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ACTH-STIMULATED RABBIT ADRENAL 17α-HYDROXYLASE. KINETIC PROPERTIES AND A COMPARISON WITH THOSE OF 3β-HYDROXYSTEROID DEHYDROGENASE

H. RICHARD FEVOLD*, PEGGY L. WILSON and SUSAN M. SLANINA
Department of Chemistry, University of Montana, Missoula, Montana 59812, U.S.A.

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SUMMARY

Initial rate values were determined for the 17α-hydroxylation of [4-^{14}C]-pregnenolone by adrenal microsomes prepared from control and ACTH-stimulated rabbits. Apparent K_M values were calculated to be 2.7 and 5.6 × 10^{-7} M in two separate experiments, while the corresponding apparent V_{max} values were 0.27 and 2.0 nmol/min per mg protein. The maximum measured rate in other experiments was 4.6 nmol/min per mg. Under the assay conditions employed no significant amount of ^{14}C-labeled product other than 17α-hydroxypregnenolone was formed. Control tissue microsomes had very low 17α-hydroxylase activity of questionable significance and equivalent to less than 5 percent of the activity in the ACTH-stimulated tissue. Apparent K_M and V_{max} values for 3β-hydroxysteroid dehydrogenase activity in microsomes from ACTH-stimulated tissue were 6.48 × 10^{-6}M and 121 nmol/min per mg. These results suggest a correlation between the lower value of the apparent K_M of the 17α-hydroxylase for pregnenolone substrate compared with that of the 3β-ol-dehydrogenase for the same substrate and the preferential formation of 17α-hydroxycorticosteroids in ACTH-stimulated rabbit adrenal tissue.

Although ACTH stimulation increased 17α-hydroxylase activity, in a typical experiment the amount of microsome cytochrome P-450 decreased from 1.08 to 0.91 nmol/mg protein and from 4.40 to 2.96 nmol/g tissue, while the amount per adrenal remained constant. The Δ_{max} values (A_{390}–A_{420}) from type I substrate binding difference spectra were equal with either progesterone or 17α-hydroxyprogesterone, and the values were not additive. This result was the same with microsomes from either control or ACTH-stimulated tissue. We were unable, therefore, to demonstrate by this technique the presence of a separate cytochrome for 17α-hydroxylation.

INTRODUCTION

17α-Hydroxylation in the biosynthesis of glucocorticosteroids is the committed step for the biosynthesis of cortisol. In species which produce and secrete primarily corticosterone, this reaction is quantitatively unimportant or nonexistant in adrenal tissue, while in species which secrete primarily cortisol it is a predominant reaction. The substrate for this reaction in cortisol secreting species is either progesterone [1] or pregnenolone [2–6]. In the former case, the predominance of cortisol over corticosterone secretion must result from more 17α-hydroxylation than 21-hydroxylation of progesterone. The result would be the conversion of more progesterone to 17α-hydroxyprogesterone than to 11-deoxycorticosterone. With pregnenolone as substrate, the competition must favor 17α-hydroxylation over 3β-ol-dehydrogenation and Δ^5-isomerization to form 17α-hydroxypregnenolone and progesterone, respectively. It has been shown previously that chronic stimulation of rabbit adrenal tissue with ACTH stimulates cortisol in preference to corticosterone formation [7] and that the 17α-hydroxylase utilizes pregnenolone in preference to progesterone as substrate [8–11]. The present experiments were designed to compare the apparent K_M and V_{max} values of the 17α-hydroxylase and 3β-hydroxysteroid dehydrogenase-Δ^5-isomerase in ACTH-stimulated rabbit adrenal tissue as a step in elucidating the reason for the preferential 17α-hydroxylation of pregnenolone, at low substrate concentrations [11]. Additionally, data are presented regarding the relationship of the ACTH-stimulated 17α-hydroxylase activity to type I substrate binding difference spectra and the contents of cytochrome P-450 in microsomes from control and ACTH-stimulated adrenals.

EXPERIMENTAL

Adult male New Zealand White rabbits were injected twice daily for three days with either 25 IU ACTH in 0.2 ml 5% beeswax in peanut oil, containing 0.5% phenol preservative, or with an equal volume of the vehicle alone. Adrenal microsomal fractions were prepared from the pooled adrenal glands of the ACTH-stimulated or the control (vehicle injected) animals essentially by the method of Estabrook, Cooper and Rosenthal[12] with the modifications described earlier [13]. Briefly, the tissue was minced and homogenized in pH 6.8, 0.1 M sodium phosphate buffer, containing 0.25 M sucrose and 0.001 M dithio-

* To whom correspondence should be sent.

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threitol [14]. The resulting 10% (w/v) homogenate was centrifuged at 9,000 *g* for 20 min. The supernatant was removed and centrifuged for 10 min at 12,000 *g*. The resulting supernatant was removed and centrifuged at 17,500 *g* for 30 min. The microsomal fraction employed in these studies was sedimented from the 17,500 *g* supernatant at 105,000 *g* for 60 min. The pellet was resuspended in approximately one third the original volume of 0.1 M sodium phosphate buffer pH 6.8 containing 0.154 M KCl and 0.001 M dithiothreitol and recentrifuged at 105,000 *g* for 60 min. The KCl wash step was repeated a second time. The final microsomal pellet was resuspended in Krebs–Ringer phosphate buffer, pH 7.0, in which $MgCl_2$ had been substituted for $MgSO_4$. Two millilitres of buffer were used per adrenal equivalent. This suspension was used as the enzyme source in the assays described.

