complaints had not been improved after the operation, and palpitation had appeared since 6 months before the present admission. Buffalo hump, weight increase, the appearance of striae, abnormal scanty menstrual bleeding with irregular cycles and hirsutism were noticed at the examination. The blood pressure was 230 – 220/170 – 120 mm Hg in supine position. The pituitary fossa was normal. The levels of plasma and urinary 17-OHCS, measured by a colorimetric method (Silber and Porter, 1954; Kambegawa, 1963), were 29.5 $\mu\text{g/dl}$ and 3.15 mg/day (normal range; 0.7 – 2.5 mg/day) respectively. By administration of ACTH 40 i.u. and then, by administration of dexamethason 2 mg/day, plasma level of 17-OHCS was increased to 105 $\mu\text{g/dl}$ and was reduced to 22.3 $\mu\text{g/dl}$. From these results adrenal hyperplasia was suspected, and left total and right subtotal adrenalectomy was performed. A globular tumor, about 1 cm in its diameter was found at the middle of the

left adrenal. The parts of the tumor and its adjacent adrenal tissues were reserved for histological examination, and the remaining tissues were subjected to biochemical studies. Histological studies revealed that the tumor was a clear cell adenoma, and its adjacent tissue and right adrenal tissue were normal as shown in Figure 1.

Fig. 1. Microscopic appearance of adrenal tissue adjacent to adenoma (1a) and adrenal adenoma (1b). Hematoxyline and eosin staining, $\times 100$.

Chemicals

All organic solvents were redistilled before use. Radioactive steroids were purchased from New England Nuclear Corp. (Boston, Mass., U.S.A.) and the Radiochemical Centre (Amersham, England). Their radiochemical purities were checked with thin-layer chromatography in a number of solvent systems shortly before use. They were diluted with the corresponding non-radioactive carriers to insure the saturation of the substrates to the enzymes.

Tissue preparation and condition of incubation

Immediately after the removal, the left adrenal was chilled on crushed ice, and kept in this state for about 2 hrs., by the time of the tissue preparation

for incubation. The tissue was rinsed with ice-cold 0.33 *M* sucrose solution and the part of tumor was separated from its adjoining adrenal tissue. The tissues were then weighed, minced and homogenized in the same ice-cold sucrose solution with a loose-fitting Teflon-glass homogenizer. The weight of the tumor tissue was 1.15 g and adjacent adrenal was 4.45 g. After centrifugation at 800 × *g* for 20 mins., the supernatant fluid was used as a cell-free homogenate of each tissue. Each homogenate was divided into 5 flasks (an equivalent amount to 0.23 g of wet tissue per flask), which contained 50 μg (20×10^4 cpm) of one of such radioactive steroids dissolved in a drop of propylene glycol as pregnenolone-4-¹⁴C, progesterone-4-¹⁴C, 17α-hydroxyprogesterone-4-¹⁴C, dehydroepiandrosterone-4-¹⁴C and 11-desoxycortisol-4-¹⁴C. By adding 1 mg of NADP⁺ into a flask containing pregnenolone-4-¹⁴C or dehydroepiandrosterone-4-¹⁴C and 0.5 mg of NADPH into other flasks dissolved in 1.0 cc of 0.33 *M* sucrose solution containing 0.05 *M* Tris buffer (pH 7.4) and 0.005 *M* MgCl₂, a final volume was adjusted to 4 ml per flask.

The incubation was performed at 37°C for 1 hr. with constant shaking, under the atmosphere of 95 % O₂ and 5 % CO₂.

Extraction and preliminary separation of the products

The incubation was terminated by rapid extraction of the steroids from incubation medium with 15 ml of methylene dichloride. After adding appropriate amounts of carrier steroids, the extraction was repeated two more times with the same amount of methylene dichloride. The combined extracts were dried over anhydrous Na₂SO₄, and filtered through glass wool. The solvent was evaporated under reduced pressure at 40°C. Up to this step, over 90 % of radioactivity initially added was recovered in these extracts.

Thin-layer chromatography of silica gel developed in solvent system of benzene: acetone (4:1, v/v, system A) was used for preliminary separation.

