

CONVERSION OF 17-HYDROXY-PREGNENOLONE TO CORTISOL BY NORMAL AND HYPERPLASTIC HUMAN ADRENAL SLICES *

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Progesterone¹ is a metabolic precursor common to cortisol and corticosterone biosynthesis in the adrenal cortex. However, incubation of radioactive progesterone with bovine adrenal homogenates (1) and human adrenal slices (2, 3) yields corticosterone with a specific activity greater than that of cortisol, even though the amount of cortisol produced is greater. Although hypotheses involving enzyme kinetics have been suggested to explain these results, other pathways of cortisol biosynthesis may exist that do not involve progesterone.

The pathway from progesterone to cortisol is believed to be via 17-hydroxyprogesterone → 11-deoxycortisol → cortisol (4). Progesterone is derived from Δ^5 -pregnenolone upon oxidation of the 3β -hydroxyl group and a shift of the double bond. Hydroxylation of Δ^5 -pregnenolone to 17α -hydroxy- Δ^5 -pregnenolone is considered the first step in adrenal androgen biosynthesis (5). Conversion of 17-hydroxy- Δ^5 -pregnenolone to 17α -hydroxyprogesterone by oxidation of the 3β -hydroxyl group and a shift of the double bond appeared to be a reasonable alternative possibility for cortisol biosynthesis. Indeed, Weliky and Engel (6) reported that 17-hydroxy- Δ^5 -pregnenolone is metabolized to cortisol by sheep adrenal homogenates and by slices of a human adrenal tumor, and the present authors in preliminary reports have shown the conversion of 17α -hydroxy- Δ^5 -pregnenolone-7- H^3

to cortisol by slices of a hyperplastic human adrenal gland (3, 7).

The present report describes the conversion of 17-hydroxy- Δ^5 -pregnenolone to cortisol by slices of normal and hyperplastic human adrenal tissue. A pathway from 17-hydroxy- Δ^5 -pregnenolone → 17-hydroxyprogesterone → 11-deoxycortisol → cortisol has been identified.

MATERIALS AND METHODS

All the adrenal glands were obtained by surgery and immediately iced until used. Adrenals were weighed, sectioned, freed of fat, reweighed, and sliced with a Stadie-Riggs microtome. Slices from different areas of each adrenal were taken for each experimental flask. Medullary tissue was not removed. Incubation of weighed slices was performed in a Dubnoff metabolic incubator for 3 hours at 37° C in 1 ml of Krebs-Ringer bicarbonate buffer per 30 mg of tissue with 200 mg of glucose per 100 ml in an atmosphere of 95 per cent oxygen—5 per cent CO₂. The incubations were begun within 3 hours after excision of the adrenals.

The four normal adrenals were obtained from two patients with inoperable carcinoma of the prostate. Patient J.C. had had a bilateral orchiectomy and had received estrogenic therapy for 1 year prior to adrenalectomy. He received no glucocorticoid prior to the removal of the first adrenal. An infusion of 200 mg cortisol-hemisuccinate was started and the second adrenal was removed. Patient M.K. underwent a bilateral orchiectomy and received estrogenic therapy for 2 years prior to adrenalectomy. Prednisone, 20 mg each day, was administered for 3 months prior to adrenalectomy. During surgery, he received infusions of cortisol-hemisuccinate. The adrenals were of normal size.

Patient A.D., a 30-year-old female, had classical Cushing's syndrome. She received glucocorticoids on the day before and on the day of surgery. Two hyperplastic adrenals were found at surgery.