All assays of enzyme activity were carried out in 25 ml Erlenmeyer flasks containing a total volume of 2.0 ml and incubated in a shaking water bath at 37.5 ± 0.25°C. Reactions were started by adding 1.0 ml of prewarmed microsomal suspension to 1.0 ml of prewarmed modified Krebs–Ringer buffer containing the substrate for the enzyme, cofactor(s) and other additives required for the specific assay being performed. Reactions were stopped by the rapid addition of 5 ml of cold CH_2Cl_2 and thorough mixing. Reaction flasks containing the 2 ml aqueous incubation media and the 5 ml dichloromethane were stored at −20°C until analyzed for product formation. Incubation durations are indicated in the results.

Conditions for specific assays

1. *Pregnenolone 17α-hydroxylase activity.* Incubations were performed with [4-^{14}C]-pregnenolone (Amersham) substrate together with final concentrations of 0.5 mM NADPH, cyanoketone equivalent to 0.1 mM, 2.0 mg glucose-6-phosphate, and 2 U glucose-6-phosphate dehydrogenase (Sigma type XV). The substrate specific activity was 51 μCi/μmol. Its purity was checked by thin-layer chromatography prior to use and it was purified by paper chromatography if necessary. The [4-^{14}C]-pregnenolone was added to each incubation flask in 200 μl of a solution in ethanol:propylene glycol (1:1, v/v) prior to the other incubation components. The cyanoketone inhibitor was added second in 50 μl of a 4.0 mM solution in a similar solvent mixture. The ethanol component of these solutions was evaporated under a stream of nitrogen at 45°C prior to the addition of the other incubation components. No evidence of dissolution of either the substrate or the cyanoketone was observed either at the time of evaporation of the ethanol or at the time of addition of the aqueous incubation components. Both NADPH and a NADPH generating system were included to assure that this cofactor was not limiting at anytime during the assays. The gas phase was air.

2. *Pregnenolone 3β-hydroxysteroid dehydrogenase-Δ^5-isomerase activity.* Incubations were performed with [4-^{14}C]-pregnenolone substrate together with a final concentration of 0.5 mM NAD. The incubation flasks were gassed for 2 min with carbon monoxide and stoppered with rubber serum stoppers. The microsomal suspension, which had also been pregassed with CO, was added to the flasks by injection through the serum stopper.

Extraction and analysis

The frozen incubation media were thawed, diluted with 3 ml water and extracted twice with double volumes (10 ml) of cold spectrograde CH_2Cl_2. The two extracts were combined and the solvent evaporated. The residues were chromatographed on paper [15] in a heptane/formamide system, developed for 1 h after the heptane had reached the end of the paper, followed by development in heptane:benzene (1:1)/formamide (3β-hydroxysteroid dehydrogenase-Δ^5-isomerase assay) or benzene/formamide (pregnenolone 17α-hydroxylase assays). Subsequent isolation and quantification of specific reaction products were performed as follows.

1. *Pregnenolone-17α-hydroxylase assay.* The 17α-hydroxypregnenolone areas of the original paper chromatograms were eluted with 15 ml EtOH. After evaporation of the solvent, one-half of each residue was chromatographed on Quanta LQDF-precoated t.l.c. plates in a cyclohexane:ethylacetate, 85:115, solvent system. The plates were developed for two successive 1 h periods, allowing them to dry between developments. The areas corresponding to 17α-hydroxypregnenolone were scraped into vials and quantified by liquid scintillation spectrometry using Aquasol (New England Nuclear) as the counting fluid.

To evaluate the effectiveness of the inhibition of the 3β-dl-dehydrogenase activity by the cyanoketone, the progesterone-pregnenolone areas of the paper chromatograms were eluted with 15 ml EtOH. After evaporation of the solvent, each residue was acetylated with a 4:1 mixture of pyridine:acetic anhydride for 12 h. 0.5 ml of ethanol was added to stop the reaction and the resulting mixture was evaporated. The products were chromatographed on paper in the heptane/formamide system. The separate areas corresponding to progesterone and pregnenolone-3β-acetate were eluted and aliquots taken for quantification by liquid scintillation spectrometry as described above.

2. *3β-Hydroxysteroid dehydrogenase-Δ^5-isomerase assay.* The progesterone-pregnenolone areas of the original paper chromatogram were eluted, acetylated, rechromatographed and the products quantified as described for the previous assay.

In later experiments the paper chromatographic and acetylation steps were avoided by chromatographing the incubation media extracts on 17.0 × 0.4 cm Lipidex (Packard Instrument Company) columns in heptane:dichloromethane (95:5, v/v). The progesterone fraction collected was the volume eluting between 5–15 ml, with the peak concentration eluting

at 10 ml. The pregnenolone fraction collected was the volume eluting between 23–44 ml, with the peak concentration eluting at approx. 33 ml. These columns are remarkably stable and the elution patterns very reproducible. Recoveries from the columns approached 100% [16].

Micromolar conversion rates were calculated from the percent of the substrate converted and from the specific activity of the added substrate. Percent recoveries from the aqueous incubation media were calculated by counting aliquots of the dichloromethane extract.

Verification of the identity of the products quantified was obtained by addition of authentic nonlabelled material and crystallization to constant specific activity, and by gas-liquid chromatographic analysis of the methoxime-trimethylsilyl ether derivatives [17]. Protein determinations were done by the Lowry modification [18] of the Folin–Ciocalteu reaction using crystalline bovine serum albumin as a standard.

