J. Steroid Biochem. Molec. Biol. Vol. 38, No. 2, pp. 165–172, 1991
Printed in Great Britain

0960-0760/91 $3.00 + 0.00
Pergamon Press plc

STEROID 21-HYDROXYLASE ACTIVITY IN EQUINE OVARIAN FOLLICLES EVIDENCED BY ISOTOPE DILUTION-MASS SPECTROMETRY

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(Received 13 September 1990)

Summary—Steroid 21-hydroxylase activity of the microsome-enriched fraction of follicular linings from equine ovaries has been demonstrated by gas chromatography–mass spectrometry. The 21-hydroxylated metabolites were quantified by isotope dilution with deuterated analogues. The two most abundant potential substrates for follicular steroid 21-hydroxylase, progesterone (P) and 17-hydroxyprogesterone (17OHP), were converted respectively to 11-deoxycorticosterone (DOC) and 11-deoxycortisol with corresponding apparent specific activities of 308 and 24 pmol/mg protein/h and apparent K_m values of 1.1 and 6.4 μM. Competitive inhibition of the P-to-DOC conversion was exerted by 17OHP and pregnenolone. Hence, the ovarian follicle of the mare is an extraadrenal site of preferential DOC biosynthesis by an enzyme having steroid 21-hydroxylase activity.

INTRODUCTION

Steroid 21-hydroxylase activity has been reported in several extraadrenal tissues in the human [1–4], in other mammalian species [5, 6] and in some teleost fishes [7, 8]. The extraadrenal conversion of P to DOC appears to be an important route to DOC biosynthesis when plasma levels of P are high, in pregnancy [9] and during the mid-luteal phase of the ovarian cycle [10].

In the adrenal cortex, the presence of different 21-hydroxylase systems, one active on 17OHP and the other on 5P, has been suggested by Gustafsson [11] and this was subsequently corroborated by other findings [12, 13]. Hepatic 21-hydroxylation of C-21 steroid sulfates was shown to be an important metabolic pathway in human pregnancy [14].

In FF aspirated from human preovulatory follicles of stimulated cycles, Dehennin *et al.* [15] have demonstrated the presence of high levels of DOC, and also other 21-hydroxylated metabolites at lower concentrations. Consecutively it has been shown that the human ovary secretes DOC into the blood circulation [16]. These two findings strongly suggest that the human ovary, and the ovarian follicle in particular, are sites of extraadrenal steroid 21-hydroxylase activity.

Since DOC has been identified and quantified in equine, bovine and porcine FF (Bijault C. and Dehennin L., unpublished data), although at concentrations lower than in the human, we have selected, for this study, the mare ovary on the basis of the large size of its mature follicles, which makes the recovery of follicular walls very convenient. In the present report, we demonstrate significant ovarian steroid 21-hydroxylase activity and we characterize some of its biochemical properties. As in previous work on the ovarian follicular steroid content [15, 17], an analytical technique based on GC–MS associated with stable isotope dilution was set-up in order to provide sufficient specificity of detection and accuracy of quantitative determination.

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Abbreviations: K_m, apparent Michaelis constant; V_{max}, apparent maximal specific activity; FF, follicular fluid; GC–MS, gas chromatography–mass spectrometry; DTT, dithiothreitol; PFP, pentafluoropropionate; MO, methyloxime; EO, ethyloxime; TMS, trimethylsilyl ether; P, progesterone; 5P, pregnenolone, 3β-hydroxy-5-pregnen-20-one; 17OHP, 17-hydroxyprogesterone, 17α-hydroxy-4-pregnene-3,20-dione; DOC, 11-deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione; S, 11-deoxycortisol, 17,21-dihydroxy-4-pregnene-3,20-dione; B, corticosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione; 17OH5P, 17-hydroxypregnenolone, 3β,17α-dihydroxy-5-pregnen-20-one; 21OH5P, 3β,21-dihydroxy-5-pregnen-20-one; 11βOHP, 11β-hydroxy-4-pregnene-3,20-dione; 19norP, 19-norprogesterone; 19norDOC, 19-nordeoxycorticosterone; Δ^{16}P, 16-dehydroprogesterone, 4,16-pregnadiene-3,20-dione; Δ^{16}DOC, 16-dehydrodeoxycorticosterone, 21-hydroxy-4,16-pregnadiene-3,20-dione; A, androstenedione, 4-androstene-3,17-dione; E_2, estradiol-17β.

EXPERIMENTAL

Steroids

Unlabelled steroids were obtained from Steraloids (Wilton, NH, U.S.A.), except 19norP and 19norDOC which were a gift from Organon International (Oss, The Netherlands) and Δ^{16}DOC which was synthesized according to Allen and Bernstein [18]. Deuterium labelled steroids were synthesized as described previously [19]. Tritium labelled steroids, used for establishing chromatographic elution profiles, were obtained from Amersham International (Les Ulis, France).

Ovarian follicles

The mare being a seasonal polyœstrus animal, ovaries were collected between April and October at a local abattoir. Follicles were selected according to the size (minimum dia was 3 cm) and the extent of vascularization. After aspiration of FF, follicular walls were removed with fine forceps from follicles bisected *in situ*, as described by Haney and Schomberg [20]. This technique yields hemifollicles with intact membrana granulosa and theca interna, and variable amounts of theca externa, visible upon microscopic examination of histological sections. Pools of follicular linings of such hemifollicles were constituted in order to allow isolation of sufficient enzymatic material for characterization.

