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STUDIES ON THE C-11 AND C-21 STEROID HYDROXYLATION SEQUENCE IN SUBCELLULAR FRACTIONS OF HUMAN ADRENALS

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SUMMARY

The possibility of cortisol biosynthesis proceeding through a C-11$\beta \rightarrow$C-21-hydroxylation sequence of 17-hydroxypregnenolone and 17-hydroxyprogesterone was investigated *in vitro* in the normal human adrenal and in an adrenal adenoma. The mitochondrial 11β-hydroxylase activity towards the C-21-hydroxy vs C-21-deoxy substrates and the microsomal 21-hydroxylase activity towards the C-11-deoxy vs C-11β-hydroxy substrates was compared.

The mitochondria converted compound S to cortisol with a high efficiency, while the 11β-hydroxylation of the C-21-deoxysteroids proceeded only to a limited extent. Moreover, when present in equimolar quantities, compound S inhibited the 11β-hydroxylation of C-21-deoxysteroids. The microsomes converted 21-deoxycortisol to cortisol, but this 21-hydroxylation was 10-fold lower than that of 17-hydroxyprogesterone to compound S. From the combined results of the respective hydroxylations by the mitochondria and microsomes it is concluded that in the human adrenal the alternative pathway to cortisol via the C-11$\beta \rightarrow$C-21-hydroxylation sequence does not seem to be significant.

INTRODUCTION

It is widely accepted that in the normal human adrenal the sequence of hydroxylations leading from pregnenolone to cortisol (F) proceeds by hydroxylation at C-17, then C-21 and finally C-11β, either before or after transformation of 3β-hydroxy-5-en-precursors into the 4-en-3-keto intermediates[1–4]. In abnormal conditions such as congenital adrenal hyperplasia, Cushing's syndrome secondary to adrenal hyperplasia or polycystic ovary syndrome, peripheral metabolites of 21-deoxycortisol (21DF) have been isolated[5–8] indicating an impairment of the normal sequence by 11β-hydroxylation of 21-deoxysteroids. This is especially relevant to studies in certain vertebrates, which showed that C-21-deoxysteroids may be hydroxylated at C-11β[9–14] which may then serve as substrates for C-21-hydroxylase[13, 15–19] suggesting an alternative pathway for biosynthesis of corticosterone and cortisol, proceeding via the $C_{17} \rightarrow C_{11\beta} \rightarrow C_{21}$ sequence of hydroxylations[13, 14].

The experiments to be reported herein were designed to determine whether in human adrenal the biosynthesis of cortisol may proceed through such an alternative pathway. If this were so, 21DF would be a normal intermediate and its concentration in blood or of its metabolite in urine (pregnanetriolone) would depend in a great measure on the relative extent of C-21 and C-11-hydroxylations to which the C-21-deoxy-precursors might have been exposed. Thus we have investigated the role of 17OHP and 21DF as possible substrates for 11β and 21-hydroxylations by mitochondria and microsomes prepared from normal human adrenal cortex and from a benign adrenal tumor causing Cushing syndrome.

EXPERIMENTAL

Radioactive and crystalline steroids. [7-^{3}H]-17-Hydroxypregnenolone (17OHΔP S.A. 12 Ci/mmol), [1,2-^{3}H]-17-hydroxyprogesterone (17OHP, S.A. 50 Ci/mmol), [4-^{14}C]-17OHP (S.A. 50 mCi/mmol and [4-^{14}C]-17-hydroxydesoxycorticosterone (Reichstein's cpd S, S.A. 50 mCi/mmol) were purchased from New England Nuclear Corporation. [1,2-^{3}H]-21DF was prepared from [1,2-^{3}H]-17OHP according to Maschler and Horn[20]. Crystalline steroids were purchased from Ikapharm (Ramat-Gan, Israel). 3β, 11β, 17α-Trihydroxy-5-pregnen-20-one and 3β, 17α-dihydroxy-5-pregnene-11,20-dione were synthesized according to Halperin and Finkelstein[21].

Adrenal tissue. Normal adrenal tissue was obtained from a 19 year old male, killed in a road accident, at the time of cadaveric renal transplantation. Adrenal adenoma tissue was obtained at adrenalectomy from a 30 year old female patient with Cushing syndrome. On operation the right adrenal was found to be occupied by 3 × 4 cm adenoma. For 6 months prior to surgery the patient excreted elevated and constantly increasing amounts of urinary F; preoperative urinary F was 600 μg/24 h. Pregnenetriolone (11-oxo-pregnanetriol), which is usually excreted in the urine of patients with Cushing syndrome secondary to bilateral adrenal hyperplasia[8] was not found,

even after stimulation with ACTH (80 IU/day for 3 days); the latter caused an increase in the excretion of urinary F to 3000 μg/24 h.