Cytochrome P-450 content was determined by the procedure of Omura and Sato[19] and hydroxylase substrate binding difference spectra by the method of Narishimhulu, Cooper and Rosenthal[20], after the addition of microliter volumes of steroid solution in propylene glycol to the sample cuvette and an equal volume of propylene glycol to the reference cuvette.

RESULTS

Initial rates of 17α-hydroxy [4-^{14}C]-pregnenolone formation at representative [4-^{14}C]-pregnenolone substrate concentrations are shown in Fig. 1. All lines were drawn from least square analyses. Our first experiments designed to measure the effect of substrate concentration used a concentration range which was too high ($2.5 \cdot 10^{-5}$–$3 \cdot 10^{-4}$ M) for incubation periods of 5, 10 and 20 min. Rates were linear for 10 min, but saturation kinetics were obtained at most, if not all, of the substrate levels. When substrate levels were reduced to $5 \cdot 10^{-7}$–$5 \cdot 10^{-6}$ M, the time periods were too long to assure initial rate measurements (Fig. 1 insert). Only when the lower substrate concentrations were used with incubation durations of 20, 40 and 60 s were linear initial rates obtained which increased with substrate concentration (Fig. 1). The rates shown in Fig. 1 and in the insert in this figure are not the same at equivalent substrate concentrations since these data are from different experiments with different microsome preparations. The degree of stimulation of the adrenal tissue and the resulting enzyme specific activity was variable from preparation to preparation.

Rate values calculated from the slopes of the lines like those in Fig. 1 are listed in Table 1. In this assay the percent of the pregnenolone substrate which was converted to 17α-hydroxypregnenolone in the 1 min time interval ranged from 2.0% at the highest substrate concentration (5 μM) to 7.0% at the lowest

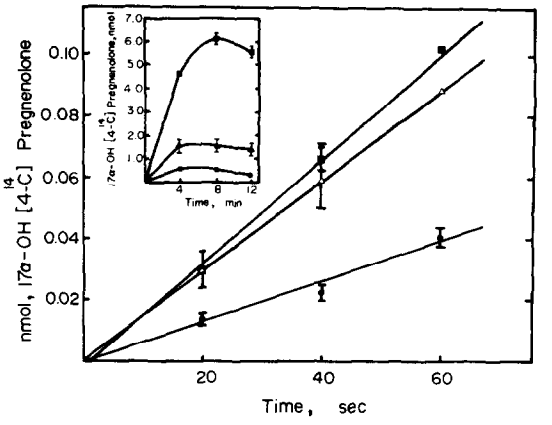

Fig. 1. Variation of the rate of 17α-hydroxylation with [4-^{14}]-pregnenolone substrate concentration. Representative results for 3 different substrate concentrations are shown: 0.50 μM —●—; 1.67 μM —△—; and 2.5 μM —□—. Assays were carried out using 0.40 mg of rabbit adrenal microsomal protein in 2.0 ml Krebs–Ringer phosphate buffer containing 0.5 mM NADPH, 0.1 mM cyanoketone, 2.0 mg glucose-6-phosphate and 2 U glucose-6-phosphate dehydrogenase. The incubation temperature was 37.5°C and the gas phase was air. Rabbits had been stimulated twice daily for 3 days with 25 U porcine ACTH prior to sacrifice. 17α-Hydroxy [4-^{14}C]-pregnenolone was isolated chromatographically and quantified by liquid scintillation spectrometry. All points represent the average of duplicate incubations. Vertical bars represent the range of the duplicate values, where the range was greater than the width of the symbol representing the experimental point. Lines were fitted by the method of least squares.

The insert shows the effect of longer assay incubation periods on 17α-hydroxylase rate measurements. Representative results for three different [4-^{14}C]-pregnenolone substrate concentrations are shown: 0.50 μM —●—; 1.5 μM —▲—; and 5.0 μM —□—. Assays were carried out using 0.38 mg of rabbit adrenal microsomal protein. Other conditions as above.

(0.5 μM). Apparent V_{max} and K_M values with 95% confidence limits of 0.27 ± 0.02 nmol/min per mg and $2.7 \pm 0.8 \times 10^{-7}$ M were determined from the double reciprocal plot of these data (Fig. 2, curve A). Each

Table 1. Rate of pregnenolone 17α-hydroxylation

Pregnenolone concn μM	v, nmol/min per mg protein
0.50	0.100
0.67	0.176
1.00	0.215
1.25	0.219
1.67	0.222
2.50	0.258

[4-^{14}C]-Pregnenolone was incubated with 0.40 mg of microsomal protein prepared from adrenal tissue of rabbits which had been stimulated twice daily for 3 days with 25 U porcine ACTH prior to sacrifice. Incubation periods were for 0, 20, 40 and 60 s at 37.5°C in a total volume of 2.0 ml Krebs–Ringer phosphate buffer containing 0.5 mM NADPH, 0.1 mM cyanoketone, 2.0 mg glucose-6-phosphate and 2 U glucose-6-phosphate dehydrogenase. The gas phase was air. 17α-Hydroxy [4-^{14}C]-pregnenolone was isolated chromatographically and quantified by liquid scintillation spectrometry. See text for details.

