DIFFERENCES BETWEEN ADRENAL ADENOMA CAUSING PRIMARY ALDOSTERONISM AND OTHER ADRENAL TISSUES IN THE INCORPORATION OF LABELED STEROID PRECURSORS INTO THEIR PRODUCTS

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ABSTRACT

The incorporation and conversion of several labeled steroid precursors into their products were examined in slices of adrenal tissue from two patients with primary aldosteronism and compared with that in "normal" adrenal tissue and adrenal tissues from a patient with Cushing's syndrome. The products of the incorporation were separated by Sephadex LH-20 column chromatography. The major products of conversion in the adenomatous tissue of primary aldosteronism were 18-hydroxycorticosterone and lesser amounts of aldosterone. Smaller amounts of 18-hydroxycorticosterone were isolated from all other adrenal tissues studied. No aldosterone could be recovered after incubating any of the adrenal tissue studied with labeled 18-hydroxy-11-deoxycorticosterone or 18-hydroxycorticosterone as precursor steroid. These in vitro results seem to suggest that there is increased 18-hydroxylation in the adenoma of primary aldosteronism compared with other tissues and that relatively more 18-hydroxycorticosterone is produced in such tissue than aldosterone.

INTRODUCTION

Numerous investigators have conducted in vitro studies of steroid biosynthesis using various animal and human adrenal tissues. Production of aldosterone by adrenal tissue from patients with primary aldosteronism (1) as well as the aldosterone content of these tissues have been measured (2-4). Some investigators have examined the incorporation of labeled steroids by adrenal tissue from such patients usually using one or two precursor steroids (5-15). In most instances a single adenoma and its surrounding tissue have been studied. The uninvolved adrenal tissue of the adenoma-bearing gland may not be the most appropriate control for

such investigations.

Our objective in the present study was to examine the patterns of incorporation of several labeled steroid precursors into their products in the adenomatous and the surrounding adrenal tissues in the patients with primary aldosteronism as well as in normal adrenal tissue in a patient with renal cell carcinoma. We also examined similarly the adenomatous and the surrounding adrenal tissues in a different adrenal disorder, namely Cushing's syndrome. These studies have provided a functional basis for the enhanced production of some of the steroids in vivo in the two adrenal disorders and help to shed light on the roles of various precursor steroids in the steroidogenesis in these adrenal tissues.

MATERIALS AND METHODS

Solvents and Reagents

All organic solvents were spectrograde (Matheson-Coleman and Bell). Ethanol, methanol and ethyl acetate were redistilled with sodium hydroxide. The radioactive steroids such as progesterone (1,2,6,7- ^{3}H), 11-deoxycorticosterone (1,2-^{3}H), corticosterone (1,2-^{3}H) and 17-hydroxyprogesterone (1,2-^{3}H) were obtained from New England Nuclear Company. 18-hydroxy-11-deoxycorticosterone (1,2-^{3}H) and 18-hydroxycorticosterone (1,2-^{3}H) were obtained from Amersham Searle, Inc. All tritiated steroids were diluted in ethanol and stored at $4^{o}C$. Aliquots of these steroids were purified just before each experiment by Sephadex LH-20 (Pharmacia, Inc.) column chromatography. Non-labeled steroids were obtained from Steraloids, Inc. (Wilton, NH) and used without further purification. Nicotinamide adenine dinucleotide phosphate, reduced type (NADPH) and periodic acid were purchased from Sigma Chemical Co.

Clinical Summary

The adrenal glands were obtained from two patients with primary aldosteronism (#1, #2), one patient with Cushing's syndrome (#3) and two patients with renal carcinoma (#4, #5). Both patients with primary aldosteronism had a typical solitary adenoma (aldosteronoma) in the adrenal gland removed during operation. They had received a triamterene-thiazide (Dyazide^{R}, Smith, Kline and French, Inc.) combination until 2 days before operation. The adrenal gland removed from the patient with Cushing's syndrome had an adenoma with a brownish-yellow color on cut surface. All of the adenomas on histological examination had clear cells. The adrenal glands removed from two patients with renal cell carcinoma were grossly and

microscopically normal and showed no evidence of tumor invasion.