Preparation of mitochondria and microsomes. Upon obtaining the adrenal tissue it was put on ice and transfered to the laboratory. It was trimmed of adhering fat and the cortical tissue separated from the medulla. All subsequent procedures were carried out in the cold. In each case, a homogenate was prepared from 3.5 g of the cortical tissue with 14 ml of 0.25 M sucrose. After sedimenting the cellular debris and nuclei at 900 *g* for 10 min, mitochondria were obtained from the resultant supernatants by centrifugation at 8500 *g* for 15 min. The 8500 *g* mitochondrial pellets were washed once by resuspension in half of the original vol. of 0.25 M sucrose and resedimented at 8500 *g* for 15 min. The pellets obtained were suspended in 0.154 M KCl and their protein content was estimated according to Lowry *et al.*[22]. The normal tissue yielded 17.5 mg mitochondrial protein and the adenoma 90 mg.

The initial 8500 *g* supernatants were centrifuged at 15,000 *g* and the pellets discarded. The microsomes were then sedimented from the resultant supernatants by centrifugation at 105,000 *g* for 60 min. The pellets were washed with half a vol. of 0.25 M sucrose and resedimented at 105,000 *g* for 60 min. The microsomes were then resuspended in 0.154 M KCl and aliquots taken for estimation of protein. The normal tissue yielded 21 mg microsomal protein and the adenoma tissue 40 mg protein. The microsomal suspensions were kept at −20°C until assayed (2 weeks).

Incubations of mitochondria. The incubation medium (total vol. of 2 ml) consisted of 0.1 M phosphate buffer pH 7.4, 50 mM neutralized sodium malate, 5 mM $MgCl_2$, 0.75 mM NADPH and the appropriate radioactive substrates dissolved in 0.1 ml propylene glycol. The incubations were performed with 1 g-equivalent of the fresh tissue i.e. for each incubation 5 mg of normal and 25 mg of adenoma mitochondria protein were used. In each case 1.5 μCi of the radioactive substrates was 'diluted' with the appropriate crystalline steroids to a concentration of 2 μg steroid/mg protein. Thus the normal adrenal mitochondria were incubated with 10 μg/1.5 μCi and tumor mitochondria with 50 μg/1.5 μCi of each of the appropriate steroid substrates. The incubations were carried out at 37°C for 45 min in an atmosphere of 95% O_2–5% CO_2. They were terminated by the addition of 10 ml of methylene chloride.

Incubations of microsomes. Microsomes were incubated according to Rosenthal and Narasimhulu[23] in 1.2 ml glycyl-glycine buffer pH 7.4 containing a NADPH generating system and 1.5 μCi of appropriate radioactive substrates dissolved in 0.1 ml propylene glycol; the amounts of the crystalline substrates per mg microsomal protein were as detailed for the particular incubations (see Tables 5 and 6). The microsomes used in each incubation were equivalent to 0.15 g of fresh tissue. The final vol. was adjusted to 2.2 ml with 0.154 M KCl. The incubations were carried out for 60 min at 37°C in an atmosphere of 95% O_2–5% CO_2. They were terminated by the addition of 10 ml of methylene chloride.

Table 1. Scheme for purification of steroid metabolites following their initial separation by chromatography in systems PC-1*, PC-2, PC-6 and TLC-8[24].

Metabolite	I	II	III	IV
17HΔP	TLC-7	TLC-9	acetylation	TLC-7
17OHP	TLC-7	PC-2 (18 h)	TLC-9	
3β, 11β, 17α-Trihydroxy-5-pregnen-20-one	PC-1 (10 h)	PC-2	acetylation	PC-2 (27 h)
3β-17α-Dihydroxy-5-pregnene-11,20-dione (as acetate)	PC-2 (8 h)	PC-1 (31/2 h)	PC-2 (10 h)	
21DF	PC-1 (6 h)	TLC-10	PC-5 (5 h)	
Cpd. S	PC-1 (51/2 h)	TLC-10	acetylation	TLC-9
F	PC-7 (31/2 h)	PC-5 (20 h)	PC-1 (36 h)	PC-8 (31/2 h)
DHA	TLC-7	PC-2 (6 h)		
Androstenedione	TLC-7	TLC-8	PC-2 (6 h)	
Testosterone (as acetate)	TLC-7	TLC-9	TLC-8	

