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Effect of Androgens on Steroid C-21 Hydroxylation*

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C-21 hydroxylation of progesterone, 17α-hydroxyprogesterone, pregnenolone, and 17α-hydroxypregnenolone was studied using the microsomal fraction of bovine adrenal cortex as the source of "steroid C-21 hydroxylase." 4-Androstene-3,17-dione, testosterone, and dehydroepiandrosterone do not ihhibit C-21 hydroxylation of progesterone and 17α-hydroxyprogesterone in contrast to the inhibitory action of the adrenal androgens on 11β-hydroxylation step in corticosteroid biosynthesis. However, C-21 hydroxylation of pregnenolone and 17α-hydroxypregnenolone was markedly inhibited by 4-androstene-3,17-dione, testosterone, and dehydroepiandrosterone. This is of particular significance since 17α-hydroxypregnenolone and not 17α-hydroxyprogesterone has been suggested to be the primary precursor of cortisol, and intraglandular modulation of steroid biosynthesis at least in part correlates certain clinical findings. Effect of pharmacological inhibitors 1,2-bis-(3-pyridyl-2-methyl-1-propanone), 3-(6-chloro-3-methyl-2-indenyl)pyridine, 3-(1,2,3,4-tetrahydro-1-oxo-2-naphthyl)pyridine, and the 7-chloro derivative of 3-(1,2,3,4-tetrahydro-1-oxo-2-naphthyl)pyridine on steroid C-21 hydroxylation has also been reported.

Intraglandular modulation of steroid biosynthesis and its possible role in physiological, and even more in pathological, conditions has recently been postulated (Mahajan and Samuels, 1962; Leventhal and Scommegna, 1963). The influence of certain steroids on 11β-hydroxylation of 11-deoxycorticosterone[1] has been reported earlier (Sharma *et al.*, 1963). In the present paper, steroid C-21 hydroxylation of various substrates has been studied and the effect of certain steroids, particularly adrenal androgens, has been investigated. The effect of certain pharmacological inhibitors has also been reported.

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[1] The following trivial names and abbreviations have been used in this work: progesterone, 4-pregnene-3,20-dione; 17α-OH-progesterone, 4-pregnen-17α-ol-3,20-dione; pregnenolone, 5-pregnen-3β-ol-20-one; 17α-OH-pregnenolone, 5-pregnene-3β,17α-diol-20-one; 11-deoxycorticosterone, 4-pregnen-21-ol-3,20-dione; 11-deoxycortisol, 4-pregnene-17α-21-diol-3,20-dione; testosterone, 4-androsten-17β-ol-3-one; dehydroepiandrosterone, 5-androsten-3β-ol-17-one; G-6-P, glucose-6-phosphate; SU 4885, 1,2-bis-(3-pyridyl-2-methyl-1-propanone); SU 8000, 3-(6-chloro-3-methyl-2-indenyl)pyridine; SU 9055 3-(1,2,3,4-tetrahydro-1-oxo-2-naphthyl)pyridine; SU 10603, 7-chloro derivative of SU 9055.

EXPERIMENTAL PROCEDURE

Triphosphopyridine nucleotide, "98% pure," as the sodium salt and glucose-6-phosphate also as the sodium salt were obtained from Sigma Chemical Co. Glucose-6-phosphate dehydrogenase was obtained from Calbiochem, Inc. SU 8000, SU 9055, and SU 10603 were generous gifts from Dr. J. J. Chart, Ciba Pharmaceutical Co., Summit, N. J. Amphenone, steroids, and all other chemicals were obtained commercially.

Enzyme Preparation.—Bovine adrenal glands were collected within 15 minutes after slaughter, freed from connective tissues and adhering fat, dipped in ice-cold 0.25 M sucrose solution, and placed in dry ice. The frozen glands were stored at −20° and processed within 8 weeks.