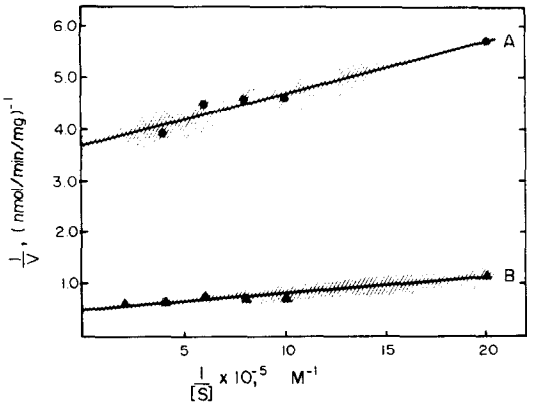

Fig. 2. Lineweaver–Burk double-reciprocal plot of the kinetic data from two 17α-hydroxylase assays. The hatched areas represent the 95% confidence limits for the lines, which were fitted by the method of least squares. Enzyme assay incubations were of 0, 20, 40 and 60 s duration. Other assay conditions as in Fig. 1 (Curve A) and in the text (Curve B).

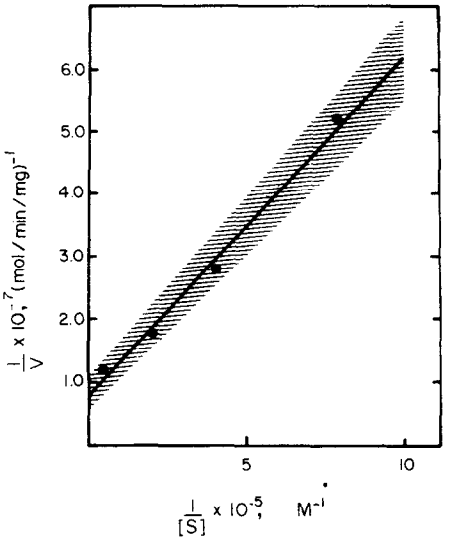

Fig. 3. Lineweaver–Burk double-reciprocal plot of the kinetic data from a 3β-hydroxysteroid dehydrogenase-Δ^5-isomerase assay. Assays were carried out using 0.23 mg of rabbit adrenal microsomal protein in 2.0 ml Krebs–Ringer phosphate buffer containing 0.5 mM NAD. The incubation times were 0, 20, 40 and 60 s, the temperature was 37.5°C, and the gas phase was carbon monoxide. Rabbits had been stimulated twice daily for 3 days with 25 U porcine ACTH prior to sacrifice. All points are the average of the results from duplicate assay flasks. The hatched area represents the 95% confidence limits for the line, which was fitted by the method of least squares.

incubation flask contained a total of 0.40 mg protein (0.20 mg/ml). The cytochrome P-450 content of this microsomal preparation was 0.98 nmol/mg. A double reciprocal plot of the data obtained in a duplicate of the above experiment, performed with a different microsomal preparation and at a different time (Fig. 2, curve B), gave apparent V_{max} and K_M values of 2.0 ± 0.3 nmol/min per mg and 5.6 ± 1.8 × 10^{-7} M. In this second assay, which had a more active enzyme preparation, each incubation flask contained a total of 0.31 mg of protein and the percent of the pregnenolone substrate converted to 17α-hydroxypregnenolone in the 1 min time interval ranged from 5.2 to 27.8% at the 5 and 0.5 μM substrate concentrations, repectively. The results of these two experiments show that the 17α-hydroxylase K_M value is in the range of 2–7 × 10^{-7} M, the variability perhaps being inherent in the procedure. The agreement of K_M values within a factor of 2–4 using enzyme preparations differing markedly in apparent V_{max} values lends credence to the accuracy of the former values. The apparent V_{max} depends, of course, on the degree of stimulation effected by the ACTH. The highest velocity observed for the 17α-hydroxylation of pregnenolone in repeated experiments has been 4.6 nmol/min per mg.

Figure 3 shows the double reciprocal plot of the initial rate data for the 3β-hydroxysteroid dehydrogenase assay. The apparent K_M and V_{max} values determined from the plot were 6.48 ± 1.75 μM and 121 ± 31 nmol/min per mg, respectively.

To be assured that the product being measured in the 17α-hydroxylase assays was 17α-hydroxypregnenolone, three other criteria were used in addition to the chromatographic identity obtained in the quantification procedure. Table 2 shows the crystallization of the ^{14}C-labelled product with added 17α-hydroxypregnenolone, and Fig. 4 shows the gas-liquid chromatographic identity of the methoxime-trimethylsilyl ether derivative. The methoxime-trimethylsilyl ether derivative of the acetylated derivative of the isolated product also had the same retention time on gas–liquid chromatographic analysis as the similarly derivatized authentic 17α-hydroxypregnenolone-3β-acetate. A comparison of peak areas between this sample and a known quantity of standard 17α-hydroxypregnenolone indicated the presence of approximately 3.6 ng in the sample. This compared with a value of 4.7 ng calculated from the percent conversion of the original substrate, demonstrating that there was little or no endogenous pregnenolone in our microsome preparation to dilute the exogenous substrate. This finding validates the method used for calculating the rate values in which it was assumed that the specific

Table 2. Crystallization of isolated 17α-hydroxy [4-^{14}C]-pregnenolone to constant specific activity

Crystallization number	DPM/MG × 10^{-3} Crystals	Supernatant	Solvent
0	2.96	—	EtOH*
1	2.95	2.96	MeOH†: EtOAC‡
2	3.58	3.78	MeOH: Et_2O§
3	3.40	2.50	ETOH: Et_2O
4	3.45	3.66	ETOH:EtOAC

* Ethanol; † methanol; ‡ ethylacetate; § diethyl ether.