Chromatographic systems:

* PC-1: light petroleum–benzene–methanol–water 50:30:80:20; (by vol.) PC-2: petroleum ether–methanol–water 100:80:20; (by vol.) PC-5: toluene–methanol–water 100:75:25; (by vol.) PC-6: petroleum ether/propylene glycol–methanol 1:4; (vol./vol.) PC-7: benzene–methanol–water 100:50:50; (by vol.) PC-8: toluene–ethyl acetate–methanol–water 90:10:50:50; (by vol.) TLC-7; benzene–methanol 9:1; (vol./vol.) TLC-8: chloroform–ethyl acetate 3:1; (vol./vol.) TLC-9: benzene–ethyl acetate 7:3; (vol./vol.) TLC-10: cyclohexane–ethylacetate–methanol 45:45:10 (by vol.).

Table 2. Identification of metabolites of labelled precursors shown in Tables 3* and 5† by crystallizations to constant specific activity.

Precursors	Metabolites isolated	Solvents§	Specific Activity d.p.m./mg Crystals		Mother Liquor	
			[^{3}H]	[^{14}C]	[^{3}H]	[^{14}C]
*[7-^{3}H]-170HΔP + [4-^{14}C]-170HP	[^{3}H]/[^{14}C]-cpd S (as acetate)	1) MeOH	120	390	142	520
		2) A	115	400	130	380
		3) EtAc	130	415	126	410
	[^{3}H]-3β, 17α-Dihydroxy-5-pregnene 11,20-dione (as acetate)	1) MeOH	2560		2650	
		2) A-PE	2560		2600	
		3) EAc-PE	2600		2570	
	[^{14}C]-21DF	1) MeOH		2920		3400
		2) EtOH		3000		2930
		3) A-PE		3080		3080
	[^{3}H]/[^{14}C]-F	1) MeOH	504	19200	505	19300
		2) EtOH	520	20200	560	19800
		3) E-Ac	500	20700	490	20400
†[1,2-^{3}H]-21DF	[^{3}H]-F	1) MeOH	390		402	
		2) A	400		380	
		3) E-Ac	415		410	

§ MeOH: Methanol; A: Acetone; PE: Petroleum Ether; E-Ac: Ethyl Acetate; EtOH: Ethanol.

Isolation of the incubation products. The steroid metabolites were extracted and separated as described earlier[24]. They were further purified by paper and t.l.c. as shown in Table 1 and identified by reversed isotopic dilution[25]. Representative crystallizations to constant S.A. are shown in Table 2.

RESULTS

Normal adrenal mitochondria. Results of the incubations of normal adrenal mitochondria are presented in Table 3. The incubations were performed with (I) [7-^{3}H]-170HΔP + [4-^{14}C]-170HP, (II) [7-^{3}H]-170HΔP + [4-^{14}C]-cpd S and (III) [1,2-^{3}H]-170HP + [4-^{14}C]-cpd S.

The results of incubation (I) indicate that 170HΔP was a poor substrate for 11β-hydroxylation (approx 1.7% was converted to 11β-hydroxylated derivatives) while 170HP was a better substrate (16.6% conversion). However, the substrate of choice for 11β-hydroxylation was cpd S, as shown in incubations (II) and (III), where 73% and 66% of the cpd S, respectively, were converted to F and only a trace of the substrate remained unconverted (0.2%).

Compound S had an inhibitory effect on the 11β-hydroxylation of 170HP to 21DF, suppressing it from 9% (I) to 3% (III). On the other hand, the presence of cpd S did not significantly affect the conversion of 170HP to F, which was 6.2% in incubation III compared to 7.6% in incubation I. This apparently conflicting result may be explained by assuming that the inhibition of 11β-hydroxylation of 170HP was dependent upon the presence of cpd S which competed for the 11β-hydroxylase. However, by being rapidly metabolized (by 11β-hydroxylation and by degradation to C_{19}-steroids) little or none of the cpd. S remained to inhibit the reaction completely. On the other hand, the conversion of 170HP to F was carried out by a two step reaction: first, conversion to cpd. S by a 21-hydroxylase present in our mitochondrial preparation and second, by a rapid 11β-hydroxylation of cpd S which was limited by the first step. Thus, the production of F was dependent on 21-hydroxylation of 170HP, a reaction which was not affected by cpd S in the concentrations used.