The substrate, 17α -hydroxy- Δ^5 -pregnenolone-7- H^3 (specific activity 2.6 mc per mg), was obtained from the New England Nuclear Corporation. It was subjected to three separate solvent partitionings (8) and then stored frozen in redistilled benzene:methanol (9:1, vol/vol) until used. An aliquot was mixed with stable 17α -hydroxy- Δ^5 -preg-

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¹ Compounds referred to are: progesterone (4-pregnene-3,20-dione); cortisol (11 β ,17 α ,21-trihydroxy-4-pregnene-3,20-dione); corticosterone (11 β ,21-dihydroxy-4-pregnene-3,20-dione); 11-deoxycortisol (17 α ,21-dihydroxy-4-pregnene-3,20-dione); 17-hydroxy-pregnenolone (3 β ,17 α -dihydroxy-5-pregnene-20-one); 17α -hydroxyprogesterone (17 α -hydroxy-4-pregnene-3,20-dione); ACTH, adrenocorticotrophic hormone.

nenolone and subjected to several different chromatography systems. A broad radioactive peak which corresponded to the broad peak of stable 17 α -hydroxy-pregnenolone was found. A second minor peak of radioactivity estimated to contain less than 5 per cent of radioactive 17 α -hydroxy-pregnenolone was noted in one system. The substrate progesterone-4-C¹⁴ was obtained from the New England Nuclear Corporation and subjected to two paper chromatographic systems prior to use.

In experiments 1, 2, 3, and 4, 17 α -hydroxy- Δ^5 -pregnenolone-H³ was added to the incubation flask either alone (experiments 1 and 2) or with stable 17 α -hydroxy-pregnenolone in ethanol:propylene glycol (experiments 3 and 4). In the "trapping" experiment 1, stable 17 α -hydroxy-progesterone in ethanol:propylene glycol was also added prior to incubation. The volatile solvents were evaporated under nitrogen or air at 37° C. In experiment 5, approximately equimolar quantities of 17 α -hydroxy-pregnenolone-7-H³ and progesterone-4-C¹⁴ were added to each flask and dried under nitrogen. Propylene glycol, 0.1 ml, was added to facilitate solubilization of the substrates.

Separation and measurement of steroids. The media were extracted twice with 2 vol of dichloromethane. The dichloromethane extract was washed once with 0.1 vol of 0.05 N NaOH, twice with 0.1 vol of distilled water, dried with anhydrous sodium sulfate, and evaporated in vacuum at 37° C. The residues were applied to Whatman no. 1 chromatography paper and developed for approximately 72 hours in a modification of the toluene:propylene glycol system, i.e., propylene glycol was diluted with methanol (1:1 vol/vol) before it was applied to paper and methanol was allowed to evaporate. The cortisol area was eluted with absolute ethanol and chromatographed successively in the Bush C system (9), then in the E₂B system (10). The effluents from the toluene:propylene glycol system were collected and chromatographed for 20 hours in the cyclohexane:dioxane:methanol:H₂O system (100:100:50:25) (11) which separated corticosterone from 11-deoxycortisol. The effluents from the latter system were collected and after addition of carrier 17-hydroxyprogesterone to all except experiment 1, chromatographed in the same system for 7 hours followed by the cyclohexane:benzene:methanol:water system (100:50:100:25) (11) for 8 hours.

Cortisol, 11-deoxycortisol, and corticosterone were acetylated with acetic anhydride:pyridine (1:1) and the monoacetate derivatives chromatographed in at least two systems. Hydrolysis of cortisol acetate was carried out with acetyl cholinesterase in glycylglycine buffer and the free alcohol extracted with methylene chloride. In experiments 1 through 4, cortisol was oxidized with 2 per cent aqueous chromic acid for 18 hours at room temperature to adrenosterone which was isolated by paper chromatography in the cyclohexane:benzene:methanol:water system. Prior to chromic acid oxidation, authentic C¹⁴-cortisol was added. H³/C¹⁴ ratios were measured as the free cortisol and its adrenosterone derivative. In experiment 5, cortisol underwent bismuth oxidation to 11 β -hydroxyandrostenedione (12) which was isolated by paper chromatography in heptane:benzene:methanol:H₂O (13) followed by a heptane:96 per cent methanol system (13).

17 α -Hydroxy-progesterone was oxidized with 2 per cent aqueous chromic acid for 20 hours at room temperature to Δ^4 -androstenedione. This derivative was isolated by chromatography in the heptane:96 per cent methanol and the cyclohexane:benzene:methanol:water systems. Specific activities were measured by alkaline fluorescence (3) or ultraviolet absorption at 240 to 242 m μ and by liquid scintillation counting.