The areas of the carrier steroids and radioactive spots on thin-layer chromatograms were detected under ultraviolet light (253 mμ) and by autoradiography with X-ray film, respectively.

Each area containing the radioactive steroid on the chromatogram was scraped off and after packing the scraped silica gel in a small column, the steroid was exhaustively eluted with a 1:1 (v/v) mixture of ethanol and chloroform. Details of the methods stated above were described previously by one of the authors (Oshima, 1967).

For further separation and purification of the

steroids, several solvent systems for thin-layer chromatography were used as follows: methylene dichloride:methanol (94:6, v/v, system B), cyclohexane:ethyl acetate:n-heptane (4:6:1, v/v/v, system C) and benzene:glacial acetic acid (9:1, v/v, system D).

Quantitation of radioactivity

Radioactivities of thus separated and purified steroid fractions were measured with a liquid scintillation spectrometer (System 725, Nuclear Chicago, Ill. U.S.A.) in the toluene-PPO-POPOP system as described previously (Shikita and Tamaoki, 1965b). The counting efficiency of radiocarbon was about 70 %

Chemical procedures

Acetylation of steroids was carried out by a mixture of pyridine and acetic anhydride (1:1, v/v), standing overnight at room temperature.

For oxidation of hydroxyl group to ketone, 0.5 ml of 0.5 % (w/v) chromium trioxide in 90 % acetic acid was used per flask. Chromium trioxide in glacial acetic acid (0.5 %, w/v) was used for the oxidative side-chain cleavage of 17α-hydroxylated C₂₁-steroids (0.5 ml per flask). One ml of 2 % (w/v) Na₂SO₄ in 1 *N* H₂SO₄ was applied for oxidation of C-21-hydroxylated steroids to the corresponding carbonic acids. After addition of the above mentioned oxidation reagents to the steroid preparation, the mixture was left to stand at room temperature for 10 – 20 mins. and then water was added to stop the reactions. The mixture was immediately extracted with appropriate amount of methylene dichloride.

Results and Discussion

Separation and identification of metabolized products

17α-Hydroxyprogesterone and 11-desoxycorticosterone: As 17α-hydroxyprogesterone and 11-desoxycorticosterone were not separated by thin-layer chromatography in system A, these fractions were subjected to further separation with thin-layer chromatography developed in system B, on which the two steroids showed distinctly different mobilities. Thus separated radioactive compounds behaved identically with the corresponding authentic preparations through the chemical reactions respectively. An aliquot of the radioactive preparation which corresponded to 11-desoxy corticoste-

rone was acetylated with acetic anhydride in pyridine. Then, recrystallization of the acetylated product was performed with authentic 11-desoxycorticosterone-21-acetate and constant specific activities of crystals and solid in mother liquor were obtained. Also, the other metabolite which corresponded to 17 α -hydroxyprogesterone was recrystallized with authentic preparation, and constant specific radioactivities were obtained.

Corticosterone, 16 α -hydroxyprogesterone and 17 α , 20 α -dihydroxypregn-4-en-3-one: As these three steroids were not separated clearly with the thin-layer chromatography in system A, these were further separated with thin-layer chromatography in system C. Radioactivities were found to be associated with the each corresponding carrier spot detected by ultraviolet light. Products after oxidation with Na₂SO₄ and acetylation with acetic anhydride of the radioactive compound which corresponded to corticosterone behaved identically to 3-oxo-11 β -hydroxy-4-etienic acid after the oxidation, and to corticosterone-21-acetate after acetylation on the chromatograms in systems A and D, respectively. Finally, adding authentic corticosterone to the corresponding radioactive compound, specific radioactivities of crystals were found constant through recrystallizations from various solvent mixtures. 16 α -Hydroxyprogesterone was tentatively identified by its characteristic chemical behaviors as described previously (Oshima *et al.*, 1967). Also, 17 α ,20 α -dihydroxypregn-4-en-3-one was tentatively identified through the chromatographic and chemical behaviors with the standard preparation as previously described (Shikita and Tamaoki, 1965a).