Incubation procedures

Immediately after removal of the adrenal gland the tissue was placed in cold Krebs-Ringer bicarbonate solution (16) in crushed ice. Adenomatous and surrounding adrenal tissue were separated, sliced into small portions (20-70 mg dry weight) and carefully washed with the solution. Adrenal slices of approximately equal size were incubated in 2 ml of Krebs-Ringer bicarbonate solution. In the experiments using adrenal homogenates the Krebs-Ringer solution additionally contained 200 mg% of glucose and 5 X 10^{-5}M NADPH. The amount of tritiated steroid added in each experiment is shown in Tables 1 and 2. Incubation were carried out in a Dubnoff shaker for 60 minutes at 37°C in an atmosphere of 95% O_2 - 5% CO_2.

A series of experiments were carried out using the normal adrenal tissue obtained from patient 5 to determine the effect of preincubation, differing incubation times, the mass of adrenal tissue incubated and the mass of labeled steroids used. In addition, incubations were conducted with homogenized tissue and, in some experiments, the tissue was homogenized after incubation, mixed with medium, and examined to determine the conversion pattern of the steroids.

Extraction and Chromatographic Purification

The medium was removed immediately after incubation with the adrenal slices and extracted with 10 times the volume of sodium hydroxide-distilled ethyl acetate. The organic solvent layer was washed with 0.1 N sodium hydroxide solution and distilled water. After drying in vacuo, an aliquot of the extract was used for recovery. Chromatographic separation was performed using glass columns (1 X 30 cm, Bio-Rad) with Sephadex LH-20. A solvent mixture containing heptane, benzene, methanol and water (65:25:10: 0.1) was run through the Sephadex LH-20 bed (17). The ethyl acetate extract was dissolved in 0.5 ml of the solvent mixture and applied to the top of the packed column. Eluates were collected in 1 ml fractions. Chromatographic separation of progesterone (10-13 ml), 11-deoxycorticosterone (17-22 ml), corticosterone (30-36 ml), aldosterone (39-47 ml) and cortisol (53-63 ml) could be obtained in this system. The more polar or cyclic hemiketal forms of 18-hydroxy-11-deoxycorticosterone and 18-hydroxycorticosterone eluted between 27 and 34 ml, and 45 and 57 ml, respectively. The more polar steroid fractions were collected in 10 ml volumes after elution with methanol. Because of the overlapping elution of 18-hydroxy-11-deoxycorticosterone and corticosterone and, to some extent, aldosterone and 18-hydroxycorticosterone, periodic acid oxidation (18) of these fractions was performed and rechromatographed using a smaller LH-20 column for more accurate separation of the steroids. Relatively specific antibodies with known binding characteristics and cross-reactivity, generated in our laboratory against corticosterone, cortisol and 18-hydroxy-11-deoxycorticosterone as well as aldosterone antibody obtained from NIH were used for further identification of the products of conversion. The antibody to 18-hydroxy-11-deoxycorticosterone also reacted with 18-hydroxycorticosterone at 10-fold lower dilution (17). The

18-hydroxy-11-deoxycorticosterone, 18-hydroxycorticosterone and aldosterone were subsequently rechromatographed after conversion to their respective gamma lactones. In our system, the elution volume of each steroid was constant in repeated chromatography using the same column and solvent mixture for weeks or months, but strict care of the column and the solvent system was required. Ethyl acetate was used for extraction because of its high and fairly similar extraction rates for all steroids involved. To monitor losses the total radioactivity was counted before and after extraction. Following chromatography the radioactivity recovered in the elution volume of each steroid peak was corrected for loss during extraction and then divided by the original count of the precursor steroid to calculate the percent recovered in the specific steroid fraction. The results of such calculations appear in tables 1 and 2.

RESULTS

a) ^{3}H-Progesterone and ^{3}H-17-hydroxyprogesterone as substrates:

Most of the radioactivity after incubation of adenomatous tissue (aldosteronoma) with ^{3}H-progesterone from patients 1 and 2 was recovered as corticosterone and 18-hydroxycorticosterone and a lesser amount as aldosterone (Table 1 and Figure 1). In contrast to the adenomatous tissue, corticosterone was the major product of incubation of surrounding adrenal tissue or normal adrenal. Cortisol was also formed from surrounding adrenal tissue (Patients 1 and 2) and the normal adrenal tissue (Patient 4). There was no 18-hydroxycorticosterone identified in the incubations of the surrounding adrenal tissue (Patients 1 and 2) or "normal" adrenal tissue from patient 4. In incubations of adenomatous tissue from patient 3 (Cushing's syndrome) the major product was cortisol, but there was a significant amount of corticosterone formed. The surrounding tissue converted ^{3}H-progesterone to corticosterone and some 18-hydroxycorticosterone. There was no detectable cortisol identified in the latter incubation (see Fig. 1 and Tables 1 and 2).

