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# STUDIES OF THE HUMAN FETAL ADRENAL GLAND—PROPERTIES OF 17α-HYDROXYLASE AND $C_{17}$-$C_{20}$ LYASE IN THE BIOSYNTHESIS OF DEHYDROEPIANDROSTERONE FROM PREGNENOLONE

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SUMMARY

The microsomal fraction of the human fetal adrenal gland showed NADPH dependent enzyme activities of 17α-hydroxylase and $C_{17}$-$C_{20}$lyase. The apparent Michaelis constants ($K_M$) of 17α-hydroxylase and $C_{17}$-$C_{20}$lyase for NADPH were $6 \times 10^{-8}$ M and $3 \times 10^{-7}$ M, respectively. The apparent $K_M$ value of 17α-hydroxylase for pregnenolone was $1.3 \times 10^{-8}$ M and that of $C_{17}$-$C_{20}$lyase for 17-hydroxypregnenolone was $1.2 \times 10^{-7}$ M. These enzymes were inhibited by most of the steroids produced in the feto-placental unit. 5-Pregnene-3β,20β-diol (apparent $K_i = 1.5 \times 10^{-8}$ M), pregnenolone-sulphate ($2.4 \times 10^{-8}$ M), 5-pregnene-3β,20α-diol ($3.1 \times 10^{-8}$ M), 17-hydroxypregnenolone ($6.1 \times 10^{-8}$ M) and progesterone ($8.1 \times 10^{-8}$ M) inhibited the activity of 17α-hydroxylase toward pregnenolone. The following steroids were the inhibitors of $C_{17}$-$C_{20}$lyase toward 17-hydroxypregnenolone; 5-pregnene-3β, 20β-diol (apparent $K_i = 0.6 \times 10^{-7}$ M), 5-pregnene-3β,20α-diol ($1.7 \times 10^{-7}$ M), progesterone ($3.4 \times 10^{-7}$ M), 17,20β-dihydroxy-4-pregnen-3-one ($5.6 \times 10^{-7}$ M), 17-hydroxyprogesterone ($7.6 \times 10^{-7}$ M), dehydroepiandrosterone ($2.3 \times 10^{-6}$ M), 5-androstene-3β,17β-diol ($3.2 \times 10^{-6}$ M), 16α-hydroxydehydroepiandrosterone ($3.2 \times 10^{-6}$ M), testosterone ($6.2 \times 10^{-6}$ M) and estradiol-17β ($9.2 \times 10^{-6}$ M). The type of inhibition of these steroids appeared to be competitive.

## INTRODUCTION

Recently, effects of naturally occurring steroids on the enzymic actions which are involved in the biosynthesis of steroid hormones have been reported by several investigators. The placental steroid 3-sulphatase is susceptible to competitive inhibition by endogenous steroids and this may provide a mechanism controlling placental estrogen synthesis from conjugated precursors [1]. Similar effects by endogenous steroids on the placental 3β-hydroxysteroid dehydrogenase were also reported [2]. Other investigators have reported that human testicular 3β-hydroxy-5-ene steroid dehydrogenase was inhibited by most of the steroids produced by the testis [3].

The human fetal adrenal gland is an important steroidogenic organ in pregnancy, producing androgens such as dehydroepiandrosterone (DHA) *in utero*. To further investigate the nature of enzymes involved in androgen biosynthesis in the human fetal adrenals, the present experiment was undertaken.

The following trivial names are used—progesterone: 4-pregnene-3,20-dione; 17-hydroxyprogesterone: 17-hydroxy-4-pregnene-3,20-dione; pregnenolone: 3β-hydroxy-5-pregnen-20-one; 17-hydroxypregnenolone: 3β,17-dihydroxy-5-pregnen-20-one; dehydroepiandrosterone: 3β-hydroxy-5-androsten-17-one; 16α-hydroxydehydroepiandrosterone: 3β,16α-dihydroxy-5-androsten-17-one.

## EXPERIMENTAL

### *Tissue preparations*

Human fetal adrenal tissue was obtained from a fetus aborted for socio-economic reasons at 22 weeks of gestation. The adrenal gland was decapsulated and homogenized with a loose fitting Teflon–glass homogenizer in an ice-cold 0.33 M sucrose solution buffered with 0.05 M Tris-HCl buffer (pH 7.4). After centrifugation of the homogenates at 800 *g* for 20 min, the supernatant fluid was centrifuged at 7,000 *g* for 20 min. The supernatant fluid obtained at 7,000 *g* was centrifuged at 10,000 *g* for 20 min, the precipitates containing the light mitochondrial fraction were discarded. The supernatant fluid thus obtained was centrifuged at 105,000 *g* for 60 min and the precipitate was resuspended in 0.33 M sucrose solution and used as the microsomal fraction [4]. This fraction was divided into aliquots of 100 mg equivalent wet tissue weight (1.87 mg protein) and they were kept at −80°C until incubations were started. The activities of 17α-hydroxylase and $C_{17}$-$C_{20}$lyase in this fraction were found to be stable under the present experimental conditions for at least 5 months.

