Journal of Steroid Biochemistry, 1976, Vol. 7, pp. 571–575. Pergamon Press. Printed in Great Britain

# PROGESTERONE AND PREGNENOLONE 17α-HYDROXYLASE: SUBSTRATE SPECIFICITY AND SELECTIVE INHIBITION BY 17α-HYDROXYLATED PRODUCTS

PIERRE KREMERS
Laboratory of Medical Chemistry, University of Liège, Institut de Pathologie, Unité de Biochimie, B-4000 Sart-Tilman par Liège 1, Belgium

(*Received* 16 *January* 1976)

SUMMARY

*In vitro* biochemical properties of steroid-17α-hydroxylase have been studied using rat testes and beef adrenal microsomes as enzyme sources.

Steroid-17α-hydroxylase has a greater affinity for pregnenolone than for progesterone. Furthermore, pregnenolone competitively inhibits the hydroxylation of progesterone and vice-versa. The first products of the reaction, 17α-hydroxypregnenolone and 17α-hydroxyprogesterone inhibit competitively the hydroxylation of both pregnenolone and progesterone with a similar efficiency. This paper indicates that cortisol and androgens are principally synthesized through the so called Δ5 pathway.

## INTRODUCTION

The hydroxylation of carbon 17 constitutes the first specific step of the metabolic pathways leading to the biosynthesis of glucocorticoids and androgens[1]. Thus, steroid-17α-hydroxylase, the enzyme responsible, is likely to play an important regulatory role. Progesterone and pregnenolone are the usual substrates of this enzyme which is detectable only in tissues involved in the production of steroid hormones, namely the testes, the ovaries and the adrenal glands[1].

The 17α-hydroxylase requires NADPH and oxygen as cofactors, is bound to the smooth endoplasmic reticulum[2] and is cytochrome P-450 dependent[10]. Most of the published works[2, 3] are devoted to the study of the overall metabolic pathway and do not specifically describe the individual enzymes involved in the biosynthesis of cortisol and androgens[4]. In particular, very few articles are directly concerned with 17α-hydroxylase[5, 6] and its biochemical and biological properties.

The preferential use of progesterone or pregnenolone as substrates for the 17α-hydroxylase remains a very controversial question. In fact, the utilization of either pregnenolone or progesterone as the substrate determines the exact metabolic pathway leading to the synthesis of cortisol and androgens. Presently, the choice between these two possibilities is still an open question.

Various works[2] support the Δ4 metabolic pathway where progesterone is the substrate for the 17α-hydroxylase, while other authors believe that pregnenolone (the Δ5 metabolic pathway) is the preferred substrate[4, 7–9].

The published works usually measure the incorporation of trace amounts of labelled precursors into the various pools formed during the enzymatic reactions. The results obtained with such a methodology are obviously difficult to interpret and make any quantitative comparison of the enzyme biochemical properties difficult. Recently, we have described a new assay allowing a specific and sensitive measurement of both progesterone and pregnenolone 17α-hydroxylase activity. During the incubation, the tritium specifically located on the 17α position is directly and specifically replaced by a hydroxyl group and incorporated into the incubation medium as a molecule of tritiated water. Counting of the tritiated water gives a direct measurement of the enzymatic activity[12].

This paper will analyze in a comparative and quantitative manner the biochemical properties of the adrenal and testicular 17α-hydroxylase.

## MATERIALS AND METHODS

The sources of the reagents and the labelled compounds used in this study have been described [12–14].

3-(6-chloro-3-methyl-2 indenyl) pyridine (SU 8000), 3-(1,2,3,4-tetrahydro-4-oxo-2-naphtyl)pyridine (Su 9055), 3-(1,2,3,4-tetrahydro-4-oxo-7 chloro-2 naphtyl)-pyridine (SU 10603) were kindly donated by Professor Neher (Basel).

150 g Sprague–Dawley rats were purchased from IFFA CREDO (Les Oncins, France) and were kept on standard commercial food UAR A04 (Villemoisson, France).

*Tissular preparation.* Beef adrenal glands were collected on ice at the slaughterhouse; the cortical part was dissected from the medular region and used to prepare a homogenate. Rat testes were also collected on ice after decapitation of the animals.

The tissues were homogenized in 4 vol. of isotonic KCl using a Potter–Elvejhem apparatus with a Teflon pestle. The homogenates were centrifuged for 10 min at 10,000 *g*; the supernatant was centrifuged for 1 h at 100,000 *g*. The microsomal pellets were then resuspended in a phosphate buffer (0.15 M; pH 7.4) in order that one ml of the final suspension contains the microsomes of 2 g of wet tissue. These tissular preparations were kept at −20°C for as long as 2 months without any significant loss of enzymatic activity.