The following operations were carried in a cold room at 4°. The cortex was removed while still frozen and was homogenized with three times its weight of ice-cold 0.25 M sucrose solution first in a Waring Blendor at 1/4 speed for 2 minutes and then in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 700 × *g* for 15 minutes at 0° to remove nuclei and cell debris; the supernatant fluid was then centrifuged at 7000 × *g* for 30 minutes to remove the mitochondrial

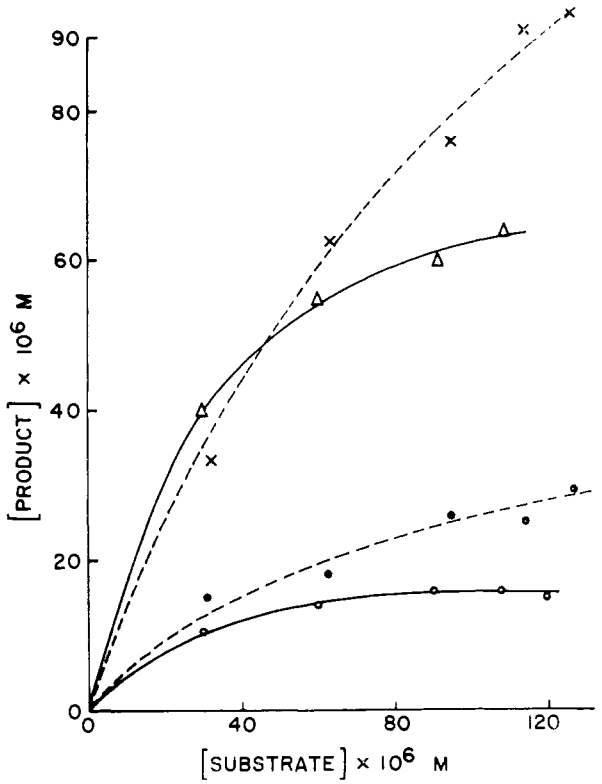

FIG. 1.—Effect of substrate concentration on C-21 hydroxylation of progesterone (X), 17α-OH-progesterone (Δ), pregnenolone (●), and 17α-OH-pregnenolone (O). Enzyme preparation equivalent to 10 mg of protein was used. Besides substrate, each incubation flask contained 17.4 μmoles of G-6-P, 2.2 μmoles of TPN, 5 μmoles of $MgCl_2$, 250 μmoles of Tris-maleate (*p*H 7.4) buffer, and 1.5 Kornberg units of G-6-P dehydrogenase in a total volume of 5.0 ml.

pellet. This was followed by centrifugation of the supernatant fluid at 105,000 × *g* for 60 minutes to sediment the microsomes. The microsomes were washed by resuspending the pellet in twice its weight of 0.25 M sucrose solution and centrifuged at 105,000 × *g* for 60 minutes. The microsomal preparation was then homogenized in three times its weight distilled water and lyophilized to obtain a light-fawn-colored powder which was stored at −20° under desiccation. On an average, 1 g of dried enzyme preparation was obtained from 190 g of whole adrenal glands. Protein was determined by the method of Lowry *et al.* (1951), using crystalline bovine serum albumin as standard. Ten mg of enzyme preparation was equivalent to 6 mg of protein.

Incubation.—All incubations were carried out in 20-ml beakers at 37° in air, with shaking in a Dubnoff Metabolic Incubator. The incubation beakers contained, besides substrate and enzyme, the following additions: 3.5 μmoles of TPN, 22 μmoles of glucose-6-P, 1 Kornberg unit of glucose-6-P dehydrogenase, 5 μmoles of $MgCl_2$, 200 μmoles of Tris buffer *p*H 7.4, in a total volume of 4 or 5 ml. The substrate was added to the beakers in 0.05 ml of propane-1,2-diol before other additions.

After incubation the steroids were extracted with 45 ml of methylene chloride and centrifuged at 0° for 10 minutes, and the lower layer was quantitatively transferred to another tube and evaporated under nitrogen. The extracted steroids were dissolved in a definite volume of methylene chloride and suitable aliquots were taken in duplicate for estimation of the C-21-hydroxylated products.