### *Chemicals*

[4-$^{14}$C]-Pregnenolone (NEC-375, Lot No. 965-063, S.A. 56.6 mCi/mmol), [7-$^{3}$H]-pregnenolone (NET-039, Lot No. 635–131, S.A. 19.8 mCi/mmol) and

[7-$^3$H]-17-hydroxypregnenolone (NET-036, Lot No. 636-117, S.A. 12.0 mCi/mmol) were purchased from New England Nuclear Corp. (Boston, MA.) and purified by thin-layer chromatography in the solvent system benzene-methanol (9:1, v/v) prior to use. Non-radioactive steroids were obtained from commercial sources (Merck, Steraloids, Ikapharm and Sigma Chemical Co.). NADPH and NADH were purchased from Oriental Yeast Co. (Japan).

*Incubation*

The microsomal fraction of adrenal tissue was incubated with the labelled pregnenolone (0.5 nmol, $4 \times 10^5$ d.p.m.) or 17-hydroxypregnenolone (1 nmol, $4 \times 10^5$ d.p.m.) dissolved in a drop of propylene glycol and 0.2 $\mu$mol of NADPH dissolved in 0.05 M Tris–HCl buffer (pH 7.4). The final vol. of the incubation medium was adjusted to 1 ml. The incubation was carried out at 37°C for 6 min with constant shaking under a gas phase of 95% $O_2$ and 5% $CO_2$. The reaction was terminated by adding 5 ml of methylene chloride and shaking vigorously. The reaction mixture without the tissue preparation was incubated under the same condition as a control.

*Extraction, separation and identification*

Steroids were extracted twice with 5 ml of methylene chloride. To the combined extract, carrier steroids were added and then it was subjected to the separation procedure. The metabolites formed from pregnenolone were acetylated first and then separated by thin-layer chromatography on silica gel $GF_{254}$ (Merck, Germany) in the solvent system benzene–ethylacetate (95:5, v/v); thus complete separation of pregnenolone–acetate ($R_F = 0.54$), 17-hydroxypregnenolone–acetate ($R_F = 0.16$) and DHA–acetate ($R_F = 0.41$) was achieved. When 17-hydroxypregnenolone was used as a substrate, the extract was immediately subjected to thin-layer chromatography in the system benzene-methanol (9:1, v/v). In this system, 17-hydroxypregnenolone ($R_F = 0.38$) and DHA ($R_F = 0.53$) were distinctly separated. The carrier steroids on the thin-layer chromatogram were detected by exposure of the plate to iodine vapor. The radioactive spots on the plate were detected by autoradiography and scanning with an Aloka Radio Chromatogram Scanner (Model TLC-4, 04R069). The final identification of each metabolite was accomplished by recrystallization to constant specific activity with authentic steroids.

Acetylation of steroids was carried out overnight at room temperature in a mixture of pyridine–acetic acid anhydride (2:1, v/v).

Protein concentration of each tissue preparation was determined by the method of Lowry *et al.* [5].

Radioactivity was measured with a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3330) in toluene-PPO-POPOP. The counting efficiency was about 90% for $^{14}$C and 50% for $^3$H. The amount of metabolites formed was quantitated from its total radioactivity found in the corresponding spots on the thin-layer chromatogram. The recoveries of extractions and chromatographic procedures were 84.0 ± 1.6% (mean ± S.E.) for the metabolism of pregnenolone and 83.3 ± 1.0% for that of 17-hydroxypregnenolone; no correction was made for experimental losses.

*Determination of enzyme activities*

The 17α-hydroxylase activity was expressed as the sum of 17-hydroxypregnenolone and DHA produced from pregnenolone, while the $C_{17}$-$C_{20}$ lyase activity was expressed as the amount of DHA produced from 17-hydroxypregnenolone.