11-Desoxycortisol: An aliquot of the radioactive compound which was associated with the carrier 11-desoxycortisol was oxidized with chromium trioxide in glacial acetic acid, and the other aliquot was acetylated with acetic anhydride in pyridine. The products which were chemically derived behaved identically to authentic androstenedione and 11-desoxycor-

tisol-21-acetate on thin-layer chromatogram in system A, respectively. Finally, this radioactive compound was recrystallized with authentic 11-desoxycortisol and constant specific radioactivities were obtained.

Cortisol: When radioactive fraction, which was associated with the carrier cortisol was subjected to oxidation and acetylation, the products showed the same mobilities as adrenosterone and cortisol-21-acetate respectively on thin-layer chromatogram in system A. After adding authentic cortisol to the initial radioactive compound, recrystallization was performed and constant specific activities of the crystals and the solid in the mother liquor were obtained.

Testosterone: The radioactive fraction which was found in the spot of authentic testosterone on thin-layer chromatogram in system A was examined on its identity to testosterone through acetylation and oxidation, and then, was recrystallized with authentic testosterone. From the results obtained, this ¹⁴C-labelled metabolite was identified as testosterone.

17 α -Hydroxypregnenolone: Radioactive fraction which was found in the spot of authentic 17 α -hydroxypregnenolone on thin-layer chromatogram in systems A and C was tentatively identified as 17 α -hydroxypregnenolone, after it was ascertained that the acetylate of the products showed the same mobility as 17 α -hydroxypregnenolone-3-acetate on thin-layer chromatogram in system A.

11 β -Hydroxyandrostenedione: A radioactive compound was eluted from the area of authentic 11 β -hydroxyandrostenedione on thin-layer chromatogram in system A. This compound was not acetyltable with acetic anhydride in pyridine, but oxidized to adrenosterone with chromium trioxide in 90 % acetic acid, judging from their mobilities on the chromatogram developed in system A. From these results, this initial radioactive compound was tentatively identified as 11 β -hydroxyandrostenedione.

Androstenedione and progesterone: Radioactive compounds were found to be associated with the authentic androstenedione and progesterone on the chromatograms developed in systems A and C. These radioactive fractions were neither acetylated nor oxidized, judging from the unchanged mobilities on the chromatograms in systems A and C, and thus tentatively identified as androstenedione and progesterone, respectively.

No significant radioactivities were detected on the spots of authentic 11β -hydroxyprogesterone, and dehydroepiandrosterone which was detected by iodine-vapor.

Thus, radioactive steroids isolated and identified in this study were 17α -hydroxypregnenolone, progesterone, 17α -hydroxyprogesterone, 11 -desoxycortisol, cortisol, 11 -desoxycorticosterone, corticosterone, androstenedione, testosterone, 11β -hydroxyandrostenedione, 16α -hydroxyprogesterone and $17\alpha,20\alpha$ -dihydroxypregn-4-en-3-one. Besides the above described metabolite, 7α - and 18 -hydroxylation and reduction of Δ^4 -steroids to 5α -saturated steroids were reported to exist in human adrenals from *in vitro* experiments (Pasqualini, 1964; Roman *et al.*, 1965; Neville and Webb, 1965; Dufau *et al.*, 1968; Charreau *et al.*, 1968).

Metabolism of steroids in vitro in adenoma and its adjacent tissue of adrenal

Quantities of the steroids produced from pregnenolone were shown in Table 1. The sum of 17α -hydroxylated products occupied about 70 % of all the metabolized products, while the sum of progesterone, 11 -desoxycorticosterone and corticosterone occupied only about 7% of the total products.

Table 2 shows quantities of the metabolites obtained from dehydroepiandrosterone which was used as the substrate. Although androstenedione was the main products in both tissues, testosterone was obtained as a minor products, indicating low activity of 17α -hydroxysteroid dehydrogenase. Sums of

Table 1. Metabolites obtained by incubation of pregnenolone-4- ^{14}C with adenoma or its adjoining tissue of human adrenal