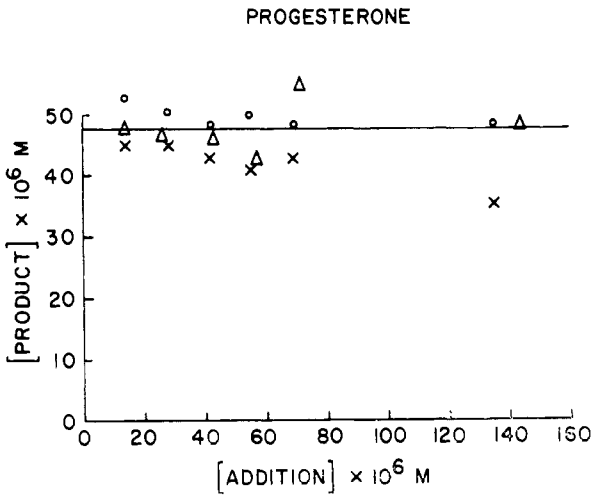

FIG. 2.—Effect of added 4-androstene-3,17-dione (Δ), testosterone (X), and dehydroepiandrosterone (O) on C-21 hydroxylation of progesterone. Substrate concentration 127.2 mμmoles/ml. Besides substrate, each incubation flask contained 13.6 μmoles of G-6-P, 2.0 μmoles of TPN, 5 μmoles of $MgCl_2$, 1.5 Kornberg units of G-6-P dehydrogenase, and 250 μmoles of Tris-maleate buffer (*p*H 7.4) in a total volume of 5.0 ml. Data calculated for enzyme preparation equivalent to 10 mg of protein.

Assay Methods.—11-Deoxycorticosterone was estimated by the blue tetrazolium reaction (Moncloa *et al.*, 1959). 11-Deoxycortisol was estimated by the blue tetrazolium as well as by the Porter-Silber method (Peron, 1962). 21-Hydroxypregnenolone and 17,21-dihydroxypregnenolone were estimated by the blue tetrazolium reaction as well as by the Petten-kofer reaction (Munson *et al.* 1948), after paper chromatographic separation in toluene–propylene glycol system.

Determination of Radioactivity.—Samples were evaporated to dryness under nitrogen in vials of low potassium content (Wheaton Glass Co., Millville, N.J.) and dissolved in 10 ml of scintillation-grade toluene containing 0.4% of 2,5-diphenyloxazole and 0.001% of 1,4-bis-2(5-phenyloxazolyl)benzene. Internal standards were used to check that no self-absorption occurred over the range of steroid weights used. Average efficiency of counting for ^{14}C was 65%.

Partition-Column Chromatography.—The system used was similar to that mentioned by Engel *et al.* (1961). The fractions were evaporated under nitrogen, the residues were dissolved in 95% aqueous ethanol, and aliquots were removed for analyses. Absorbance at 240 mμ was determined on a Zeiss spectrophotometer (PMQ II). Radioactivity measurements were carried out in Packard automatic Tri-Carb liquid scintillation spectrometer.

RESULTS

Effect of Substrate Concentration.—The effect of substrate concentration on the formation of C-21-hydroxylated products is shown in Figure 1. The per cent conversion for progesterone and 17α-OH-progesterone is much higher than those for pregnenolone and 17α-OH-pregnenolone.

Effect of Dehydroepiandrosterone, Testosterone, and 4-Androstene-3,17-dione.—Figures 2 and 3 show the effect of Dehydroepiandrosterone, testosterone, and 4-androstene-3,17-dione on the conversion of progesterone and 17α-OH-progesterone into 11-deoxycorticosterone and 11-deoxycortisol, respectively. No appreciable effect on C-21 hydroxylation of progesterone and 17α-

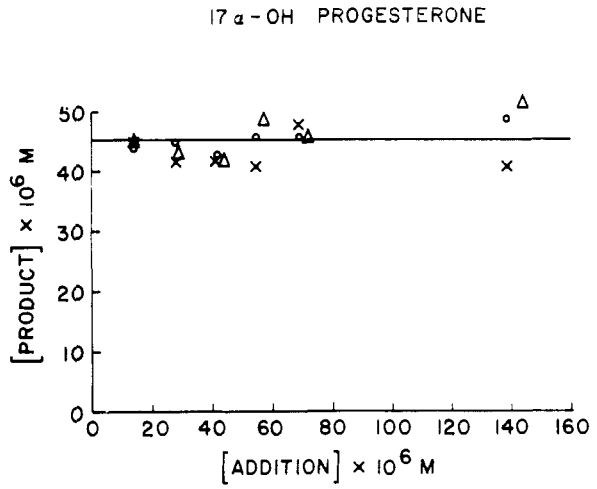

FIG. 3.—Effect of added 4-androstene-3,17-dione (Δ), testosterone (X), and dehydroepiandrosterone (O) on C-21 hydroxylation of 17α-OH-progesterone. Substrate concentration 121.1 mμmoles/ml. Besides substrate, each incubation flask contained 13 μmoles of G-6-P, 1.8 μmoles of TPN, 5 μmoles of $MgCl_2$, 1.5 Kornberg units of G-6-P dehydrogenase, and 250 μmoles of buffer in a total volume of 5 ml. Data calculated for enzyme preparation equivalent to 10 mg of protein.