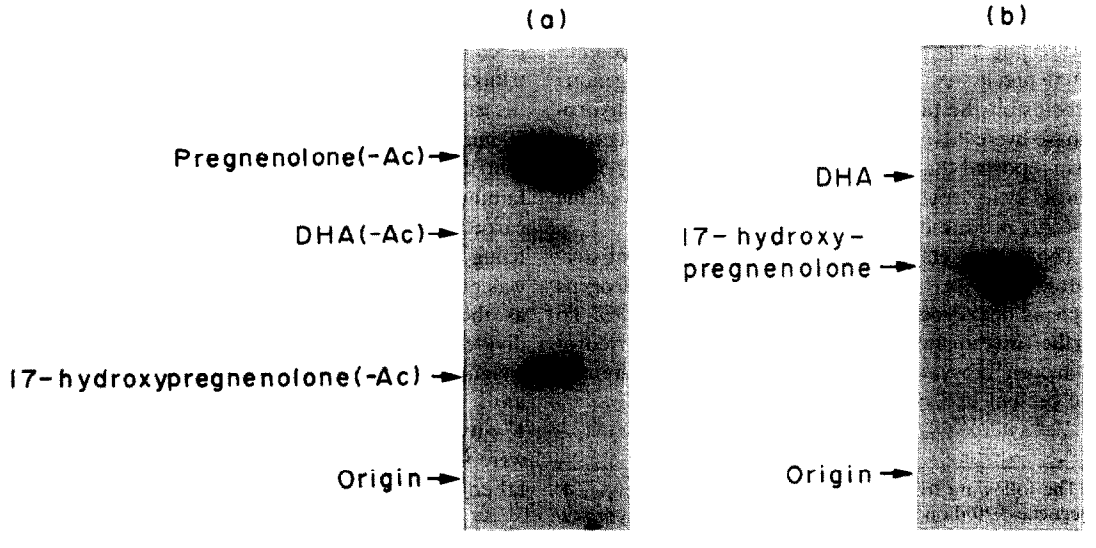


Fig. 1. Autoradiograms of the thin-layer chromatograms of the metabolites formed from pregnenolone (a) or 17-hydroxy-pregnenolone (b) by the microsomal fraction of the fetal adrenal tissue. The amount of protein used was 1.87 $\mu$g in a, and 7.48 $\mu$g in b.

Table 1. Crystallization data of the radioactive steroid metabolites with the authentic preparations

| Substrates | Metabolites | Specific activities (d.p.m./mg) Before crystallization | 1st crystal | 2nd crystal | 3rd crystal | 4th crystal | Mother liquor |
|---|---|---|---|---|---|---|---|
| Pregnenolone | 17-Hydroxypregnenolone* | 1173 | 1107 | 1043 | 1118 | 1080 | 1078 |
| | DHA* | 1174 | 1571 | 1489 | 1575 | — | 1657 |
| 17-Hydroxypregnenolone | DHA | 1150 | 1076 | 1031 | 953 | 948 | 927 |

* Crystallization was performed after acetylation.

Table 2. Intracellular distribution of the 17α-hydroxylase and $C_{17}$-$C_{20}$lyase activities in fetal adrenal gland: each subcellular fraction was incubated with pregnenolone or 17-hydroxypregnenolone under conditions described in the text

| Subcellular fraction | 17α-Hydroxylase* | $C_{17}$-$C_{20}$Lyase† |
|---|---|---|
| Cell-free (sup. at 800 *g*) | 4.92 | 3.06 |
| Mitochondria (800–7,000 *g*) | 1.93 | 1.76 |
| Microsome (10,000–105,000 *g*) | 17.14 | 11.59 |
| Cytosol (sup. at 105,000 *g*) | 0 | 0.21 |

* Activity is expressed as nmol of 17-hydroxypregnenolone and DHA produced from pregnenolone in 6 min per mg protein.

† Activity is expressed as nmol of DHA produced from 17-hydroxypregnenolone in 10 min per mg protein.

## RESULTS

### *Reaction products*

Autoradiograms of the thin-layer chromatograms of the metabolites formed from pregnenolone by the microsomal fraction revealed two major products; 17-hydroxypregnenolone and DHA. A single radioactive product, DHA was formed from 17-hydroxypregnenolone (Fig. 1). All reaction products were isolated and identified by their mobility on the thin-layer chromatograms, acetylation and crystallizations to constant specific radioactivity (Table 1).

Each subcellular fraction was incubated with pregnenolone or 17α-hydroxypregnenolone as a substrate. As shown in Table 2, both activities of 17α-hydroxylase and $C_{17}$-$C_{20}$lyase were localized in the microsomal fraction of human fetal adrenal tissue.