Incubations of adenomatous tissue and surrounding adrenal tissue

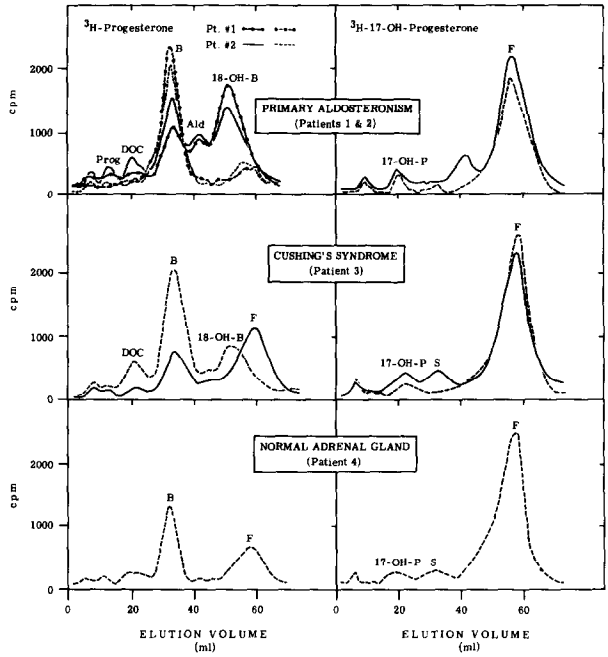

Figure 1. Shows the patterns of conversion of ^{3}H-progesterone and ^{3}H-17-hydroxyprogesterone by adenoma (_____) and surrounding tissue or normal tissue (-----).

from Patient 2 (with primary aldosteronism) and from Patient 3 (with Cushing's syndrome) and normal adrenal tissue from Patient 4 with ^{3}H-17-hydroxyprogesterone resulted in the formation of cortisol as the main product (Fig. 1). During incubation with each of the tissues some unidentified steroids more polar than cortisol, were detected (Table 1 and 2), in a relatively greater proportion in the incubations of aldosteronomas.

b) ^{3}H-11-deoxycorticosterone and ^{3}H-corticosterone as substrates:

Incorporation of tritiated 11-deoxycorticosterone by the aldosteronoma from patients 1 and 2 resulted in the formation of corticosterone and 18-hydroxycorticosterone as the major products

(Figures 2 and 3, Table 1). However, detectable amounts of aldosterone were produced. In the surrounding normal adrenal tissue from these patients, as well as the tissues of patients 3 and 4, the predominant steroid was corticosterone, with a smaller amount of 18-hydroxycorticosterone being formed. None of the latter tissues produced detectable amounts of aldosterone. When tritiated corticosterone was used as substrate, the adenomatous tissue from patients 1 and 2 produced predominantly 18-hydroxycorticosterone and a lesser proportion of aldosterone. In the other tissues studied, small amounts of 18-hydroxycorticosterone were discerned but no aldosterone (see Figures 2, 3 and Table 1).

c) ^{3}H-18-hydroxy-11-deoxycorticosterone and ^{3}H-18-hydroxy-corticosterone as substrates:

When ^{3}H-18-hydroxy-11-deoxycorticosterone was incubated with the adenomatous tissue of patients 1 and 2 some of the tritiated steroid was recovered as 18-hydroxycorticosterone but the rest of it remained unconverted (Tables 1 and 2 and Figure 2). No aldosterone could be recovered from the medium. In all other tissues studied 18-hydroxy-11-deoxycorticosterone was not converted to other steroids except in the case of the adenomatous tissue from patient 3 where major conversion to 18-hydroxycorticosterone was seen (Tables 1 and 2).

No other steroid formation could be demonstrated when tritiated 18-hydroxycorticosterone was used in any tissues studied (Tables 1 and 2 and Figure 3). In these studies the unconverted 18-hydroxy-11-deoxycorticosterone and 18-hydroxycorticosterone remained as the more polar or cyclic hemiketal form in the medium.