OH-progesterone is exerted by the C-19 steroids, even when present in equimolar concentration.

In contrast, Figures 4 and 5 clearly show a marked inhibition of the C-21 hydroxylation of pregnenolone and 17α-hydroxypregnenolone by dehydroepiandrosterone, testosterone, and 4-androstene-3,17-dione. That the inhibition is not owing to limitation of cofactors was checked earlier. It is interesting to note that with rising concentration of 4-androstene-3,17-dione, there is a reversal of the inhibition. No such reversal is apparent in cases of dehydroepiandrosterone and testosterone.

In order to study the fate of 4-androstene-3,17-dione during its role as an inhibitor of C-21 hydroxylation of pregnenolone, 4-androstene-3,17-dione-[4-^{14}C] was used.

To each of the two incubation flasks containing 200 μg of the substrate pregnenolone were added 4-androstene-3,17-dione-[4-^{14}C] (312 × 10^3 dpm) and enough cold 4-androstene-3,17-dione to make a total of 300 μg of the addition. Besides the substrate and the added steroid, each incubation flask contained 3 μmoles of TPN, 21 μmoles of G-6-P, 5 μmoles of $MgCl_2$, 1.5 Kornberg units of G-6-P dehydrogenase, and 200 μmoles of Tris-maleate (*p*H 7.4) buffer in a total volume of 4.0 ml. Enzyme preparation equivalent to 6.6 mg of protein was added and the incubation was stopped after 1 hour. After the incubation, the steroids were extracted and an aliquot fourth was taken for estimation of 21-hydroxypregnenolone. In the controls, 26% of the substrate was hydroxylated at C-21. The remaining three-fourths aliquots from two identical flasks, in which 4-androstene-3,17-dione was added before incubation, were pooled and the steroids were separated by partition chromatography on a Celite column (Engel *et al.*, 1961). Coincidence of the elution curves of radioactivity and the steroid having a Δ^4-3-keto group (240-mμ absorbance peak in alcohol) is apparent from Figure 6. Fractions 80–90 were pooled and subjected to isotopic-dilution analyses. The data are summarized in Table I. Since out of 375 × 10^3 dpm put on the Celite column 319 × 10^3 dpm were recovered as radioactive 4-androstene-3,17-dione and 2345 dpm were obtained in the strippings, the inhibitory effect observed in presence of 4-androstene-3,17-dione is probably not owing to any metabolic product of 4-androstene-3,17-dione, but to the steroid itself.

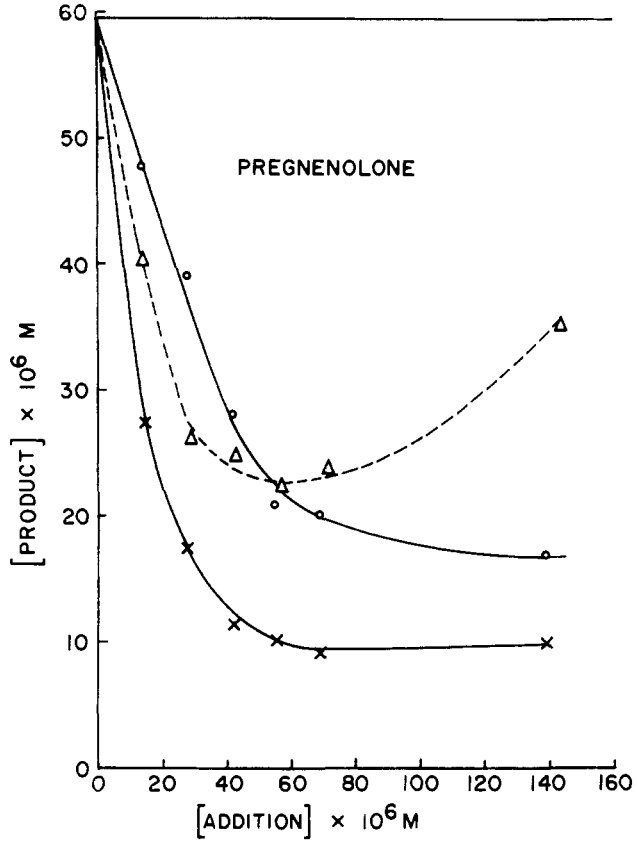

FIG. 4.—Effect of added 4-androstene-3,17-dione (Δ), testosterone (X), and dehydroepiandrosterone (O) on C-21 hydroxylation of pregnenolone. Substrate concentration 126.4 mμmoles/ml. Besides substrate each incubation flask contained 17 μmoles of G-6-P, 2.2 μmoles of TPN, 5 μmoles of $MgCl_2$, 1.5 Kornberg units of G-6-P dehydrogenase, and 250 μmoles of Tris-maleate buffer (*p*H 7.4) in a total volume of 5.0 ml. Enzyme preparation equivalent to 18 mg of protein was used.