### *Effect of cofactors*

In the absence of cofactors, no significant amount of metabolite was formed. Figure 2 shows the effect of varying concentrations of NADPH on the activities of 17α-hydroxylase and $C_{17}$-$C_{20}$lyase. The apparent Michaelis constants ($K_M$) for NADPH calculated by the method of Hofstee [6] were $6 \times 10^{-8}$ M for 17α-hydroxylase and $3 \times 10^{-7}$ M for $C_{17}$-$C_{20}$lyase. As shown in Table 3, NADH was less effective in increasing the activities of these enzymes.

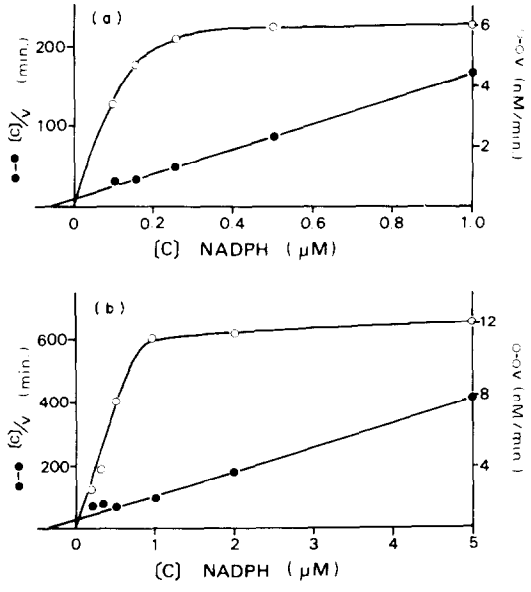


Fig. 2. Effect of varying NADPH concentrations on the activities of 17α-hydroxylase and $C_{17}$-$C_{20}$lyase. (a) Pregnenolone (0.5 μM) was incubated with the microsomal fraction containing 1.87 μg of protein in the presence of varying concentrations of NADPH. (b) 17-Hydroxypregnenolone (1 μM) was incubated in the same conditions except that the amount of protein used was 7.48 μg. [*C*] = NADPH concentration in μM. *v* = reaction rate expressed in nM of product per min.

Table 3. Effect of cofactors on the activities of 17α-hydroxylase and $C_{17}$-$C_{20}$lyase; experimental conditions were the same as described in Fig. 2

| Cofactors | 17α-Hydroxylase* | $C_{17}$-$C_{20}$Lyase† |
|---|---|---|
| None | 0.63 | 0.16 |
| 0.2mM NADPH | 11.66 | 12.05 |
| 0.2mM NADH | 3.84 | 3.12 |

* Activity is expressed as nmol of 17-hydroxypregnenolone and DHA produced from pregnenolone in 6 min per mg protein.

† Activity is expressed as nmol of DHA produced from 17-hydroxypregnenolone in 10 min per mg protein.

*Effect of pH*

The effect of pH on the activities of 17α-hydroxylase and $C_{17}$-$C_{20}$lyase were examined in 0.05 M sodium acetate buffer between pH 5.0 and 5.9, in 0.05 M phosphate buffer of pH 6.2 to 7.3, and in 0.05 M Tris-HCl buffer of pH 7.1 to 8.5. The optimal pH for these enzymes was found to be between 6.6 and 7.5 (Fig. 3).

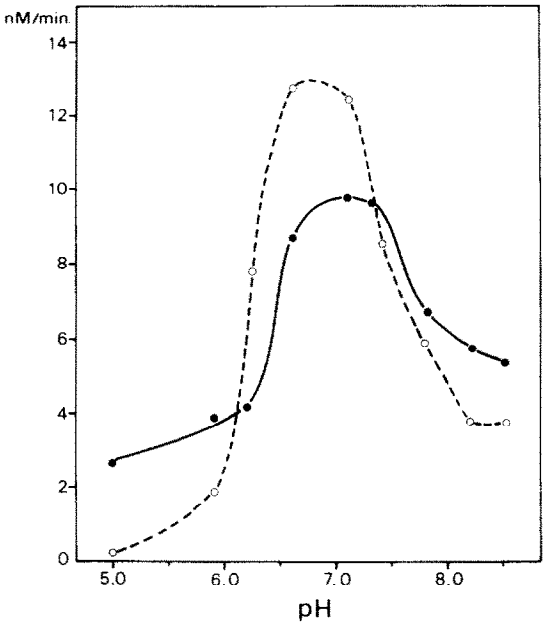


Fig. 3. Effect of pH on the activities of 17α-hydroxylase and $C_{17}$-$C_{20}$lyase. Pregnenolone (0.5 μM) was incubated with the microsomal fraction containing 1.87 μg of protein in the presence of 0.2 mM NADPH in the varying pH buffer. 17-Hydroxypregnenolone (1 μM) was incubated in the same conditions except that the amount of protein used was 7.48 μg. The amount of metabolites (nM/min.) formed from pregnenolone (●——●) or 17-hydroxypregnenolone (O----O) was plotted against varying pH.