25 Mg of authentic 17α-hydroxypregnenolone were added to the isolated ^{14}C-labeled material and the resulting mixture was crystallized four successive times from the solvents indicated.

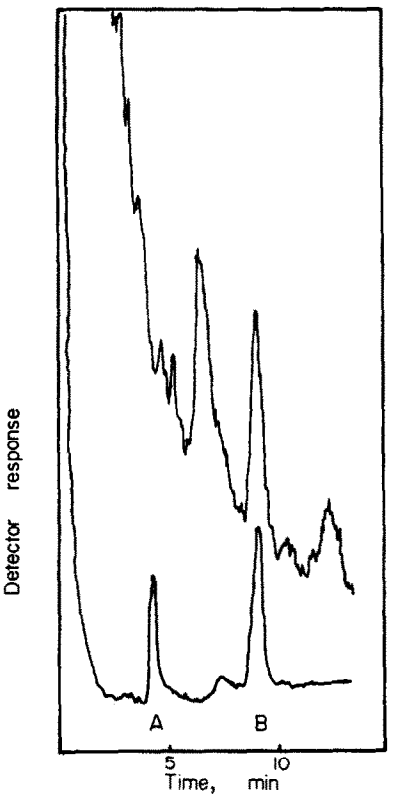

Fig. 4. Gas chromatographic identification of 17α-hydroxypregnenolone isolated from ACTH-stimulated rabbit adrenal microsome 17α-hydroxylase assays. The methoxime-trimethylsilylether derivative of the isolated material (upper curve) and a standard containing 0.8 μg each of androstenedione (Peak A, lower curve) and the methoxime-trimethylsilylether derivative of authentic 17α-hydroxypregnenolone (Peak B, lower curve) were chromatographed on a 4 ft × 0.5 cm glass column containing Gas Chrom Q solid support bearing 0.5% OV-1 liquid phase. The method of detection was flame ionization. Temperatures were 252, 212 and 230°C for the injector, column and detector, respectively. Attenuation was 1 × 2 and 1 × 8 for the upper and lower curves. Carrier gas was helium at a flow rate of approximately 40 ml per min.

Table 3. Effect of NADPH concentration on the rate of [4-^{14}C]-pregnenolone 17α-hydroxylation

	Pregnenolone conc. mM	NADPH conc. mM	Rate, nmol/min per mg protein
Exp. 1	0.1	0.5	4.42
		1.0	4.42
Exp. 2	0.015	0.5	3.64
	0.05	0.5	3.11
		1.0	3.67

[4-^{14}C]-Pregnenolone was incubated with 0.32 (Exp. I) or 0.38 (Exp. II) mg of adrenals microsomal protein prepared from rabbits which had been stimulated twice daily for 3 days with 25 U porcine ACTH prior to sacrifice. Other conditions as in Table 1.

activity of the product was the same as that of the added substrate. The data in Table 3 from several microsome incubations shows that the 0.5 mM concentration of NADPH routinely used in these experiments was sufficient to assure a maximal rate of 17α-hydroxylation.

Progesterone formation in the presence of the cyanoketone inhibitor was not detectable in the 17α-hydroxylase assays represented in Figs 1 and 2, curve A, and in Table 1. In the more active preparation (Fig. 2, curve B), a small amount of progesterone formation was observed. At the 1 min interval and 0.5 μm substrate concentration, for instance, the progesterone isolated amounted to 0.36% of the added substrate compared with 27.8% formation of 17α-hydroxypregnenolone. As shown in the tracing of the paper and thin-layer chromatographic isolation of the 17α-hydroxypregnenolone product (Figs 5 and 6), there was no evidence for the formation of significant amounts of any other product under these incubation conditions.

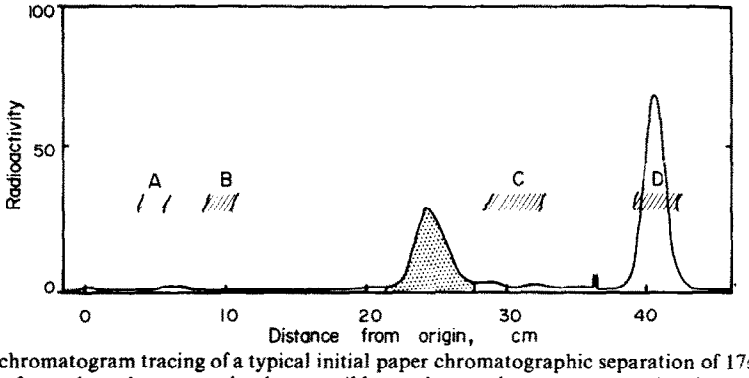

Fig. 5. Radiochromatogram tracing of a typical initial paper chromatographic separation of 17α-hydroxypregnenolone from the substrate and other possible products subsequent to the incubation of rabbit adrenal microsomes with [4-^{14}C]-pregnenolone in the 17α-hydroxylase assays. The stippled area was eluted for further purification and quantification. The hatched areas show the location on the chromatogram of 50 μg each of four non-radioactive internal standards added to the sample prior to chromatography: corticosterone, A; 11-dehydrocorticosterone, B; 17α-hydroxyprogesteone, C; and progesterone, D. The [4-^{14}C]-pregnenolone substrate and progesterone standard are not separated on this chromatogram. Samples were chromatographed on 2 cm strips of Whatman no. 1 filter paper and developed in a heptane:formamide system for 1 h after the mobile phase had reached the end of the strip, followed by development in a benzene:formamide system. The counting scale for the first 36 cm of the chromatogram was 10 K and for the last 10 cm, 30 K. See text and Fig. 1 for assay details.