TABLE I
PURIFICATION OF ANDROST-4-ENE-3,17-DIONE

Step	Radioactivity (dpm × 10^{-4})	Weight (mg)	Specific Activity (dpm/mg × 10^{-3})
Pool off Celite column (fractions 79–91)	31.91		
Dilute with androst-4-ene-3,17-dione		31.4	
First crystals	24.16	24.8	9.7
First mother liquor	4.46	6.3	7.1
Second crystals	19.91	20.8	9.6
Second mother liquor	2.69	3.3	8.2
Third crystals	13.64	14.5	9.4
Third mother liquor	4.70	5.2	9.0
Fourth crystals	9.84	10.2	9.6
Fourth mother liquor	3.15	3.4	9.3

Effect of Some Other C-19 *Steroids.*—An attempt was made to investigate the functional group necessary in a C-19 steroid for exerting the inhibitory effect. The five compounds shown in Figure 7 were tried.

From Table II it is apparent that compound C had practically no effect, while compounds A, B, D, and E exerted varying degrees of inhibition of C-21 hydroxylation. It can be interpreted that presence of a Δ^5-3β-ol or Δ^4-3-keto group is necessary and that the Δ^4-

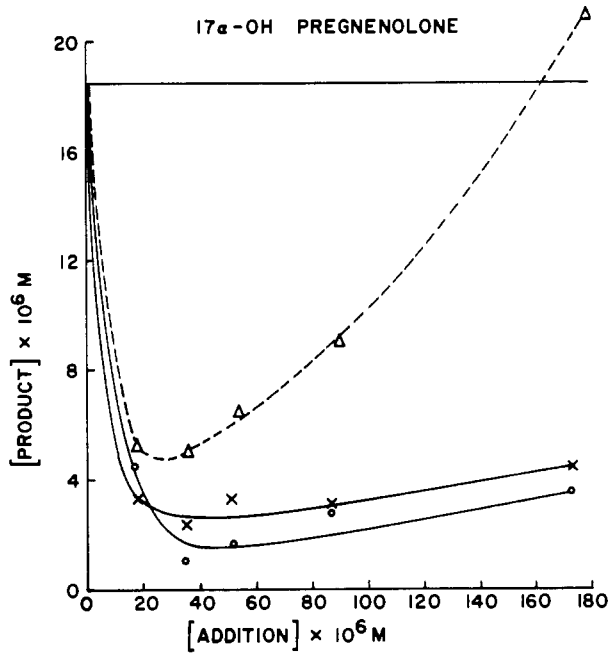

FIG. 5.—Effect of added 4-androstene-3,17-dione (Δ), testosterone (X), and dehydroepiandrosterone (O) on C-21 hydroxylation of 17α-OH-pregnenolone. Enzyme preparation equivalent to 10 mg of protein was used. Other incubation conditions as described in the text.

TABLE II
EFFECT OF ADDED Δ⁴-ANDROSTENE-3,17-DIONE, TESTOSTERONE, DEHYDROEPIANDROSTERONE, Δ⁴-ANDROSTENE-3β,17β-DIOL, AND Δ⁵-ANDROSTENE-3β,17β-DIOL ON CONVERSION OF PREGNENOLONE TO 21-HYDROXYPREGNENOLONE[a]

Addition	Amount Added (μg)	C-21 Hydroxylation of Pregnenolone (%)
None		26
Δ⁴-Androstene-3,17-dione	20	18
	80	12
	200	19
Testosterone	200	8
Dehydroepiandrosterone	200	5
Δ⁴-Androstene-3β,17β-diol	20	21
	60	23
	80	23
	200	23
Δ⁵-Androstene-3β,17β-diol	20	21
	60	10
	80	9
	100	7
	200	6

[a] As well as the additions indicated, each incubation flask contained 200 μg of pregnenolone as substrate, 3.5 μmoles of TPN, 28 μmoles of glucose-6-P, 1.5 Kornberg units of glucose-6-P dehydrogenase, 200 μmoles of Tris-maleate buffer, pH 7.4, 5 μmoles of $MgCl_2$, and 6.6 mg of enzyme protein preparation. Total volume was 4 ml. Incubation was at 37° for 60 minutes in air.