*Effect of incubation time and protein concentrations of the tissue preparation*

Figure 4 shows the time course of the conversion of pregnenolone or 17-hydroxypregnenolone with the fetal adrenal microsomal fraction. The amount of the product formed as the result of the enzyme reactions increased proportionately to the protein concentrations of the tissue preparation (Fig. 5).

*Effect of substrate concentration*

Figure 6A shows the relation between concentrations of pregnenolone and the amount of the product formed by 17α-hydroxylase. The apparent $K_M$ value was estimated by the graphical method of Lineweaver and Burk [7] and by the computerized statistical analysis [8]. The latter value of 17α-hydroxylase for pregnenolone was $1.3 \pm 0.3 \times 10^{-8}$ M. The result of the graphical method was essentially the same as that obtained by the statistical analysis of enzyme kinetic data. A similar study on $C_{17}$-$C_{20}$lyase

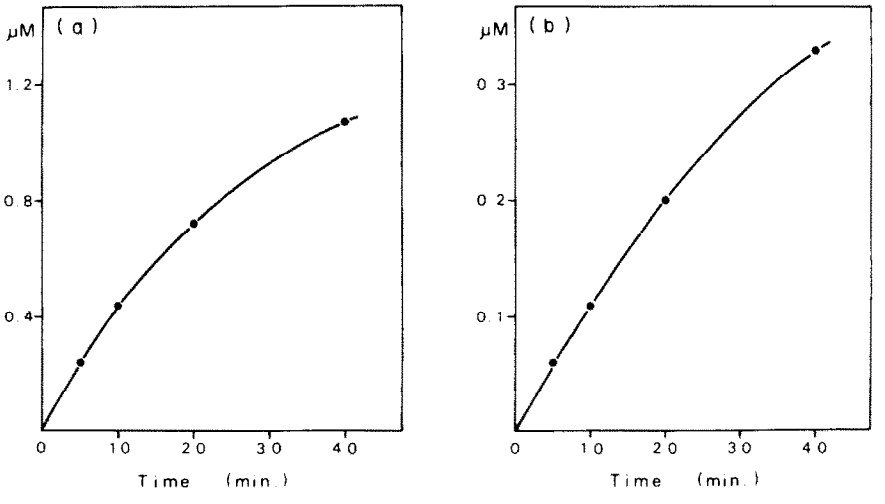


Fig. 4. The time course of the product formation with the fetal adrenal tissue preparation. (a) Pregnenolone (2 μM) was incubated with the microsomal fraction containing 7.48 μg of protein in the presence of 0.2 mM NADPH. (b) 17-Hydroxypregnenolone (2 μM) was incubated with the microsomal fraction containing 7.48 μg of protein in the presence of 0.2 mM NADPH. The amount of metabolites (μM) formed was plotted against incubation time.

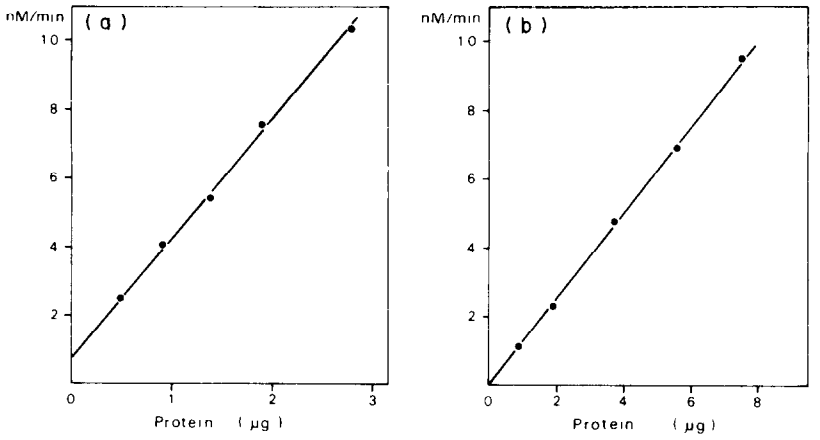


Fig. 5. Effect of protein concentrations of the fetal adrenal tissue preparation on the product formation. (a) Pregnenolone (0.5 $\mu$M) was incubated with the microsomal fraction containing varying amounts of protein as indicated in the presence of 0.2 mM NADPH. (b) 17-Hydroxypregnenolone (1 $\mu$M) was incubated in the same conditions. The amount of metabolites (nM/min.) formed was plotted against protein concentration.