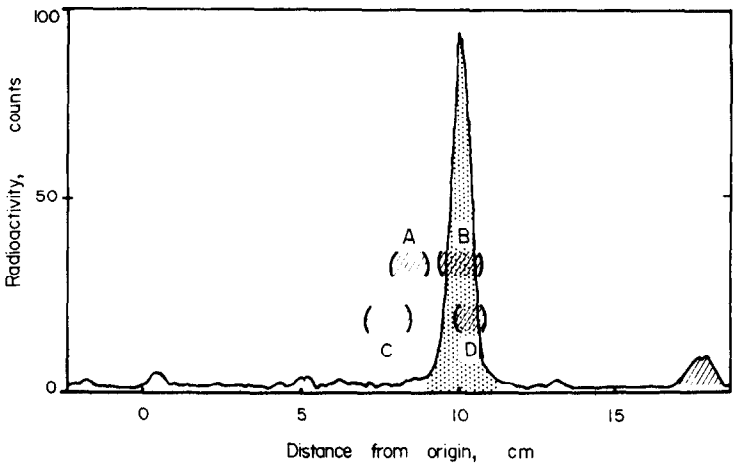

Fig. 6. Radiochromatogram tracing of a typical silica gel thin-layer chromatographic purification of 17α-hydroxy [4-^{14}C]-pregnenolone isolated from a 17α-hydroxylase assay with [4-^{14}C]-pregnenolone substrate and rabbit adrenal microsomes. The stippled area was scraped from the plate for final quantification by liquid scintillation spectrometry. The hatched areas under the curve indicate radioactive markers applied for the purpose of aligning the tracing and the chromatogram. The hatched areas marked A and B indicate the location of 21-hydroxypregnenolone and 17α-hydroxypregnenolone chromatographed on a lane adjacent to the sample and visualized with a sulfuric acid:ethanol (1:1) spray. The hatched areas marked C and D indicate the location of 11β-hydroxyprogesterone and 17α-hydroxyprogesterone which were added to the sample prior to chromatography. The chromatogram was developed for two successive 1 h periods in a cyclohexane:ethylacetate, 85:15, solvent. The counting scale was 3 K. See text and Fig. 1 for assay details.

Table 4 shows the values for 17α-hydroxylase activities in microsomes prepared from ACTH-stimulated and non-stimulated control rabbits. As has been reported for cortisol production using whole animals [7] or whole adrenal homogenates [8], the 17α-hydroxylase activity in the stimulated microsomes is much greater than in the control microsomes. The amount of 17α-hydroxy [4-^{14}C]-pregnenolone formed by the control microsomes in 40 sec was usually less than twice the zero-time values.

Table 4. The rate of formation of 17α-hydroxypregnenolone and the cytochrome P-450 content in rabbit adrenal microsomes as a function of ACTH-stimulation

	ACTH	Control
17α-hydroxypregnenolone, nmol/min per mg		
[4-^{14}C]-pregnenolone-0.5 μM	1.57	0.06
1.6 μM	1.35	0.06
5.0 μM	1.32	0.03
Cytochrome P-450		
nmol/mg protein	0.91	1.08
nmol/g tissue	2.96	4.40
nmol/adrenal	0.27	0.28

17α-Hydroxylase assays were performed with [4-^{14}C]-pregnenolone substrate as described in Table 1 and the text. Adrenal microsomes were prepared from control, non-stimulated rabbits and from rabbits which had been stimulated twice daily for 3 days with 25 U porcine ACTH. Control tissue incubations contained 0.20 mg of microsomal protein and ACTH-stimulated tissue incubations contained 0.11 mg of microsomal protein. Cytochrome P-450 determinations were made as described in the text.

As has been frequently but not invariably found, the cytochrome P-450 content of the control microsomal preparation was slightly higher than that of the ACTH-stimulated microsomes (Table 4), suggesting no preferential increase of total cytochrome P-450(s) in rabbit adrenal microsomes after ACTH stimulation. This is emphasized by the greater than 30% decrease of the cytochrome per gram of adrenal tissue following ACTH stimulation, and the virtually constant amount per adrenal gland. Average paired adrenal weights rose from 126.4 to 179.3 mg after ACTH stimulation in this experiment. The possible effect of the cyanoketone on the rate of 17α-hydroxylation was also determined. In incubations with microsomes from ACTH-stimulated animals, the omission of the cyanoketone 3β-hydroxysteroid dehydrogenase inhibitor resulted in a rate increase from 1.32 to 1.55 nmol/min per mg, while in control microsome incubations the rate increased from 0.03 to 0.05 nmol/min per mg. While this is not a marked increase in overall rate due to the absence of the cyanoketone, it was a consistent observation, suggesting a small inhibitory effect of cyanoketone on 17α-hydroxylase activity as well as on the 3β-hydroxysteroid dehydrogenase-Δ^5-isomerase activity. Such an inhibition could have affected the apparent K_M and V_{max} values determined for the 17α-hydroxylase. However, since the cyanoketone has a basic C_{19} steroid carbon skeleton and already has a substituent in the 17α-position, there is no apparent reason to suspect that it would significantly bind to and inhibit the 17α-hydroxylase system.