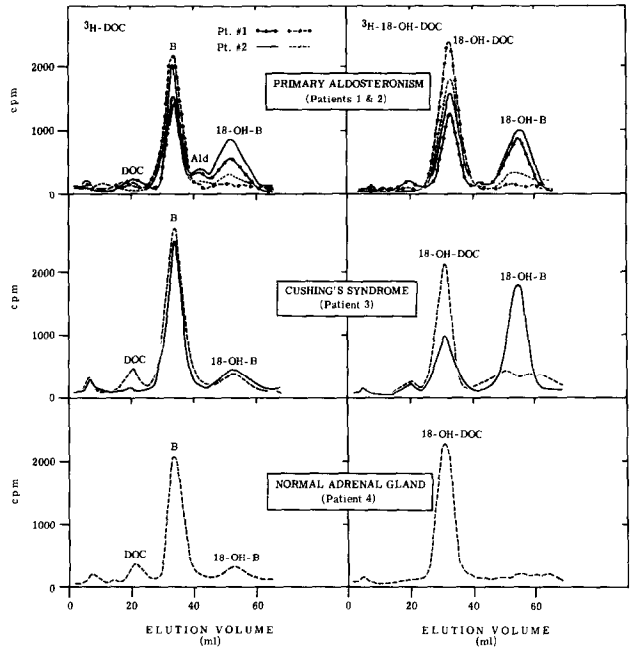

Figure 2. Shows the patterns of conversion of ^{3}H-11-deoxycorticosterone and ^{3}H-18-hydroxy-11-deoxycorticosterone in the same patients.

d) Effect of preincubation, duration of incubation, amount of substrate and tissue mass:

Results of the studies using the adrenal tissue from patient 5 showed no significant differences in the patterns or rates of conversion of precursors with or without preincubation, with a larger mass of labeled steroid, longer incubation time or with homogenized tissue. Significant changes in conversion profile were seen only with marked differences in tissue mass.

Except when progesterone was used as precursor the losses of radioactivity during incubation and extraction were minimal. Incubations of adrenal tissue from patient 5 suggested that losses during incubation with labeled progesterone resulted probably from

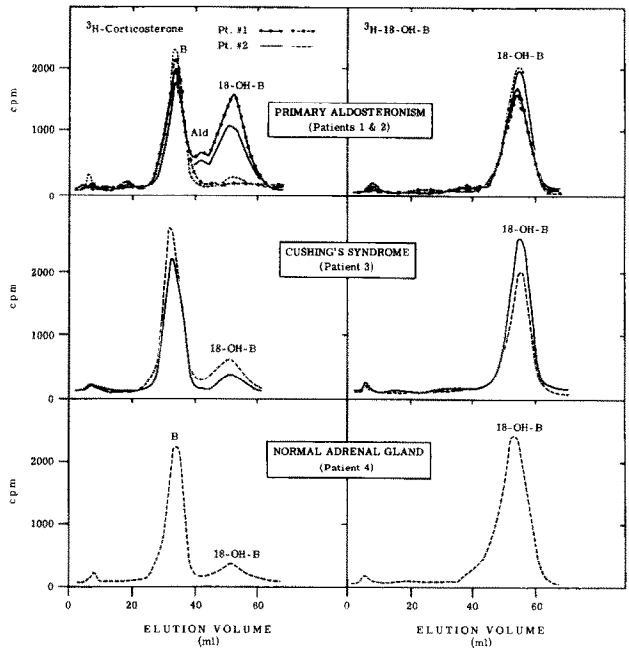

Figure 3. Shows the steroid profile using ^{3}H-corticosterone and ^{3}H-18-hydroxycorticosterone in the same patients.

unconverted progesterone remaining in the tissue. The overall recovery of radioactivity usually ranged between 36 and 84%. The lowest recovery was seen with ^{3}H-progesterone. For other substrates the overall recoveries were consistently greater than 50%, including those of 18-hydroxycorticosterone, 18-hydroxy-11-deoxycorticosterone and aldosterone.