RESULTS

The conversion of 17 α -hydroxy- Δ^5 -pregnenolone to cortisol by human adrenal slices is illustrated in Table I. The specific activity of cortisol was not measured until after it was subjected to three successive chromatography systems as the free alcohol (toluene:propylene glycol \rightarrow Bush C \rightarrow E₂B). Previous experience with cortisol-H³ derived from progesterone-16-H³ indicated that this was sufficient to insure radiochemical purity (3). Table II illustrates that this holds true for cortisol derived from 17 α -hydroxy- Δ^5 -pregnenolone-7-H³. The per cent conversion represented is a minimum, since no corrections were made for losses in the purification of cortisol. In our laboratory the losses involved in extractions and purification

TABLE I
Conversion of 17 α -hydroxy- Δ^5 -pregnenolone-7-H³ to cortisol by human adrenal slices

Exp.	Pt	Pathology	Tissue Wt		Substrate		Cortisol produced		Conversion*
			Total	Incubated	S.A.	Amt	$\mu\text{g/g/3 hrs}$	$\mu\text{c}/\mu\text{mole}$	
			g	g	$\mu\text{c}/\mu\text{mole}$	μc			%
1	J.C.	Normal, rt	3.6	2.93	8.58×10^2	4.76†	389†	0.15†	9.8†
2	J.C.	Normal, lt	4.7	2.52	8.58×10^2	3.96	42.5	2.61	19.3
3	M.K.	Normal, rt	8.7	4.94	1.19	2.86	53.5	0.32	8.2
4	M.K.	Normal, lt	7.0	4.00	1.19	2.86	67.2	0.33	8.8

* Per cent conversion of the radioactivity of the substrate cortisol.

† Approximately 3 mg of stable 17 α -hydroxy-progesterone was added to the media prior to incubation.

TABLE II
Radiochemical purity of cortisol- H^3 derived from 17α -hydroxy- Δ^5 -pregnenolone- $7-H^3$

Chromatography system *	Exp. 1 S.A.	Exp. 2 S.A.	Exp. 3 S.A.	Exp. 4 S.A.
	$\mu\text{c}/\mu\text{mole}$		$\mu\text{c}/\mu\text{mole}$	
E ₂ B	0.15	2.61	0.32	0.33
	Carrier cortisol ↓ 0.78			
	Acetylation to cortisol monoacetate			
Dioxane: cyclohexane: methanol: H ₂ O	0.15	0.83	0.34	0.36
Benzene: cyclohexane: methanol: H ₂ O	0.16	0.84	0.29	0.29
	Hydrolysis to cortisol			
	Addition of authentic C ¹⁴ -cortisol			
E ₂ B	H ³ /C ¹⁴ ratios 8.64	H ³ /C ¹⁴ ratios 17.2	H ³ /C ¹⁴ ratios 12.0	H ³ /C ¹⁴ ratios 12.5
	Chromic acid oxidation to adrenosterone			
Benzene: cyclohexane: methanol: H ₂ O	7.95	15.4	11.3	11.1

* The cortisol was chromatographed in the toluene:propylene glycol and in the Bush C system prior to the E₂B system.

tion of cortisol to this degree are from 30 to 50 per cent. As might be expected, addition of large quantities of stable 17α -hydroxyprogesterone to the incubation media of experiment 1 led to a

marked increase in cortisol production. Incubation, under similar conditions, of tissue from the left and right adrenals of the same patient (experiments 3 and 4) led to similar results.