Adrenal adenoma mitochondria. Results of incubations of adrenal adenoma mitochondria with [1,2-^{3}H]-170HP (IV), [4-^{14}C]-cpd S. (V) and

Table 3. Incubations of normal human adrenal mitochondria with 17-hydroxypregnenolone (170HΔP), 17-hydroxyprogesterone (170HP) and Reichstein's Compound S (Cpd S.)*

Metabolite identified	% conversion from I [7-^{3}H] 170HΔP + [4-^{14}C] 170HP		II [7-^{3}H] 170HΔP + [4-^{14}C] Cpd S		III [1,2-^{3}H] 170HP + [4-^{14}C] Cpd S	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
170HΔP	61.2	—	58.1	—	—	—
170HP	6.0	42.0	5.6	—	70.0	—
Cpd S	0.07	0.29	0.18	0.24	0.17	0.17
3β, 11β, 17α-Trihydroxy-5-pregnen-20-one	0.13	—	<0.05	—	—	—
3β, 17α-Dihydroxy-5-pregnene-11,20-dione	1.25	—	1.0	—	—	—
21DF	<0.05	9.0	<0.05	—	3.0	—
F	0.36	7.6	<0.05	73.0	6.2	66.0
Androgens†	6.9	2.2	5.4	0.42	6.5	0.6
Unidentified polar fraction	24.0	38.0	26.5	25.0	14.0	33.0

* The incubations were performed with 1.5 μCi/2 μg of each of the substrates per mg of mitochondrial protein. Each incubation was performed with 5 mg of mitochondrial protein which was equivalent to 1 g of fresh tissue.

† Androgens encompass C_{19}-steroids identified as DHA, androstenedione and testosterone.

Table 4. Incubations of mitochondria prepared from human adrenal adenoma with 17-hydroxyprogesterone (170HP) and Reichstein's compound S (Cpd S)*

Metabolite identified	% conversion from IV [1,2-^{3}H] 170HP	V [4-^{14}C] Cpd S.	VI [1,2-^{3}H] 170HP + [4-^{14}C] Cpd S.	
	[^{3}H]	[^{14}C]	[^{3}H]	[^{14}C]
170HP	76.0		80.0	
CpdS	1.3	14.9	5.0	28.9
21DF	2.0		<0.2	
F	11.1	60.0	1.0	36.1
Androgens†	5.0	17.5	5.0	17.9
Unaccounted polar fraction	1.4	6.0	1.0	10.1

* The incubations were performed with 1.5 μCi/2 μg of each of the substrates per mg of mitochondrial protein. Each incubation was performed with 25 mg of mitochondrial protein which was equivalent to 1 g of fresh tissue.

† Androgens encompass C_{-19}-steroids identified as androstenedione and testosterone.

[1,2-^{3}H]-170HP + [4-^{14}C]-cpd S (VI) are summarized in Table 4. In incubation IV 2% of the 170HP was 11β-hydroxylated to 21-DF; 1.3% was converted to cpd S and 11.1% to F, indicating that 21-hydroxylating activity was present also in this preparation. When cpd S was incubated alone (V) 60% was converted to F and 14.9% remained unconverted.

On incubation of both substrates together (VI), cpd S inhibited almost entirely the 11β-hydroxylation of 170HP and no conversion was found to 21DF (<0.2%), whereas 170HP inhibited the 11β-hydroxylation of cpd S only by approximately 40% (from 60% conversion to 36.1%). The increase in the conversion of 170HP to cpd S (from 1.3% to 5%) and the simultaneous decrease in the conversion of 170HP to F (11.1% to 1%) could be due to the inhibitory effect of 170HP on the 11β-hydroxylation of cpd S. Part of this inhibition could, however, be more apparent than real since any cpd S deriving from 170HP is diluted by the added cpd S, resulting in a decrease in the percent conversion of 170HP to F.

The main result of the simultaneous incubation of equimolar quantities of 170HP and cpd S with mitochondria from the adrenal adenoma is that the 11β-hydroxylation of 170HP to 21DF is to all practical purposes not functional. This result is similar to that obtained with normal adrenal.

Normal adrenal microsomes. The results of 21-hydroxylation of 170HP and 21DF are summarized in Table 5. The substrates were incubated in increasing quantities (from 1.3 ng to 12.5 μg steroid/mg protein) either separately or simultaneously. Over the whole range of substrates concentration 21-hydroxylation of 170HP was much higher (10 to 20 fold) than that of 21DF. At a concentration of 6 ng steroid/mg protein, 170HP did not inhibit the conversion of 21DF to F whereas at a concentration of 12.5 μg steroid/mg protein, 170HP partially inhibited the 21-hydroxylation of 21DF (from 0.37 to 0.25%) but the conversion of 170HP to cpd S was virtually unaffected (from 4.4 to 4.0%).