3β-ol group is ineffective. Between 17-keto and 17β-ol grouping there is not much difference.

Effect of Certain Pharmacological Inhibitors.—Earlier we have reported (Sharma and Dorfman, 1963) the effect of certain pharmacological inhibitors on C-21 hydroxylation of progesterone and 17α-OH-progesterone. The studies were repeated and the results are summarized in Tables III and IV. Conversion of

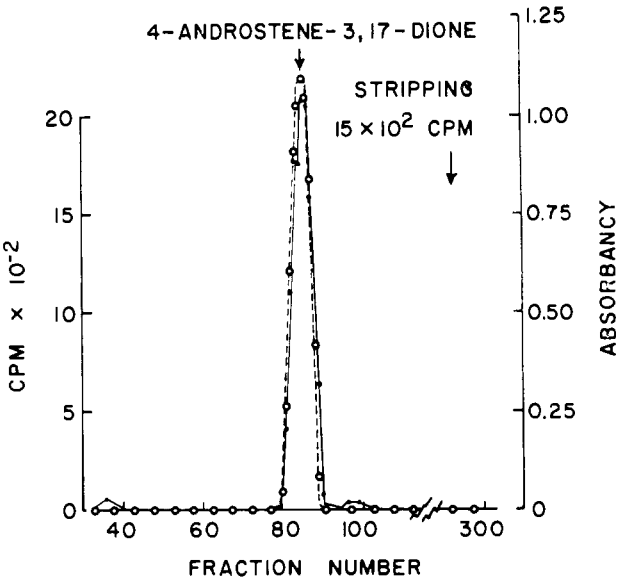

FIG. 6.—Recovery of added 4-androstene-3,17-dione-[4-^{14}C] by gradient-elution partition chromatography. Column: 1-cm diameter; 25 g of Celite; 15 ml of 80% aqueous methanol as stationery phase; 2,2,4-trimethylpentane as mobile phase. An exponential gradient of 1,2-dichloroethane was started at fraction 49. Two hundred ninety fractions, each 1.3 ml, were collected. Flow rate was 1.9 ml/10 minutes. Radioactivity, (●); absorbance at 240 mμ, (O).

TABLE III
EFFECT OF ADDED SU 4885, SU 9055, AND SU 10603 ON CONVERSION OF PROGESTERONE TO 11-DEOXYCORTICOSTERONE[a]

Addition	Amount Added (μg)	C-21 Hydroxylation of Progesterone (%)
None		32
SU 4885	20	23
	40	27
	60	25
	80	30
SU 9055	20	25
	40	7
	60	13
	80	17
SU 10603	20	12
	40	9
	60	9
	80	9

[a] As well as the additions indicated, each incubation flask contained 200 μg progesterone as substrate, 3.5 μmoles of TPN, 28 μmoles of glucose-6-P, 1.5 Kornberg units of glucose-6-P dehydrogenase, 250 μmoles of Tris-maleate buffer, pH 7.4, 5 μmoles of $MgCl_2$, and 7.8 mg of enzyme protein preparation. Total volume was 5 ml. Incubation took place at 37° for 60 minutes.

17α-OH-progesterone into 11-deoxycortisol is not affected by SU 9055 and SU 10603; however, C-21 hydroxylation of progesterone is inhibited by SU 10603. Effect of SU 9055 is not very marked; SU 4885 seems to exert some inhibitory effect at lower concentrations on C-21 hydroxylation of both progesterone and 17α-OH-progesterone.

DISCUSSION

In agreement with earlier studies (Ryan and Engel, 1957; Hayano and Dorfman, 1952; Plager and Samuels,

TABLE IV
EFFECT OF ADDED SU 4885, SU 9055, AND SU 10603 ON CONVERSION OF 17α-HYDROXYPROGESTERONE TO 11-DEOXYCORTISOL[a]

Addition	Amount Added (μg)	C-21 Hydroxylation of 17α-OH-progesterone (%)
None		36
SU 4885	20	27
	40	29
	60	30
	80	46
SU 9055	20	33
	40	40
	60	35
	80	35
	200	38
SU 10603	20	38
	40	36
	60	39
	80	36
	200	51

[a] As well as the additions indicated, each incubation flask contained 200 μg of 17α-hydroxyprogesterone as substrate, 3.5 μmoles of TPN, 28 μmoles of glucose-6-P, 1.5 Kornberg units of glucose-6-P dehydrogenase, 250 μmoles of Tris-maleate buffer, pH 7.4, 5 μmoles of $MgCl_2$, and 8.2 mg of enzyme protein preparation. Total volume was 5 ml. Incubation took place at 37° for 60 minutes.