TABLE III
Conversion of 17α -hydroxy- Δ^5 -pregnenolone- $7-H^3$ to 11-deoxycortisol- $7-H^3$

Chromatography system*	Exp. 1		Exp. 2		Exp. 3		Exp. 4	
	$\mu\text{c}/\mu\text{mole}$	% conv.	$\mu\text{c}/\mu\text{mole}^\dagger$	% conv.	$\mu\text{c}/\mu\text{mole}^\dagger$	% conv.	$\mu\text{c}/\mu\text{mole}^\dagger$	% conv.
Dioxane: cyclohexane: methanol: H ₂ O	0.15	2.62	0.16	2.1	0.039	0.69	0.042	0.74
	Acetylation to 11-deoxycortisol monoacetate							
Dioxane: cyclohexane: methanol: H ₂ O	0.14		0.14		0.033		0.031	
Cyclohexane: benzene: methanol: H ₂ O	0.12		0.14		0.028		0.028	

* The 11-deoxycortisol was chromatographed in the toluene:propylene glycol, then in the dioxane:cyclohexane:methanol:H₂O system prior to rechromatography in the latter system.

† Specific activities were measured after the addition of 150 μg of carrier 11-deoxycortisol.

TABLE IV
Conversion of 17 α -hydroxy- Δ^5 -pregnenolone-7- H^3 to 17 α -hydroxy- Δ^4 -progesterone-7- H^3

Chromatography system*	Exp. 1		Exp. 2		Exp. 3		Exp. 4	
	$\mu\text{c}/\mu\text{mole}$	% conv.†	$\mu\text{c}/\mu\text{mole}^\ddagger$	% conv.†	$\mu\text{c}/\mu\text{mole}^\ddagger$	% conv.†	$\mu\text{c}/\mu\text{mole}^\ddagger$	% conv.†
Dioxane: cyclohexane: methanol: H ₂ O	0.14	6.7	0.26	2.5	0.0156	0.18	0.24	1.2
Cyclohexane: benzene: methanol: H ₂ O	0.035		0.25		0.0092		0.056	
Dioxane: cyclohexane: methanol: H ₂ O	0.038				0.0085		0.054	
Heptane: methanol: H ₂ O	0.040		Oxidation to Δ^4 -androstenedione		0.0094		0.059	
Cyclohexane: benzene: methanol: H ₂ O	0.040							

* The 17 α -hydroxyprogesterone was chromatographed in the toluene:propylene glycol, then in the cyclohexane:dioxane:methanol:H₂O system prior to rechromatography in the latter system.

† The actual amount of radioactivity measured after the third chromatogram was corrected for the final specific activity.

‡ Specific activities were measured after addition of carrier 17 α -hydroxyprogesterone.

Conversion to 11-deoxycortisol. In Table III, the results of conversion of 17 α -hydroxy- Δ^5 -pregnenolone-7- H^3 to 11-deoxycortisol are shown. Since only a few micrograms of 11-deoxycortisol were produced by the adrenals, the specific activities were measured after the addition of approximately 150 μg of carrier 11-deoxycortisol in experiments 2, 3, and 4. In experiment 1 the addition of 17 α -hydroxyprogesterone increased the

production of 11-deoxycortisol sufficiently so that addition of carrier was unnecessary. Adrenal tissue from the same patient (experiments 1 and 2, 3 and 4) incorporated approximately the same amount of radioactivity. Radiochemical purity was demonstrated after acetylation and chromatography in two systems.

Conversion to 17 α -hydroxyprogesterone. The results are shown in Table IV. Except in experi-

TABLE V
Incorporation of 17 α -hydroxy- Δ^5 -pregnenolone-7- H^3 and progesterone-4- C^{14} to cortisol by hyperplastic human adrenal slices

Pt	Exp.	Tissue wt incubated	Substrate				Cortisol produced *			
			Type mixture†	μg	μc	Ratio H^3/C^{14}	S.A.	% conv.‡	Ratio H^3/C^{14}	
				$\mu\text{g}/\text{g}/3\text{ hrs}$	$\mu\text{c}/\mu\text{mole}$					
A.D.	Control	2.433	17-hydroxy-pregnenolone- H^3 progesterone- C^{14}	406 549	5.67 2.75	2.06	37.4 1.34 0.995	5.9 9.0	1.34	
A.D.	ACTH§	1.946	17-hydroxy-pregnenolone- H^3 progesterone- C^{14}	325 439	4.54 2.20	2.06	43.2 0.840 0.785	4.1 8.3	1.03	

* Measurements made after second chromatography system (Bush C).