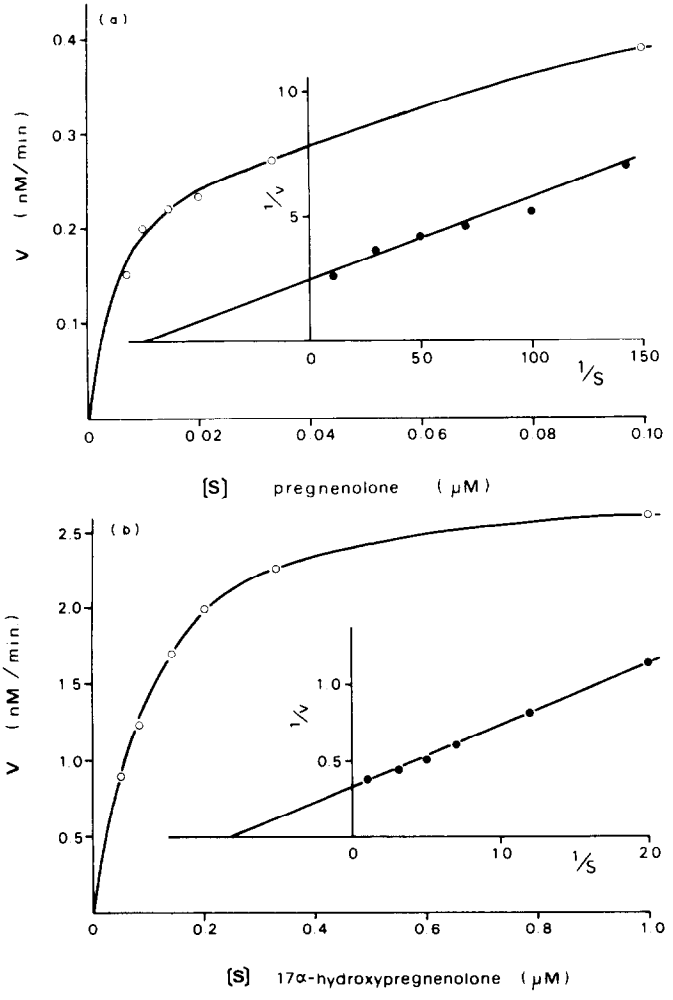


Fig. 6. Effect of varying substrate concentrations on the activities of 17$\alpha$-hydroxylase and $C_{17}$-$C_{20}$lyase. (a): Varying concentrations of pregnenolone were incubated with the microsomal fraction containing 0.094 $\mu$g of protein in the presence of 0.2 mM NADPH. (b): Varying concentrations of 17-hydroxypregnenolone were incubated in the same conditions except that the amount of protein used was 1.87 $\mu$g.

[$S$] = substrate concentration in $\mu$M. $v$ = reaction rate expressed in nM product per min.

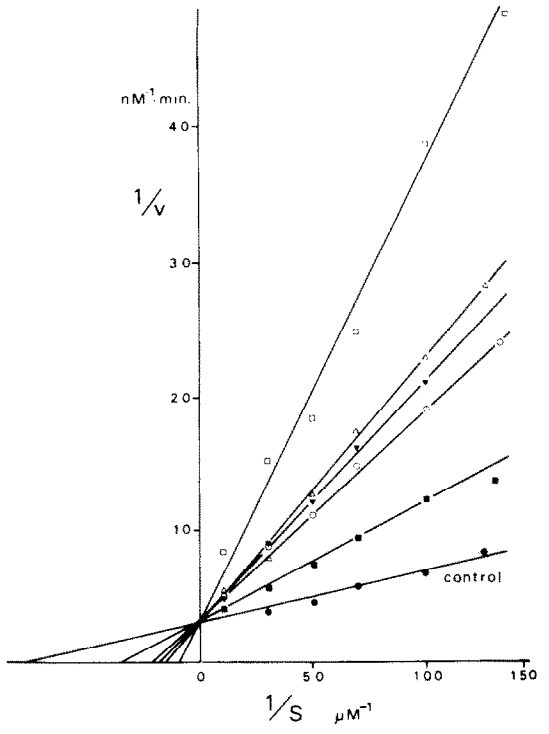


Fig. 7. Inhibition by $10^{-7}$ M of steroids of 17α-hydroxylase toward pregnenolone. Experimental conditions were the same as described in Fig. 6A except that $10^{-7}$ M of unlabelled steroids were included during incubations. Steroids are as follows; 5-pregnene-3β,20β-diol (□), pregnenolone-sulphate (△), 5-pregnene-3β,20α-diol (▼), 17-hydroxypregnenolone (○), progesterone (■) and no additional steroid (●).

using 17-hydroxypregnenolone as a substrate was carried out (Fig. 6B). The apparent $K_M$ value was $1.2 \pm 0.2 \times 10^{-7}$ M.