Steroids isolated	Quantities of products recovered ($\mu\text{g}/0.23\text{g tissue/hr.}$)	
	Adenoma	Adjacent adrenal
Pregnenolone (unconverted)	1.1	1.6
17α -Hydroxypregnenolone	0.8	0.8
Progesterone	0.9	0.3
17α -Hydroxyprogesterone	7.0	5.9
11 -Desoxycortisol	8.8	9.8
Cortisol	1.0	1.2
11 -Desoxycorticosterone	0.1	0.7
Corticosterone	1.3	1.2
Androstenedione	1.0	2.0
Testosterone	0.3	0.4
16α -Hydroxyprogesterone	2.6	2.9
$17\alpha,20\alpha$ -Dihydroxypregn-4-en-3-one	0.2	0.2
Products not identified	3.7	3.2
Sum of Δ^4 -3-ketosteroids	23.2	24.6

Table 2. Metabolites obtained by incubation of dehydroepiandrosterone-4- ^{14}C with adenoma or its adjoining tissue of human adrenal

Steroids isolated	Quantities of products recovered ($\mu\text{g}/0.23\text{g tissue/hr.}$)	
	Adenoma	Adjacent adrenal
Dehydroepiandrosterone (unconverted)	4.6	2.4
Androstenedione	25.8	26.6
Testosterone	0.2	0.4
11β -Hydroxyandrostenedione	0.7	0.5
Products not identified	0.8	1.2
Sum of Δ^4 -3-ketosteroids	26.7	27.5

Δ^4 -3-ketosteroids produced from dehydroepiandrosterone by homogenates of the adenoma and its adjacent adrenal tissues were respectively alike to those produced from pregnenolone by homogenates of both tissues.

Quantities of the steroids produced from progesterone were shown in Table 3.

Table 3. Metabolites obtained by incubation of progesterone-4-¹⁴C with adenoma or its adjoining tissue of human adrenal

Steroids isolated	Quantities of products recovered ($\mu\text{g}/0.23\text{g tissue/hr.}$)	
	Adenoma	Adjacent adrenal
Progesterone (Unconverted)	4.2	2.1
17 α -Hydroxyprogesterone	12.3	11.3
11-Desoxycortisol	3.9	4.5
Cortisol	0.3	0.4
11-Desoxycorticosterone	0.7	0.8
Corticosterone	1.1	1.3
Androstenedione	0.2	0.4
Testosterone	0.3	0.2
16 α -Hydroxyprogesterone	7.2	7.7
17 α ,20 α -Dihydroxypregn-4-en-3-one	0.2	0.3
Products not identified	0.9	0.9
Sum of 17 α -hydroxylated products	17.2	17.1

Table 4. Metabolites obtained by incubation of 17 α -hydroxyprogesterone-4-¹⁴C with adenoma or its adjoining tissue of human adrenal

Steroids isolated	Quantities of products recovered ($\mu\text{g}/0.23\text{g tissue/hr.}$)	
	Adenoma	Adjacent adrenal
17 α -hydroxyprogesterone (unconverted)	5.0	3.5
11-Desoxycortisol	19.5	19.5
Cortisol	0.6	1.5
Androstenedione	0.6	1.5
Testosterone	0.3	0.4
17 α ,20 α -Dihydroxypregn-4-en-3-one	5.5	7.4
Products not identified	0.4	2.0
Sum of 21-hydroxylated products	20.1	21.0
Sum of C ₁₉ -steroids	0.9	1.9

17 α -Hydroxyprogesterone was one of the main metabolites, but the quantities of 11-desoxycortisol and cortisol produced from progeste-

rone were far less than those produced from pregnenolone. 16 α -Hydroxyprogesterone was the other main product from progesterone.

Table 4 shows quantities of metabolites obtained from 17 α -hydroxyprogesterone.

11-Desoxycortisol and 17 α , 20 α -dihydroxypregn-4-en-3-one were the two main metabolites produced from 17 α -hydroxyprogesterone. As very limited amount of C₁₉-steroids which were derived from the substrate were obtained, the activity of C-17-C-20 lyase was considered to be low in the adenoma and its adjacent adrenal tissues used in this study.