† Mixture refers to the incubation of 17 α -hydroxy- Δ^5 -pregnenolone-7- H^3 and progesterone-4- C^{14} simultaneously with the same adrenal slices.

‡ Per cent conversion of the radioactivity of the substrate to cortisol.

§ ACTH-lyophilized Armour adrenocorticotrophin, 10 U/g.

TABLE VI
Radiochemical purity of cortisol derived from 17α -hydroxy- Δ^5 -pregnenolone-7- H^3 and progesterone-4- C^{14}

Chromatography system	Exp.	Specific activity		Ratio H^3/C^{14} *
		H^3	C^{14}	
		$\mu c/\mu Mole$	$\mu c/\mu Mole$	
E ₂ B	Control	1.14	0.866	1.30
	ACTH	0.626	0.583	1.07
Acetylation to cortisol monoacetate				
CCl ₄ : methanol: H ₂ O	Control			1.47†
	ACTH			1.05†
Dioxane: cyclohexane: methanol: H ₂ O	Control	1.27	1.01	1.25
	ACTH	0.695	0.536	1.03
Benzene: cyclohexane: methanol: H ₂ O	Control	1.19	0.98	1.22
	ACTH	0.80	0.572	1.11
Hydrolysis to cortisol				
E ₂ B	Control			1.27
	ACTH			0.93
Bismuthate oxidation to 11β -hydroxy- Δ^4 androstenedione ‡				
Ligroin: benzene: methanol: H ₂ O	Control			
	Control			1.04†
Heptane: methanol: H ₂ O				

* Ratio H^3/C^{14} of substrate before incubation was 2.06.

† Only a single aliquot was measured. All other measurements were done in triplicate.

‡ Carrier cortisol, 50 μg , was added prior to oxidation.

ment 1, where carrier 17α -hydroxyprogesterone was added to the media prior to incubation, no 17α -hydroxyprogesterone was detected on the chromatograms by ultraviolet scanning. Specific activities were measured after the addition of carrier 17α -hydroxyprogesterone. In 3 of 4 experiments radiochemical purity was demonstrated as the free alcohol, and after oxidation Δ^4 -androstenedione. The highest per cent incorporation of 17α -hydroxy- Δ^5 -pregnenolone to 17α -hydroxyprogesterone occurred in the "trapping" experiment 1.

Comparison of conversion of progesterone-4- C^{14} and 17α -hydroxy-pregnenolone-7- H^3 to cortisol by slices of a hyperplastic gland. The results of the simultaneous incubation of equimolar concentrations of progesterone-4- C^{14} and 17α -hydroxy- Δ^5 -pregnenolone-7- H^3 with slices of a hyperplastic human adrenal gland obtained from patient with Cushing's syndrome are shown in Table V. The addition of ACTH *in vitro* did not sig-

nificantly alter the production of cortisol or the conversion of the precursor. Slightly more progesterone was converted to cortisol than 17α -hydroxy- Δ^5 -pregnenolone. Table VI demonstrates the radiochemical purity of the derived cortisol.

As was expected, little or none of the tritium in 17α -hydroxy- Δ^5 -pregnenolone was converted to corticosterone. In experiment 2 the corrected per cent conversion was 0.09, but in experiment 5 no tritium was detected in the corticosterone isolated from either the control or ACTH experiment.

DISCUSSION

The present experiments have demonstrated that 17α -hydroxy- Δ^5 -pregnenolone-7- H^3 is converted to cortisol by slices of normal and hyperplastic human adrenal glands. A pathway from 17α -hydroxy- Δ^5 -pregnenolone through 17α -hydroxyprogesterone \rightarrow 11-deoxycortisol \rightarrow cortisol has been identified (Figure 1). Other pathways,

of course, have not been excluded. Welicky and Engel (6) have demonstrated that 17 α -hydroxy- Δ^5 -pregnenolone is a major precursor for cortisol

biosynthesis in sheep adrenals and in one human adrenal adenoma *in vitro*. In a preliminary communication, Lipsett and Hökfelt (14) reported that