1953), steroid C-21-hydroxylase activity was located in the microsomal fraction (105,000 × g sediment) of the adrenal cortex, and required TPNH as a cofactor. Progesterone was converted to 11-deoxycorticosterone, and 11-deoxycortisol could not be detected among the products, indicating that 17α-hydroxylation is not a prerequisite for C-21 hydroxylation of progesterone. Based on experimental evidence from Neher and Wettstein (1960) and Ortel and Eik-Nes (1961), Berliner *et al.* (1962) concluded that both pregnenolone and 17α-hydroxypregnenolone may be considered to be normal products of the adrenal cortex.

Recently, several workers (Weliky and Engel, 1962; Berliner *et al.*, 1962; Lipsett and Hökfelt, 1961) have suggested a key role for pregnenolone and 17α-hydroxypregnenolone in the biosynthesis of cortisol. Weliky and Engel (1962) have proposed that the major route to cortisol biosynthesis utilizes 17α-hydroxypregnenolone as substrate. A strong product inhibition has been observed in the enzymatic transformation of 5-pregnen-3,20-dione to progesterone (H. J. Ringold, E. Forchielli, and H. Kruskemper, paper in preparation). Since in the two-step transformation of pregnenolone to progesterone the isomerase step is faster, the overall rate will be controlled by the latter step. Also, since between Δ⁵-3β-ol and Δ⁵-3-keto the eqᵐ is in favor of Δ⁵-3β-ol, the observation lends support to the Weliky and Engel (1962) hypothesis that 17α-hydroxycorticoids come primarily from 17α-hydroxypregnenolone and not from 17α-hydroxyprogesterone.

Berliner *et al.* (1962) obtained 11-deoxycorticosterone and 11-deoxycortisol in good yield (60%) from incubations of bovine adrenals using 21-hydroxypregnenolone and 17α,21-dihydroxypregnenolone as substrates. It was suggested that if the adrenal is capable of forming these two compounds they would be excellent precursors for 11-deoxycorticosterone and 11-deoxycortisol, which in turn are readily converted to corticosterone and cortisol.

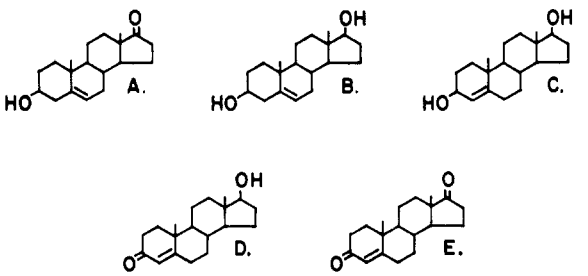

FIG. 7.—Additional C_{19} steroids studied to correlate molecular structure and inhibitory effect exerted by androgens on C-21 hydroxylation of pregnenolone and 17α-OH-pregnenolone.

Present studies demonstrate that the adrenal is capable of converting pregnenolone and 17α-hydroxypregnenolone into 21-hydroxypregnenolone and 17α,21-dihydroxypregnenolone, although the per cent conversion is much less as compared to the substrates with Δ⁴-3-keto group (Fig. 1). The C-21-hydroxylated products show a positive Petten-kofer reaction, indicating that Δ⁵-3β-ol grouping remains intact.

If 21-hydroxypregnenolone and 17α,21-dihydroxypregnenolone do play a major role in the biosynthesis of corticosterone and cortisol, the fact that dehydroepiandrosterone, testosterone, and 4-androstene-3,17-dione inhibit C-21 hydroxylation of pregnenolone and 17α-hydroxypregnenolone in contrast to the hydroxylation of progesterone and 17α-hydroxyprogesterone can be of considerable importance, particularly in certain clinical disorders like the Stein-Leventhal syndrome (Gallagher *et al.*, 1958; Perloff and Channick, 1959; Leventhal and Scommegna, 1963) and congenital adrenal hyperplasia (Sharma *et al.*, 1963).

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