DISCUSSION

A systematic investigation of steroidogenesis using labeled steroids of adrenal tissue from patients with primary aldosteronism has rarely been undertaken. Most investigators have chosen to

compare the steroidogenesis of the adenomatous tissue (usually from a single patient) with that of the surrounding adrenal tissue. The latter tissue may not be the ideal tissue for such comparison. Besides, a limited number of precursor steroids have been used for such studies. We have attempted to examine the relative conversion patterns of adenomatous and its surrounding adrenal tissue, normal adrenal tissue and adrenal tissue from a patient with a different disorder, in a comprehensive fashion using a number of early and late labeled precursor steroids. The results of our studies show relatively greater conversion of tritiated progesterone, deoxycorticosterone or corticosterone to 18-hydroxycorticosterone and aldosterone in the adenomas of primary aldosteronism compared to the other tissues, presumably reflecting increased enzyme activity of the late steps of aldosterone synthesis, particularly 18-hydroxylation. Such an observation is in agreement with the suggestions of other investigators (7-9, 12, 13). The conversion of each of the steroids to aldosterone in the adenoma of primary aldosteronism was less than its conversion to 18-hydroxy-corticosterone. Ulick and Vetter (19) have shown that the normal secretory rate of 18-hydroxycorticosterone *in vivo* is about twice that of aldosterone. Hypokalemia may further favor the production of proportionately greater amount of 18-hydroxycorticosterone (20). The present *in vitro* observations are consistent with those findings. The surrounding tissue adjacent to aldosteronomas, in contrast to normal tissue from patients 3 and 4, produced less 18-hydroxycorticosterone, a finding which may support the concept that there is suppression of 18-hydroxylation (7).

18-hydroxycorticosterone has long been considered to be the precursor of aldosterone (21, 22). However, in studies in vitro, various investigators have shown greater rates of conversion of corticosterone to aldosterone than when 18-hydroxycorticosterone was used (21, 23). Ulick has further suggested that a mixed function oxidase system may be involved in the final steps of conversion of corticosterone to aldosterone (24). In our experiments no detectable conversion of 18-hydroxycorticosterone to aldosterone could be demonstrated. Such a finding is in agreement with previous studies.

In primary aldosteronism it has been postulated that 18-hydroxy-11-deoxycorticosterone may be an intermediate in aldosterone biosynthesis (25). In our experiments using adrenal adenomatous tissue which incorporated progesterone, deoxycorticosterone and corticosterone into aldosterone, we were unable to recover any aldosterone using tritiated 18-hydroxy-11-deoxycorticosterone as the substrate. Since we used adrenal slices rather than homogenates it is possible that the permeability of the 18-hydroxylated steroids into the cells might be different from that of the other steroids. Therefore, the role of 18-hydroxycorticosterone or 18-hydroxy-11-deoxycorticosterone in aldosterone biosynthesis cannot be answered definitively from these experiments. Significantly more 18-hydroxy-11-deoxycorticosterone appeared to be converted to 18-hydroxycorticosterone in the aldosteronoma than in other adrenal tissues. This observation suggests increased 11 beta-hydroxylase activity in these tumors as well. Such a finding is compatible with the concept of parallelism of 11 beta-hydroxylase and 18-hydroxylase

activities (26, 27).

When progesterone was used as substrate in aldosteronoma most of it was converted to 18-hydroxycorticosterone and aldosterone and no detectable amounts of cortisol were found. The adenomatous tissue of Cushing's syndrome converted most of the progesterone to cortisol. However, when 17-hydroxyprogesterone was used as substrate in aldosteronoma some cortisol was formed. Such findings suggest that aldosteronoma tissue may lack 17 alpha-hydroxylase activity or that progesterone is preferentially utilized in aldosterone biosynthesis.

In vitro studies may not always reflect the state of in vivo secretion of steroids. Our studies indicate qualitative differences in the various adrenal tissues. One may be tempted to extrapolate the observations to suggest that there is increased activity of 18-hydroxylase and of distal enzyme(s) involved in the aldosterone biosynthesis in primary aldosteronism in vivo. If such a premise is correct our observations suggest that the patients with primary aldosteronism secrete much greater quantities of 18-hydroxy-corticosterone than aldosterone and the former may be a useful marker for primary aldosteronism as has been recently suggested by Biglieri et al (20).

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NOMENCLATURE

The following trivial names have been used: aldosterone;

corticosterone (B); cortisol (F); 11-deoxycorticosterone (DOC); 18-hydroxy-11-deoxycorticosterone (18-OH-DOC) = 18, 21-dihydroxy-pregn-4-ene-3, 20-dione-20, 18-hemiketal; 18-hydroxycorticosterone (18-OH-B) = 11β, 18, 21-trihydroxypregn-4-ene-3, 20-dione-20 18-hemiketal; 17-hydroxyprogesterone (17-O-HP) = 17-hydroxy-pregn-4-ene-3, 20-dione.