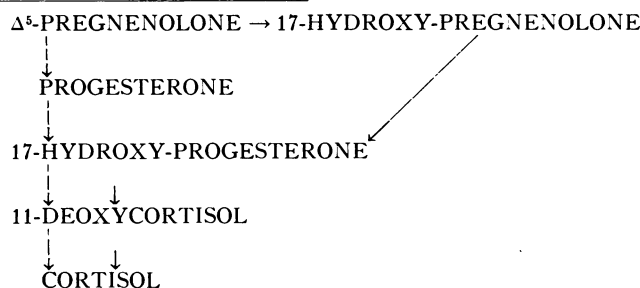


FIG. 1. PATHWAYS OF CORTISOL BIOSYNTHESIS.

slices of human, guinea pig, and rat adrenals metabolized 17 α -hydroxy- Δ^5 -pregnenolone- H^3 to cortisol via a pathway identical to one described in the present study. They found that 9 per cent of the labeled 17 α -hydroxy- Δ^5 -pregnenolone was converted to cortisol.

The extent of conversion of 17 α -hydroxy- Δ^5 -pregnenolone to cortisol *in vitro* compares favorably with the conversion of progesterone to cortisol. In a previous study (3), the present authors found that slices of a normal adrenal gland converted 6.8 per cent of tracer quantities of progesterone-16- H^3 , whereas slices of hyperplastic or adenomatous adrenal tissue converted 5.8 to 16.2 per cent. In experiment 5 of the present study, incubation of an equimolar concentration of 17 α -hydroxy- Δ^5 -pregnenolone-7- H^3 and progesterone-4- C^{14} with slices of a hyperplastic adrenal resulted in a greater per cent conversion of progesterone-4- C^{14} than 17 α -hydroxy- Δ^5 -pregnenolone- H^3 . Welicky and Engel (6) in a similar type of experiment found that a human adrenal adenoma converted 50 per cent of 17 α -hydroxy- Δ^5 -pregnenolone and 15 per cent of progesterone- C^{14} to cortisol. This type of experiment is difficult to interpret, however, since the results may be influenced by many factors, such as rates of penetration of the two substrates into their enzyme sites, size of intracellular pools of intermediates in the respective pathways, and activity of the enzymes *in vitro*. Moreover, *in vitro* experiments may not reflect the biosynthetic events occurring *in vivo*. Since progesterone and 17 α -hydroxy- Δ^5 -pregnenolone are derived from a common precursor, pregnenolone, the relative importance of the two pathways of corti-

sol biosynthesis depends upon: 1) the rates of conversion of pregnenolone to either progesterone or 17 α -hydroxy- Δ^5 -pregnenolone, and 2) their rates of conversion to 17-hydroxyprogesterone. The enzyme systems involved in the latter reactions would have to compete with the enzyme systems which metabolize progesterone and 17 α -hydroxy- Δ^5 -pregnenolone to compounds other than 17-hydroxyprogesterone.

Nevertheless, since a pathway from 17 α -hydroxy- Δ^5 -pregnenolone to cortisol has been demonstrated to exist *in vitro*, it is a possibility that one reason for the lower specific activity of cortisol compared to corticosterone when radioactive progesterone is incubated with human adrenal tissue is the presence of an alternative pathway of cortisol synthesis. Production of cortisol by way of this alternative pathway might "dilute" the cortisol derived from radioactive progesterone.

SUMMARY

Conversion of 17 α -hydroxy- Δ^5 -pregnenolone-7- H^3 to cortisol by slices of four normal adrenals and one hyperplastic adrenal has been demonstrated. The slices of normal adrenal tissue converted from 9 to 19 per cent of the 17 α -hydroxy- Δ^5 -pregnenolone-7- H^3 to cortisol, whereas slices of the hyperplastic gland incorporated 5 per cent. A pathway from 17 α -hydroxy- Δ^5 -pregnenolone \rightarrow 17 α -hydroxyprogesterone \rightarrow 11-deoxycortisol \rightarrow cortisol has been identified.

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