*Steroid 17α-hydroxylase assay.* [17α-$^3$H]-pregnenolone (or progesterone) (0.5 μCi, 200 nmol) were incubated for 30 min at 37°C in 1 ml $NaKH\ PO_4$ 0.15 M at pH 7.4 containing: 1 μmol NADP, 5 μmol glucose-6-phosphate, 1 I.U. glucose-6-phosphate deshydrogenase, 4 μmol $MgCl_2$ and the 9000 *g* supernatant corresponding to 20 mg of fresh adrenal or testicular tissue.

After incubation, the reaction is stopped by addition of 1.5 ml of a 20% trichloracetic acid solution. After a short centrifugation (20 min at 2000 *g*) the supernatant was distilled under reduced pressure, to separate the tritiated water from the other components of the incubation medium. 1 ml of distilled water is counted by liquid scintillation using Instagel (Packard instrument) as the scintillator, and allows a direct calculation of the enzymatic activity as described in detail in a methodology paper[12].

Protein content of the different enzymatic preparations was determined following the method of Lowry[15].

The enzymatic activities are expressed in nmoles of substrate transformed per hour and per mg of protein.

The different steroids were dissolved in the incubation medium with the substrate by means of 0.5 mg of Tween 80.

## RESULTS

1. *Preferential utilization of pregnenolone as an enzymatic substrate.* Tween 80 was used as a solubilizing agent to introduce saturating concentrations of progesterone or pregnenolone in the enzymatic incubation. By this means, it was possible to compare the affinity of these substrates for the steroid 17α-hydroxylase. Apparent $K_M$ were measured by the graphical method of Lineweaver–Burk (Table 1). Very significant differences were found for the two substrates, the affinity of the microsomal enzyme being very significantly higher for pregnenolone compared to progesterone. The same difference was found both in the adrenal glands and in the testes.

To further study this phenomenon, progesterone 17α-hydroxylase activity was measured in the presence of increasing amounts of pregnenolone and vice-versa. The results of this experiment (Fig. 1) clearly demonstrated that each substrate competitively inhibited the hydroxylation of the other one, and that

Table 1. $K_M$ values for pregnenolone and progesterone 17α-hydroxylase. Values are given as mean ± standard deviation ($n$ = 5)

| Source of enzyme | Substrate | |
|---|---|---|
| | Pregnenolone | Progesterone |
| Beef adrenal | 97 ± 9 μM | 620 ± 36 μM |
| Rat testes | 25 ± 6 μM | 100 ± 10 μM |

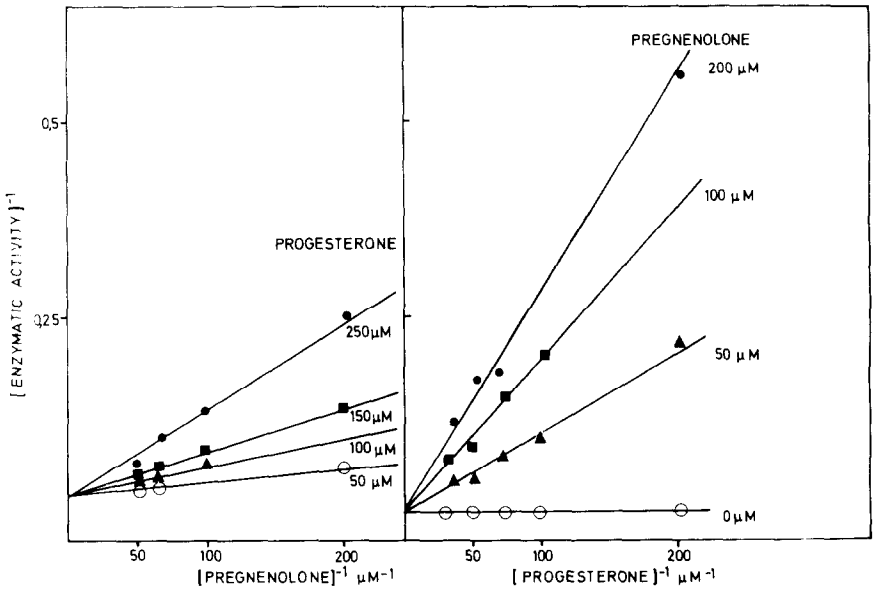


Fig. 1. Inhibition of pregnenolone hydroxylation by progesterone (left) and of progesterone hydroxylation by pregnenolone (right). Lineweaver–Burk plots: substrate concentrations are given in nmol/ml and the enzymatic activity is expressed in nmol/h × mg of proteins.

again pregnenolone was much more efficient in inhibiting the hydroxylation of progesterone than the other way around.