Adrenal adenoma microsomes. Table 6 summarizes the results of incubating increasing quantities of 170HP and 21DF separately or simultaneously.

At the concentrations of 5.8 μg and 30.0 μg steroid/mg protein, the 21-hydroxylation of 170HP to cpd S was 5-fold higher than that of 21DF to F and no mutual inhibition was found; however, all of the 170HP added was utilized. At the concentration of 200 μg substrate/mg protein, about 90% of both substrates remained unconverted; in separate incubations twice as much 170HP was 21-hydroxylated as compared to 21DF, but when both substrates were incubated together, the ratio increased to 10:1. This was a result of a decrease in the conversion of 21DF to F (from 5.2 to 0.8%) whereas the 21-hydroxylation of 170HP was only slightly decreased (from 10.2 to 8.1%).

The main result of the incubations both with normal and with adrenal adenoma microsomes is that in the presence of equivalent quantities of 170HP and of 21DF the conversion of the latter to F is reduced and becomes negligible at a relatively, high substrates concentration. Furthermore, the relatively high activity of the 21-hydroxylase in the adenoma micro-

Table 5. 21-Hydroxylation of 17-hydroxyprogesterone (170HP) and of 21-deoxycortisol (21-DF) by normal human adrenal microsomes*

Substrate	(μg/mg protein)	Unchanged substrate (%)	Product (%)
[1,2-^{3}H] 170HP	0.0013	34.3	48.8 (S)
[1,2-^{3}H] 21DF	0.0013	97.0	2.3 (F)
[1,2-^{3}H] 170HP	0.0025	62.4	37.0 (S)
[1,2-^{3}H] 21DF	0.0060	97.0	2.6 (F)
[1,2-^{3}H] 170HP† + [1,2-^{3}H] 21DF	0.0060 0.0060	72.6 97.7	23.9 (S) 2.5 (F)
[1,2-^{3}H] 170HP	0.185	62.8	26.0 (S)
[1,2-^{3}H] 21DF	0.185	97.0	1.3 (F)
[4-^{14}C] 170HP	12.5	80.0	4.4 (S)
[1,2-^{3}H] 21DF	12.5	92.2	0.37 (F)
[4-^{14}C] 170HP + [1,2^{3}H] 21DF	12.5 12.5	89.0 98.0	4.0 (S) 0.25 (F)

* Each incubation was performed with 0.9 mg of microsomal protein equivalent to 0.15 g of fresh tissue.

† In the low steroid concentrations only tritiated substrates have been used. This did not interfere with the results since each of the substrates was exclusively converted to a single specific product, e.g. 170HP to S and 21DF to F.

Table 6. 21-Hydroxylation of 17-hydroxyprogesterone (170HP) and of 21-deoxycortisol (21DF) by microsomes prepared from adrenal adenoma*

Substrate	(μg/mg protein)	Unchanged substrate (%)	Product (%)
[1,2-^{3}H] 170HP	0.09	0.7	89.6 (S)
[1,2-^{3}H] 21DF	0.09	65.5	34.4 (F)
[4-^{14}C] 170HP	5.8	1.2	90.6 (S)
[1,2-^{3}H] 21DF	5.8	68.9	17.8 (F)
[4-^{14}C] 170HP + [1,2-^{3}H] 21DF	5.8	1.0	88.2 (S)
	5.8	77.0	12.0 (F)
[4-^{14}C] 170HP	30	1.0	88.0 (S)
[1,2-^{3}H] 21DF	30	65.3	17.9 (F)
[4-^{14}C] 170HP + [1,2-^{3}H] 21DF	30	0.9	87.0 (S)
	30	72.9	14.7 (F)
[4-^{14}C] 170HP	200	89.3	10.2 (S)
[1.2-^{3}H] 21DF	200	86.0	5.2 (F)
[4-^{14}C] 170HP + [1.2-^{3}H] 21DF	200	91.1	8.1 (S)
	200	90.3	0.8 (F)

* Each incubation was performed with 1.7 mg of microsomal protein, equivalent to 0.15 g of fresh tissue.

somes suggests that an increase in this activity was essential in the overproduction of cortisol in the patient *in vivo*.