*Inhibition of enzymes by steroids*

Varying concentrations of the substrate pregnenolone and steroids to be tested were incubated with the fetal adrenal microsomal fraction, and the amounts of the reaction products of 17α-hydroxylase were compared with that in duplicate control incubations without inhibitor. The statistical analysis of enzyme kinetic data [8] and Student's *t*-test were performed to evaluate statistical significance of the inhibitory effect of added steroids. Addition of $10^{-7}$ M progesterone, pregnenolone-sulphate, 17-hydroxypregnenolone, 5-pregnene-3β,20β-diol or 5-pregnene-3β,20α-diol to the incubation medium, suppressed the formation of 17-hydroxypregnenolone and DHA at the fixed concentration of the substrate (statistically significant, $P < 0.05$; Fig. 7); the apparent inhibition constants ($K_i$) of these steroids were 8.1, 2.4, 6.1, 1.5 and $3.1 \times 10^{-8}$ M, respectively. The type of inhibition of these steroids seemed to be competitive.

The enzyme reaction of $C_{17}$-$C_{20}$lyase toward 17-hydroxypregnenolone was inhibited by $10^{-6}$ M 5-pregnene-3β,20β-diol, 5-pregnene-3β,20α-diol, progesterone, 17-hydroxyprogesterone, 17,20β-dihydroxy-4-pregnen-3-one or DHA (statistically significant, $P < 0.05$; Fig. 8); the apparent $K_i$ values of these steroids were $0.6 \times 10^{-7}$ M, $1.7 \times 10^{-7}$ M, $3.4 \times 10^{-7}$ M, $7.6 \times 10^{-7}$ M, $5.6 \times 10^{-7}$ M and $2.3 \times 10^{-6}$ M. Likewise, $10^{-5}$ M 5-androstene-3β,17β-diol, 16α-hydroxy-DHA, testosterone or estradiol-17β inhibited the enzyme reaction of $C_{17}$-$C_{20}$lyase (statistically significant, $P < 0.05$; Fig. 9); the apparent $K_i$ values were $3.2 \times 10^{-6}$ M, $3.2 \times 10^{-6}$ M, $6.2 \times 10^{-6}$ M and $9.2 \times 10^{-6}$M, respectively. The type of inhibition of these steroids on $C_{17}$-$C_{20}$lyase seemed to be also competitive.

## DISCUSSION

The presence and properties of steroid 17α-hydroxylase in various mammalian organs [4, 9–12], and of $C_{17}$-$C_{20}$lyase of testis in rat [10, 13] and guinea pig [14] were well documented. The present investigation characterized enzyme properties of 17α-hydroxylase and $C_{17}$-$C_{20}$lyase of the human fetal adrenal gland. The activities of these enzymes were localized in the microsomal fraction of human fetal adrenal tissue, and were dependent on NADPH; this is similar to those enzymes of other species.

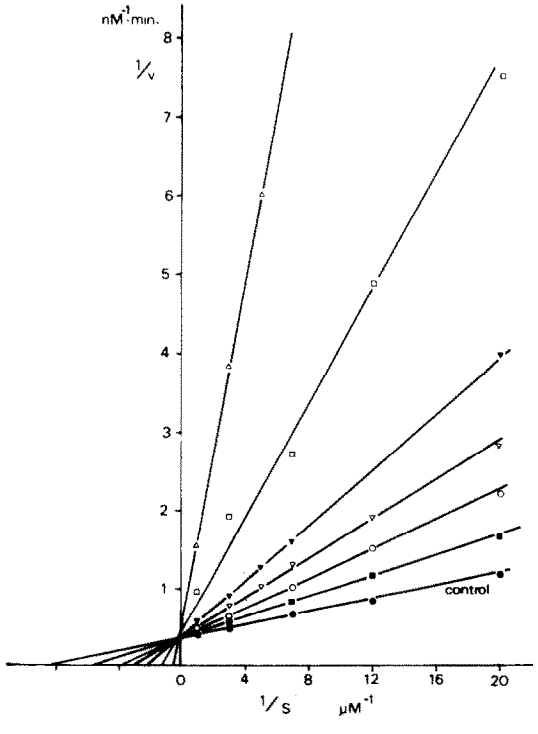


Fig. 8. Inhibition by $10^{-6}$ M of steroids of $C_{17}$-$C_{20}$lyase toward 17-hydroxypregnenolone. Experimental conditions were the same as described in Fig. 6B except that $10^{-6}$ M of unlabelled steroids were included during incubations. Steroids are as follows; 5-pregnene-3β,20β-diol (△), 5-pregnene-3β,20α-diol (□), progesterone (▼), 17,20β-dihydroxy-4-pregnen-3-one (▽), 17-hydroxyprogesterone (○), DHA (■) and no additional steroid (●).