This observation raises the question of whether there are two different enzymes or a single enzyme hydroxylating pregnenolone more actively than progesterone.

2. *Inhibitory action of various compounds.* In order to approach this problem we have further investigated the biochemical properties of 17α-hydroxylase and particularly the effect of some inhibitors on the enzymatic activity.

(a) *Thiol complexing agents.* The addition of heavy metal cations such as Hg, Ag or Cu, in the incubation medium very significantly inhibited the activity of progesterone and pregnenolone-17a-hydroxylase (Table 2). In this respect, other cations such as $Pb^{2+}$, $Fe^{3+}$, $Al^{3+}$, $Co^{2+}$, $Ni^{2+}$ had no effect on 17α-hydroxylase activity.

That the effect of $HgCl_2$ was mediated through a modification of the protein thiol functions, was further supported by the inhibitory effect of several other thiol blocking agents.

Iodobenzoic and parachloromercuribenzoic acids particularly inhibit pregnenolone and progesterone 17α-hydroxylase. After plotting the inverse of enzymatic activity as a function of inhibitor concentration (Dixon plots), it seems that these products were competitive inhibitors.

(b) *Indenyl-pyridine derivatives.* Kahnt and Neher (1962)[16] have described that a number of indenylpyridine derivatives are inhibitors of the steroid-17α-hydroxylase. Using our new assay, we were in a position to determine more precisely the exact mode of action of these derivatives. SU 8000, SU 9055 and SU 10603 inhibited the adrenal gland progesterone and pregnenolone 17α-hydroxylase in a competitive way. The inhibitory constants were calculated (Table 3) and are in the same order of magnitude as the $K_i$ described by Shikita (1967) for the testicular enzyme of the rat[17].

Table 2. Effect of heavy metal ions and thiol blocking agents on 17α-hydroxylase. Enzymatic activities are given in % of the control. A microsomal preparation of beef adrenal cortex was used as the enzyme source. Concentrations given are final concentrations in the incubation medium

| Product | Concentration | Enzymatic activity: Pregnenolone | Enzymatic activity: Progesterone |
|---|---|---|---|
| — | | 100 | 100 |
| $HgCl_2$ | $10^{-4}$ M | 57 | 35 |
| | $10^{-3}$ M | 0 | 0 |
| $CuCl_2$ | $10^{-4}$ M | 77 | 83 |
| | $10^{-3}$ M | 35 | 47 |
| $AgNO_3$ | $10^{-4}$ M | 75 | 100 |
| | $10^{-3}$ M | 34 | 55 |
| Iodobenzoic acid | $10^{-4}$ M | 70 | 60 |
| | $10^{-3}$ M | 40 | 30 |
| P-chloromercuribenzoic acid | $10^{-4}$ M | 70 | 35 |
| | $10^{-3}$ M | 0 | 0 |

Table 3. Inhibitory constant (in nmol/ml) of SU 8000, SU 9055 and SU 10603 for pregnenolone and progesterone 17α-hydroxylase of beef adrenal cortex

| Enzymatic substrate | SU 8000 | SU 9055 | SU 10603 |
|---|---|---|---|
| Pregnenolone | 1.6 | 0.7 | 1.3 |
| Progesterone | 2.3 | 2.0 | 2.1 |

(c) In vitro *action of closely related steroids.* Feedback inhibition of the first enzymatic reaction of a metabolic pathway by the end products of this pathway is a very common mechanism of control in biology. Thus, the first product, several intermediates, as well as the end products of the metabolic pathways were added to the *in vitro* incubation medium and their action on the progesterone and pregnenolone 17α-hydroxylase was measured. Table 4 shows the percentage of inhibition of the hydroxylase activity when the different compounds were added to the medium at the same concentration as the enzymatic substrate. In fact, 17-hydroxyprogesterone and 17-hydroxypregnenolone were the only two products among the products tested displaying a significant inhibition of the enzymatic activity.

We were also able to demonstrate that the products of the enzymatic reaction, the 17-hydroxylated progesterone and pregnenolone, inhibited the 17α-hydroxylase in a competitive manner (Fig. 2, Table 5).

None of the end products of the metabolic pathways affected the hydroxylase activity of the adrenal glands, as well as that of the testes.