Substrate binding difference spectra in which pro-

Table 5. Adrenal microsome Δ_{max}, $A_{390-420}$, from substrate binding difference spectra

	Δ_{max}, $A_{390-420}$/nmol Cyt. P-450	
	Control	ACTH
Expt I		
Progesterone	0.099	0.087
17α-Hydroxyprogesterone	0.094	0.082
Expt II		
Progesterone	0.117	0.092
17α-Hydroxyprogesterone	0.122	0.091
Progesterone + 17α-hydroxy-progesterone	0.120	0.094

ACTH-stimulated tissue was obtained from animals injected twice daily with 25 U ACTH in 0.2 ml 5% beeswax in peanut oil. Control animals were injected with the vehicle only. The microsomal fraction was prepared as described in the text. Steroid substrates were added to the sample cuvettes in microliter volumes of propylene glycol; equal volumes of propylene glycol were added to the sample cuvettes. Protein concentrations were 0.46 and 0.44 mg/ml for the control and ACTH-stimulated microsomal preparations in Experiment I and 0.37 and 0.46 mg/ml, respectively, for Experiment II.

gesterone and 17α-hydroxyprogesterone were used as substrates were determined with microsomal preparations from both ACTH–stimulated and non-stimulated rabbit adrenal glands. It was assumed that the difference spectra with progesterone substrate represented the sum of the binding due to 17α- and 21-hydroxylase activities, since progesterone is a substrate for both enzymes, while 17α-hydroxyprogesterone should produce a difference spectrum representative primarily of 21-hydroxylase activity. Results from two such experiments are shown in Table 5. In experiment 1 the cytochrome P-450 content of the control and ACTH-stimulated microsomal preparations were 1.44 and 1.04 nmoles/mg protein, respectively [13]. In experiment 2 these values were 0.93 and 1.14. It is apparent from the Δ_{max} values that both control and ACTH-stimulated microsomes bind progesterone and 17α-hydroxyprogesterone to the same extent and that the binding of these two substrates is not additive.

Table 6. Effect of carbon monoxide on the rate of 17α-hydroxylation of [4-^{14}C]-pregnenolone by microsomes from ACTH-stimulated rabbits

Gas phase	$CO:O_2$ ratio	Rate nmol/min per mg protein	Percent inhibition
$95\%N_2:5\%O_2$	0	3.78	0
$90\%N_2:5\%CO:5\%O_2$	1	4.09	(8%)*
$85\%N_2:10\%CO:5\%O_2$	2	1.52	58
$45\%N_2:50\%CO:5\%O_2$	10	1.44	62

* Apparent stimulation.

Microsome preparations and assay conditions as described in Table 1 and in the text. All assay flasks were gassed with the appropriate mixture for 2 min and a flow rate of between 440 and 575 cm^3/min, and the flasks stoppered prior to the addition of the microsomal suspension by injection through the stopper. Microsomal protein concentration was 0.46 mg per assay flask. The gas phase was allowed to equilibrate with the microsomes for 5 min prior to measurement of the hydroxylation rate from 5 to 10 min after the microsome addition. [4-^{14}C]-Pregnenolone substrate concentration was 100 μM.

The effect of carbon monoxide on the 17α-hydroxylation of pregnenolone at a constant P_{O_2} is shown in Table 6. Ratios of the CO/O_2 greater than one were inhibitory, although perhaps not to the degree that was expected from literature values for the inhibition of microsomal hydroxylases by CO [12, 21–23].

DISCUSSION

The apparent K_M values between 2.7 and 5.5 × 10^{-7} M reported here for the ACTH-stimulated rabbit adrenal 17α-hydroxylase with pregnenolone substrate are somewhat lower than those reported for the same enzyme in other tissues with either progesterone or pregnenolone substrates [24–26]. Kremers[25] beautifully demonstrated a strong product inhibition of 17α-hydroxylase by 17α-hydroxypregnenolone. A similar inhibition in rabbit adrenal tissue could have explained our difficulties in obtaining linear rates when using enzyme assay durations much longer than one minute (Fig. 1, insert).

The 6.5 × 10^{-6} K_M value determined in the present experiments for pregnenolone substrate and 3β-Hydroxysteroid dehydrogenase-Δ^5-isomerase enzyme activity agree well with the value reported by Neville, Orr and Engel[27] for bovine adrenal microsomal enzyme with either pregnenolone or dehydroepiandrosterone substrate. Other authors have reported K_M values ranging from 1.5 × 10^{-3} M in rat adrenal microsomes with pregnenolone substrate [28] to 2.7 × 10^{-7} M with this same substrate in porcine corpora luteal tissue [29]. In measuring K_M values it is more likely to err on the high side in the determination due to underestimates of initial rate values. This can result from many factors such as non-linearity of reaction rates due to excessively long assay times, unrecognized product inhibition of the reaction being measured, competition by more than one enzyme for the same substrate, and suboptimal concentrations of cofactors and cosubstrates, to name only a few.