As shown in Table 5, androstenedione

Table 5. Metabolites obtained by incubation of 11-desoxycortisol-4-¹⁴C with adenoma or its adjoining tissue of human adrenal

Steroids isolated	Quantities of products recovered ($\mu\text{g}/0.23\text{g tissue/hr.}$)	
	Adenoma	Adjacent adrenal
11-Desoxycortisol (unconverted)	40.8	43.7
Cortisol	2.9	3.6
Androstenedione	4.0	1.0
Testosterone	0.7	0.2
Products not identified	1.1	2.0
Sum of 11 β -hydroxylated products	2.9	3.6

and testosterone were produced from 11-desoxycortisol, demonstrating that the side-chain of 17 α ,21-dihydroxylated steroids could be cleaved by adrenal and adenoma tissues. Cortisol was obtained in low yield, so that the activity of 11 β -hydroxylase was considered to be poor in comparison with the activities of Δ^5 -3 β -hydroxysteroid dehydrogenase with the isomerase, 17 α -hydroxylase and 21-hydroxylase in these tissues used in this study.

Judging from quantities of metabolized products from the substrates, Δ^5 -3 β -hydroxysteroid dehydrogenase, 17 α -hydroxylase and 21-hydroxylase in adenoma and its adjoining tissue were highly active, but the activity of

11 β -hydroxylase was low, possibly building up a neck step of corticosteroid hormone biosynthesis in the adenoma and its adjoining adrenals used in this study. Enzyme activities other than those described above were also recognized from the obtained metabolites, namely 16 α -hydroxylase, C-17-C-20 lyase, 17 β -hydroxysteroid dehydrogenase and 20 α -hydroxysteroid dehydrogenase. The activity of 18-hydroxylase, a key enzyme for biosynthesis of aldosterone was not confirmed, as at least 18,21-dihydroxypregn-4-ene-3, 20-dione could not be detected in this study.

Among the steroids produced during incubation, 21-hydroxylated steroids produced from progesterone were significantly less than those derived from pregnenolone. Cortisol and 11-desoxycortisol were the main metabolites from pregnenolone, while corticosterone and 11-desoxycorticosterone were the two of the main metabolites from progesterone. These results demonstrate that pregnenolone is preferable to progesterone as a precursor for cortisol biosynthesis in human adrenal. The same results were obtained by incubation studies of adrenals of new born and of patient with breast cancer (Vilee and Loring, 1965; Cameron *et al.*, 1968; Whitehouse and Vinson, 1968), and also, obtained by *in vitro* studies of adrenal hyperplasia and adrenocortical tumor manifesting Cushing's syndrome (Welikey and Engel, 1962 and 1963; Cameron and Griffiths, 1968). In adrenocortical tumor giving rise to Conn's syndrome or virilizing syndrome, stimulation or suppression of certain enzyme activities related to steroid metabolism was demonstrated, causing an excessive production of aldosterone or adrenal androgens by these tissues (Dufau *et al.*, 1968; Vilee *et al.*, 1967).

Quantities of the products obtained by incubation with adrenal tissue adjacent to the adenoma were almost equivalent to those obtained by incubation with the adenoma performed under the same condition. To express this similarity of the steroid metabolism be-

tween adenoma and its adjoining adrenal tissue from enzymological viewpoint, sums of Δ^4 -3-ketosteroids derived from Δ^5 -3 β -hydroxysteroid, 17 α -hydroxylated steroids from progesterone, 21-hydroxylated steroids from 17 α -hydroxypregesterone and 11 β -hydroxylated steroids from 11-desoxycortisol were calculated individually and indicated as the note of each Table. No significant differences were found between these two corresponding values obtained by incubations of adenoma and histologically normal adrenal tissue adjacent to it. But the patient taken for this study showed hypersecretion of 17-OHCS, judging from clinical signs and data. An explanation of these unexpected inconsistent results will be suggested. The biosynthetic activities of corticosteroid hormones in the adrenal gland adjacent to adenoma might be considered to be in hyperfunctional state, as this adrenal tissue was histologically not atrophied and an adrenal obtained from another patient with Cushing's syndrome caused by adrenal hyperplasia showed similar comparative activity of steroid biosynthesis to the adrenal gland used for this study (Sarada, in preparation). It would be suggested that this stimulated state might have promoted a part of adrenal cells to formation of an adenoma, and at the time of the resection of adrenal, steroid biosynthetic activities had been enhanced in both tissues of adenoma thus formed and its adjoining adrenal.

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