## DISCUSSION

In the adrenal glands, the biosynthesis of cortisol can theoretically proceed by one of the following two pathways: (a) the Δ4 pathway by which pregnenolone is the first transformed in progesterone and then hydroxylated in 17α; (b) the Δ5 pathway by which pregnenolone is hydroxylated in 17α before being isomerized into Δ4 3 keto compounds. None of the data presently available in the literature rules out the existence of one of these pathways or establishes their relative importance. In most of the published works, trace amounts of substrate were incubated *in vitro*, permitting only qualitative conclusions. Tamaoki[18, 2] and Inano[19] concluded the existence of only one synthetic pathway, the Δ4 way.

Fevold[7], on the other hand, demonstrated that the Δ5 pathway might, in fact, be the most important one.

With the new assay recently set up[12, 13] we were in a position to measure in a comparative way, the affinity of the 17α-hydroxylase enzymatic complex towards progesterone or pregnenolone. Thus based on the apparent $K_M$ of the enzyme, it was possible to demonstrate that the Δ5 metabolic pathway is more likely the principal way of cortisol biosynthesis. The affinity of the 17α-hydroxylase is indeed 4–6 fold

Table 4. Effect of different steroids on 17α-hydroxylase activity. The different steroids as well as the substrates were maintained in solution by means of Tween 80

| Product | Concentration μM | 17α-Hydroxylation of Pregnenolone | 17α-Hydroxylation of Progesterone |
|---|---|---|---|
| — | | 100 | 100 |
| 17α-hydroxypregnenolone | 200 | 59 | 46 |
| 17α-hydroxyprogesterone | 200 | 87 | 59 |
| Cortisol | 200 | 100 | 95 |
| 17α-hydroxy-11-deoxycorticosterone | 200 | 100 | 95 |
| 11-deoxycorticosterone | 200 | 100 | 95 |
| Testosterone | 200 | 95 | 94 |
| Androstenedione | 200 | 96 | 95 |
| Oestradiol | 200 | 98 | 103 |

higher for pregnenolone than for progesterone, indicating that 17-hydroxypregnenolone must be an important metabolic intermediate in the biosynthesis of glucocorticoids and androgens.

Using a different assay, Fan *et al.* have reported identical $K_M$ values for both Progesterone and pregnenolone 17α-hydroxylase in human testes. They also described the existence of a competitive inhibition between the two substrates, but surprisingly enough, the $K_i$ of pregnenolone on the progesterone hydroxylation was 4 times lower than the other way around. These results are obviously hard to compare with our own experiments, as the methodological approach as well as the enzyme sources are different.

While progesterone is a final product which itself displays important hormonal activity, pregnenolone seems to be exclusively an intermediate in the biosynthesis of cortisol and androgens. Thus, pregnenolone, after being synthesized from cholesterol in the mitochondria should be expected to serve as the preferential substrate for the microsomal 17α-hydroxylase. In fact, Vikho and Ruokonen[4] concluded from the measure of the testicular steroids that the 3β-hydroxy-5-ene intermediates are quantitatively the most important. In addition, Strott *et al.*[20] found a greater production of 17α-hydroxy pregnenolone than 17α-hydroxyprogesterone in the adrenal cortex. Our results favor the hypothesis that pregnenolone is the preferential physiological substrate of 17α-hydroxylase and that the Δ5 pathway should presumably be the normal physiological biosynthetic way leading to androgens and cortisol. However, in some *in vitro* experimental circumstances, progesterone can also serve as a substrate for the enzyme.

Rather than supporting the hypothesis that progesterone and pregnenolone 17α-hydroxylase are two different enzymes, our results on the effects of various inhibitory agents suggest that there is only one enzymatic complex.

A very unspecific inhibitor, like the thiol complexing agents or the more specific inhibitors of steroid hydroxylase, the idenyl pyridine, affect both the progesterone and the pregnenolone 17α-hydroxylase activity in a quantitatively comparable way.

The immediate products of the enzymatic reaction, 17α-hydroxypregnenolone and 17α-hydroxyprogesterone, also inhibit the enzymatic activity very significantly. The great affinity of 17α-hydroxylase for these compounds ($K_i = K_M/5$) suggests that they may have a regulatory role in the biosynthesis of cortisol.