Our original hypothesis was that the K_M value of 17α-hydroxylase for pregnenolone in the ACTH-stimulated rabbit adrenal microsomes might be lower than the K_M value of 3β-Hydroxysteroid dehydrogenase for the same substrate. Simplistically, if we assume that K_M values are an estimate of enzyme-substrate dissociation constants, a lower K_M of the 17α-hydroxylase could explain the preferential formation of cortisol over corticosterone [7, 11]. The results, at first glance, seem to substantiate the hypothesis. The 17α-hydroxylase K_M for pregnenolone is lower than that of the 3β-Hydroxysteroid dehydrogenase for this same substrate. However, it is also obvious from a comparison of Fig. 2 with Fig. 4 (note different

ordinate units) that the initial rate of 17α-hydroxylation was never greater than 3β-hydroxydehydrogenation at any of the substrate concentrations used. In fact, it can be calculated from these data that there is no pregnenolone substrate concentration at which the initial rate of 17α-hydroxylation would exceed that of 3β-dl-dehydrogenation. This is true for both sets of 17α-hydroxylase apparent K_M and V_{max} values reported. The situation would differ if greater stimulation of the tissue resulted in a higher specific activity of the 17α-hydroxylase and, therefore, a higher apparent V_{max} value. If this value approached that of the 3β-dl-dehydrogenase, then the comparative slope of the two lines in Lineweaver–Burk plots would be under the primary influence of the K_M values. Thus, if the maximally stimulated adrenal 17α-hydroxylase apparent V_{max} value was somewhat less than that of the 3β-dl-dehydrogenase, the intercept would be higher on the ordinate. However, if the lower K_M value of the 17α-hydroxylase was low enough to result in the slope, K_M/V_{max}, of the 17α-hydroxylase plot being less than that of the 3β-dl-dehydrogenase plot, the lines would cross. At values of $1/[S]$ higher (lower substrate concentrations) than at this cross-over point, the rate of 17α-hydroxylation would exceed 3β-dl-dehydrogenation. This would also be true, of course, at *all* substrate concentrations if the apparent V_{max} of the 17α-hydroxylase exceeded that of the 3β-dl-dehydrogenase. If the 17α-hydroxylase in the tissue used in these experiments was sub-maximally stimulated by ACTH, the apparent V_{max} could have been lower than it would have been after maximum stimulation—conditions which result in greater cortisol than corticosterone production in intact animals [7] and in whole adrenal tissue homogenates [11]. Previous experiments have shown, however, that a three day stimulation period gives rise to maximum cortisol production [11], indicating that the tissue used in these experiments should have been close to maximally stimulated. It would seem possible that some reason other than the kinetic properties of the competing enzymes for pregnenolone could account for the preferential 17α-hydroxylation and resulting cortisol formation in ACTH-stimulated rabbit adrenal tissue. Perhaps inhibition of pregnenolone 3β-hydroxysteroid dehydrogenase activity by cAMP [30–33] may play a role. The necessity in this latter case for a different effect of cAMP on the 3β-ol-dehydrogenation of 17α-hydroxypregnenolone is obvious, however, since this latter step would be required for cortisol synthesis [9].

The data regarding cytochrome P-450 measurements (Table 4) and substrate binding difference spectra (Table 5) show that there is generally no measurable increase in cytochrome P-450 on any basis, i.e. on a mg of protein, on a wet weight of tissue, or on the total gland content. This has been a rather consistent finding in numerous replicate experiments. Coupled with the fact (Table 5) that in both control and ACTH-stimulated microsomal preparations there was no evidence for additional binding of a 17α-hydroxylase substrate (progesterone) over that observed with a 21-hydroxylase substrate (17α-hydroxyprogesterone), the stimulation of 17α-hydroxylation does not appear to require a measurable increase in cytochrome P-450 content. This does not imply that cytochrome P-450 is not involved in 17α-hydroxylation. Indeed, since carbon monoxide has an inhibitory effect (Table 6), the data suggest the necessity of cytochrome P-450 but that it is not the limiting component in the enzyme system catalyzing this reaction.

Other reports of tropic hormone stimulation of 17α-hydroxylation have either not included measurements of cytochrome levels [34, 35] or have found increases [36, 37]. Menard and Purvis[36] reported increased cytochrome P-450 and 17α-hydroxylase activity in chick testis microsomes in response to luteinizing hormone stimulation. Purvis and coworkers [37] also demonstrated that cytochrome P-450 levels and 17α-hydroxylase activity decay in a parallel fashion in rat testicular tissue microsomes following hypophysectomy and that enzyme and cytochrome respond similarly to human chorionic gonadotropin stimulation. Whether our inability to measure a consistent increase in microsomal cytochrome P-450 after ACTH-treatment in parallel with the increase in 17α-hydroxylase activity is due to a basic difference in tropic hormone action or to some other difference in the system used when compared with those of Menard and Purvis[36] and Purvis *et al.*[37] is not known.

The apparent formation of a single product from pregnenolone under the 17α-hydroxylase assay conditions employed, in which the 3β-hydroxysteroid dehydrogenase activity was inhibited by cyanoketone (Figs. 5 and 6), is significant because it indicates that the rabbit adrenal 21-hydroxylase is unable to utilize pregnenolone as a substrate. This result shows that 21-hydroxypregnenolone is probably not an intermediate in corticosterone synthesis from pregnenolone in rabbit adrenal tissue as has been suggested for human [38] and sheep [39] tissues. It also demonstrates that there is no significant competition for substrate, which would invalidate the kinetic constants determined for the pregnenolone 17α-hydroxylase [40].

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