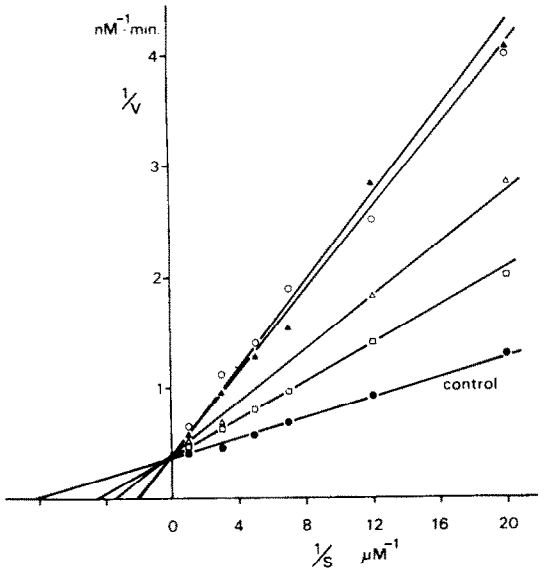


Fig. 9. Inhibition by $10^{-5}$ M of steroids of $C_{17}$-$C_{20}$lyase toward 17-hydroxypregnenolone. Experimental conditions were the same as described in Fig. 6B except that $10^{-5}$ M of unlabelled steroids were included during incubations. Steroids are as follows; 5-androstene-3β,17β-diol (▲), 16α-hydroxy-DHA (○), testosterone (△), estradiol-17β (□) and no additional steroid (●).

In recent years, inhibitory effects of naturally occurring steroids on steroidogenic enzymes of the human testis [3] and placenta [1, 2] have become of considerable interest. The regulatory mechanism by steroids of human fetal adrenal steroidogenesis *in vitro* is not elucidated yet. The present study demonstrates the inhibition of 17α-hydroxylase and $C_{17}$-$C_{20}$lyase activities in human fetal adrenal tissue by naturally occurring steroids. The most potent inhibitors for both enzymes were 20-hydroxylated steroids; 5-pregnene-3β,20β-diol, 5-pregnene-3β,20α-diol and 17, 20β-dihydroxy-4-pregnen-3-one. It was proposed that the 20α-hydroxysteroid dehydrogenase might regulate androgen formation in the testis by competing with 17α-hydroxylase and $C_{17}$-$C_{20}$lyase to utilize common substrates, and by inhibiting these enzyme activities by its reaction products [10, 11]. Our results confirm that a similar mechanism is involved in steroid biosynthesis in the human fetal adrenal gland. Alternative substrates such as pregnenolone-sulphate and progesterone, which are present in the fetal circulation in the order of $10^{-6}$ M, competed with pregnenolone for 17α-hydroxylase. In the same way, 17-hydroxyprogesterone competed with 17-hydroxypregnenolone for $C_{17}$-$C_{20}$lyase. The reaction products, 17-hydroxypregnenolone and DHA inhibited 17α-hydroxylase and $C_{17}$-$C_{20}$lyase, respectively. Steroid intermediates in androgen or estrogen synthesis, such as 16α-hydroxy-DHA, 5-androstene-3β,17β-diol and testosterone, were effective inhibitors of $C_{17}$-$C_{20}$lyase. These results suggest that there is a feedback mechanism of steroids upon the steroidogenic organs regulating the steroid synthesis. Estrogen treatment of the animals suppressed the enzymic activity of $C_{17}$-$C_{20}$lyase [15, 16, 17]. The present experimental results indicate that estradiol-17β possesses a relatively weak inhibitory effect on the steroidogenic enzymes in the fetal adrenal gland *in vitro*. It may be postulated from these results that the autoregulatory mechanism of steroid biosynthesis exists in fetal adrenal tissue; physiological levels of naturally occurring steroids in the fetal circulation or the products of the adrenal gland suppress the enzymic action, with the result of a decreased secretion of steroid hormones from the gland.

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