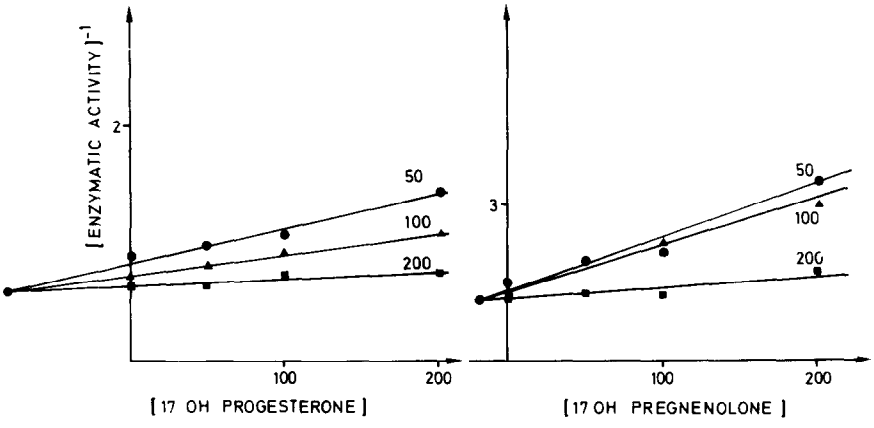


Fig. 2. Competitive inhibition of 17α-hydroxylase by 17α-hydroxyprogesterone and 17α-hydroxypregnenolone. Dixon's plots: enzymatic activities in nmol/h × mg protein, steroid concentration in nmol/ml. Pregnenolone is used as the substrate.

Table 5. Inhibitory constants ($K_i$) in μmol. for the inhibition of adrenal steroid 17α-hydroxylase by the substrates and reaction products

| Substrate | Inhibitor | $K_i$ |
|---|---|---|
| Progesterone | 17α-hydroxyprogesterone | 86 |
| | 17α-hydroxypregnenolone | 25 |
| | Pregnenolone | 25 |
| Pregnenolone | 17α-hydroxyprogesterone | 80 |
| | 17α-hydroxypregnenolone | 20 |
| | Progesterone | 45 |

Further metabolic intermediates and active hormones, have no effect *in vitro* on 17α-hydroxylase. It is thus likely that a 17α-hydroxylase is not the rate lending support to a direct feedback control by cortisol or androgens. The fact that 17α-hydroxypregnenolone is a stronger inhibitor than the 17α-hydroxyprogesterone supports even more our conclusion if one considers that pregnenolone is the physiological substrate.

## REFERENCES


1. Baulieu E. E., Milgrom E. and Robel P.: *Glandes Endocrines* (Edited by E. E. Baulieu, H. Bricaire and J. Leprat). Flammarion Medicine (1972), Paris, p. 57–88.
2. Tamaoki B. I.: *J. steroid Biochem.* **4** (1973) 89–118.
3. Tamaoki B. I., Inano H. and Nakano H.: In *The Gonads*. McKerns, NHPC, (1963) 547–613.
4. Vihko R. and Ruokonen A.: *J. steroid Biochem.* **5** (1974) 843–848.
5. Fan D. F., Oshima H., Troen B. R. and Troen P.: *Biochim. biophys. Acta* **360** (1974) 88–89.
6. Nelson E. B., Bryan G. T.: *J. clin. Endocr. Metab.* **41** (1975) 7–12.
7. Fevold A. R.: *Science* **156** (1967) 1753–1755.
8. Bermudez J. A. and Lipsett M. B.: *J. clin. Endocr. Metab.* **34** (1972) 241–243.
9. Fevold A. R. and Drummond H. B.: *Biochim. biophys. Acta* **313** (1973) 211.
10. Betz G., Tsai P. and Weakley A.: *Steroids* **25** (1975) 791–798.
11. Eik-Nes K. B.: In *The Androgens of the Testes* (Edited by K. B. Eik-Nes). M.D., Inc. (1970) p. 1–47.
12. Kremers P.: *Eur. J. Biochem.* **61** (1976) 481–486.
13. Kremers P.: *C.r. hebd. Séanc. Acad. Sci., Paris* D. **276** (1973). 1021–1024.
14. Kremers P., Denoel J. and Lapiere C. L.: *Steroids* **23** (1974) 603–613.
15. Lowry O. H., Rosenbrough N. D., Farr A. L. and Randall R. T.: *J. biol. Chem.* **193** (1951) 265.
16. Kahnt F. W. and Neher R.: *Experientia* **18** (1962) 499–504.
17. Shikita M., Ogiso T. and Tamaoki B. I.: *Biochim. biophys Acta* **105** (1965) 516–522.
18. Tamaoki B. I. and Shikita M.: In *Steroid Dynamics* (Edited by G. Pincus) (1966) pp. 493–530.
19. Fan D. R., Oshima H., Troen B. R. and Troen P.: *Biochim biophys Acta* **360** (1974) 88–99.
20. Strott C. A., Bermudez J. A. and Lipsett M. B.: *J. clin Invest.* **49** (1970) 1999–2007.