Table 1: Conversion Profiles in Adenomatous and Surrounding Adrenal Tissue from Patients with Primary Aldosteronism

	Substrate			Percent Recovered as					
	Steroids	Amount x10^6 dpm	tissue dry weight (mg)	18-OH-DOC	Cortico-sterone	Aldo-sterone	18-OH-B	Cortisol	Polar Steroids
Primary Aldosteronism (Patient 1)									
Adenoma	^{3}H-Progesterone	2.1	38.4		19.8	15.9	44.4		5.8
	^{3}H-DOC	3.4	24.3		32.9	10.2	23.6		2.0
	^{3}H-Corticosterone	3.7	25.4		49.4	9.1	28.6		1.9
	^{3}H-18-OH-DOC	1.8	38.2	43.3			42.7		4.6
	^{3}H-18-OH-B	3.2	67.2				77.6		2.2
Surrounding Tissue	^{3}H-Progesterone	2.1	22.9		58.0			16.7	5.4
	^{3}H-DOC	3.4	24.9		95.2				1.1
	^{3}H-Corticosterone	3.7	25.0		86.3		2.0		0.9
	^{3}H-18-OH-DOC	1.8	24.7	88.2			3.3		5.5
	^{3}H-18-OH-B	3.2	50.7				89.5		1.4
Primary Aldosteronism (Patient 2)									
Adenoma	^{3}H-Progesterone	2.0	26.4		39.8	8.0	23.0		10.3
	^{3}H-DOC	1.6	28.6		29.7	11.2	31.2		4.9
	^{3}H-Corticosterone	1.8	23.9		46.4	18.6	32.4		2.7
	^{3}H-18-OH-DOC	2.4	30.1	39.2			44.6		7.7
	^{3}H-18-OH-B	2.0	21.7				64.8		2.1
	^{3}H-17-OH-Progesterone	2.0	32.0					57.2	34.2
Surrounding Tissue	^{3}H-Progesterone	2.0	21.2		63.8			17.6	11.2
	^{3}H-DOC	1.6	20.5		74.9		10.2		14.9
	^{3}H-Corticosterone	1.8	26.6		85.3		2.0		1.9
	^{3}H-18-OH-DOC	2.4	29.9	37.9			21.3		14.7
	^{3}H-18-OH-B	2.0	32.1				87.9		3.1
	^{3}H-17-OH-Progesterone	2.0	29.7					69.8	8.7

Table 2: Conversion Profiles in Adenomatous and Surrounding Tissues from a Patient with Cushing's Syndrome and a Normal Adrenal

	Substrate			Percent Recovered as				
	Steroids	Amount x 10^6 dpm	tissue dry weight (mg)	18-OH-DOC	Cortico-sterone	18-OH-B	Cortisol	Polar Steroids
Cushing's Syndrome (Patient 3)	^{3}H-Progesterone	2.8	81.5		16.1		34.1	18.1
	^{3}H-DOC	3.5	58.8		82.9			8.2
Adenoma	^{3}H-Corticosterone	3.7	55.1		72.3			5.1
	^{3}H-18-OH-DOC	4.7	84.7	21.8		35.7		28.1
	^{3}H-18-OH-B	3.2	71.1			83.4		2.8
	^{3}H-17-OH-Progesterone	3.4	80.7				56.7	10.1
	^{3}H-Progesterone	2.8	28.7	9.7	40.5	20.8		6.1
	^{3}H-DOC	3.5	21.6	7.5	68.5	9.5		2.6
Surrounding Tissue	^{3}H-Corticosterone	3.7	41.1		58.3	10.8		2.5
	^{3}H-18-OH-DOC	4.7	27.8	67.6		2.7		5.7
	^{3}H-18-OH-B	3.2	32.5			79.5		3.4
	^{3}H-17-OH-Progesterone	3.4	42.9				49.8	9.6
Normal Adrenal Gland (Patient 4)	^{3}H-Progesterone	3.9	66.1		40.6		35.7	7.5
	^{3}H-DOC	3.0	37.6		82.6	7.2		2.7
	^{3}H-Corticosterone	3.5	85.4		82.7	6.5		2.3
	^{3}H-18-OH-DOC	4.5	66.6	77.2		5.0		10.6
	^{3}H-18-OH-B	3.6	30.8			73.3		1.3
	^{3}H-17-OH-Progesterone	3.3	95.1				79.0	11.9