DISCUSSION

It is reasonably well established that in the most common type of congenital adrenal hyperplasia and in the polycystic ovary syndrome, 11β-C-21-deoxysteroid metabolites are present both in plasma and urine samples but are not present in detectable amounts in samples from normal subjects[5–7, 26]. Two explanations have been offered for the presence of these compounds. One of them relates specifically to congenital adrenal hyperplasia and claims that the condition arises due to a deficiency of 21-hydroxylase[27,28], and the other encompasses both congenital adrenal hyperplasia and polycystic ovary syndrome and postulates the presence of an additional 11β-hydroxylase[24, 29–31]. While the occurence of 11β-hydroxy-21-deoxysteroids in polycystic ovary syndrome has been explained on the basis of the second theory by demonstrating the presence of an ovarian 11β-hydroxylase specific for 21-deoxysteroids[24, 30, 31], the mechanism of the aberrant biosynthesis in congenital adrenal hyperplasia is still uncertain. The concomitant presence of F and urinary metabolite of 21DF in Cushing's syndrome secondary to adrenal hyperplasia [8] and the presence of cpd S and 21DF in patients with a not well characterized variant of congenital adrenal hyperplasia [7, 28, 32, 33], cast doubts on the validity of the theory ascribing the biosynthetic effect to a deficiency in 21-hydroxylase. On the other hand, the presence of an aberrant 11β-hydroxylase specific for 21-deoxysteroids has never been positively proven in congenital adrenal hyperplasia and its presence is inferred from circumstantial evidence.

To arrive at a better comprehension of the possible defects in the biosynthesis of F in congenital adrenal hyperplasia we felt that more data were needed on the normal sequence of 11β and 21-hydroxylations of the steroid intermediates between 170HΔP and F. Specifically, two questions had to be resolved for the human adrenal: first, whether 21-deoxysteroids are suitable substrates for 11β-hydroxylation and second, whether the 11β-hydroxylated steroids may serve as substrates for 21-hydroxylation.

In an attempt to answer these questions, we tried to separate the human adrenal 11β and 21-hydroxylases from each other. This, however, was achieved only with a partial success: isolated 21-hydroxylase activity was obtained in the microsomal fraction, but the mitochondria preparations always contained some 21-hydroxylase activity. This activity complicated the study of 11β-hydroxylation of 21-deoxysteroids since 21-hydroxysteroids were formed during the incubations and probably competed with the primary substrate. Despite this limitation, the results obtained from the incubations of adrenal mitochondria from both normal and adenoma tissue demonstrated that cpd S was by far the best substrate for 11β-hydroxylation and that it inhibited the 11β-hydroxylation of 170HP when incubated together (Tables 3, 4). Since the *in vivo* 21-hydroxylation is believed to be primarily extra mitochondrial[16], it is conceivable that only small quantities of 21-deoxysteroids are available for the mitochondrial 11β-hydroxylase. Moreover, the most abundant substrate for 11β-hydroxylase is cpd S and it interferes with the 11β-hydroxylation of the earlier intermediates. This inhibition was evident when mitochondria from normal tissue and particularly from the adrenal adenoma were incubated with the various steroid intermediates, either alone or in pairs. In the latter case, the 11β-hydroxylation of 170HP was almost completely suppressed in the presence of cpd S, whereas 170HP only partially inhibited the 11β-hydroxylation of cpd S. These results suggest, that the 11β-hydroxylase can hydroxylate 170HP, should this intermediate be available in mitochondria at a concentration relatively high to cpd S. If this were so, the 11β-hydroxylated derivative of 170HP (i.e. 21-deoxycortisol) could become an intermediate in the biosynthesis of cortisol, if it were also a suitable substrate for C-21-hydroxylase. To clarify this point we compared the rates of 21-hydroxylation of 11β-hydroxy and 11-deoxysteroids.

In the incubations with microsomes from normal human adrenals the 21-hydroxylation of 170HP proceeded more efficiently than that of 21DF, and at the concentrations used, the substrates did not interfere with the hydroxylation of each other (Table 5). On the other hand, when adenoma microsomes were incubated with higher concentrations of the substrates, and the substrate/enzyme ratio was no longer a factor, the hydroxylation of 21DF was inhibited to a large extent by 170HP, whereas the 21-hydroxylation of the latter was only slightly affected (Table 6).

These results suggest that 21DF is not a significant intermediate, and that only small quantities of F can be formed from 21DF, particularly in the presence of 17OHP. Thus, the 'major' pathway for biosynthesis of cortisol in the human adrenal seems to proceed via the $C_{17} \rightarrow C_{21} \rightarrow C_{11\beta}$ hydroxylation sequence.

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