Enzyme preparation

Follicular linings were homogenized in phosphate buffer (50 mM, pH 7.4) containing 100 μM DTT and 100 μM EDTA. After 10 min sonication, the homogenate was centrifuged at 800 ***g*** (10 min at 4°C) to remove cellular debris. Mitochondria were removed by centrifugation at 9000 ***g*** (30 min at 4°C). A microsomal pellet, obtained after centrifugation at 105,000 ***g*** (1 h at 4°C) was suspended in phosphate buffer (50 mM, pH 7.4) containing 0.3% (w/v) sodium cholate, 0.5% (w/v) Emulgen 913 (a nonionic detergent donated by Kao Corporation, Tokyo, Japan), 100 μM DTT and 100 μM EDTA, for another homogenization and sonication. After a second centrifugation at 105,000 ***g*** (1 h at 4°C), a final supernatant was obtained containing solubilized microsomal enzymes. The preparation was assayed for protein concentration, diluted to a final concentration of 5 mg protein per ml, divided in aliquots and stored at −80°C.

DETERMINATION OF STEROID 21-HYDROXYLASE ACTIVITY

Standard assay with progesterone as the substrate

Progesterone (concentration range 0.2–40 μM), NADPH (0.3 mM) and 0.1 ml enzyme preparation (=0.5 mg protein) were incubated in 1 ml phosphate buffer (50 mM, pH 7.4) for 30 min at 37°C in open tubes. At the end of the incubation period, 10 ng of [1α,2α-$^2H_2$]DOC (DOC-d_2) dissolved in 20 μl aqueous ethanol (20%, v/v) were added. The enzyme reaction was immediately stopped by mixing the incubation mixture vigorously with 6 ml extraction solvent (*n*-hexane-diethyl ether, 4:1, v/v). The organic phase was evaporated to dryness, the residue was dissolved in 0.1 ml chromatography eluent and applied on top of a column (100 × 5 mm) packed with Lipidex-5000 swollen in a mixture of *n*-hexane–chloroform, 9:1 (v/v), which was also the mobile phase. The first 5.5 ml were discarded and DOC was eluted in the next 4 ml, which were evaporated to dryness. The residue was treated with pentafluoropropionic anhydride in acetonitrile and the 3,21-bis(PFP) derivative of DOC was analyzed at ion masses *m/z* 622 and 624. Experimental conditions of GC–MS analysis were those outlined in detail previously [15]. The only difference was the nature of the GC column: a fused silica column (25 m × 0.32 mm) coated with chemically bound OV-73 stationary phase (film thickness 0.12 μm) and displaying 90,000 theoretical plates. Concentrations were calculated according to equations outlined previously [21] and corrected, if necessary, for contribution of endogenous DOC present in the enzyme preparation.

Assays with other substrates

19-Norprogesterone and 16-dehydroprogesterone. With these substrates, conditions of incubation, extraction and chromatography were similar to those of the standard assay. Substrate concentration ranges were 0.2–40 μM for 19norP and 0.2–6.4 μM for Δ^{16}P. DOC-d_2 was used as internal standard. The 3,21-bis(PFP) derivatives were analyzed by GC–MS at ion masses *m/z* 608, 620 and 624 characteristic of 19norDOC, Δ^{16}DOC and deuterated DOC, respectively.

17-Hydroxyprogesterone and 11β-hydroxyprogesterone. Incubation conditions were similar to those of the standard assay, with substrate concentration ranges of 5–160 μM for 17OHP and 5–40 μM for 11βOHP. At the end of the

incubation period, 10 ng of each [1α,2α-$^2H_2$]S (S-d_2) and [1α,2α-$^2H_2$]B (B-d_2) were added and the enzymatic reaction was immediately stopped by mixing the incubation mixture with 6 ml dichloromethane. Chromatography of the extraction residues was performed on columns (120 × 4 mm) packed with Sephadex OH-20 swollen in a mixture of *n*-hexane–ethanol–acetic acid, 80:20:1 (v/v), which was also the mobile phase. The first 4 ml were discarded and S and B were eluted in the next 4.5 ml. The MO-TMS derivatives were analyzed by GC–MS at ion masses *m/z* 517 and 519.

Pregnenolone. The conditions of incubation were similar to those of the standard assay, with a substrate concentration range of 20–320 μM and a NADPH regenerating system consisting of 0.3 mM NADPH, 20 mM glucose-6-phosphate and 1.2 U/ml glucose-6-phosphate dehydrogenase. Ten ng of [17,21,21-$^2H_3$]21OH5P (21OH5P-d_3) were added at the end of the incubation period and subsequent extraction by dichloromethane was performed. The extraction residues were separated by chromatography on columns (120 × 4 mm) of Lipidex-5000 swollen in a mixture of *n*-hexane–chloroform 8:2 (v/v), which was also the mobile phase. The first 8.5 ml of eluent were discarded and the next 4 ml contained 21OH5P, which was derived to the EO-TMS and ion masses at *m/z* 519 and 522 were used for quantification.

Determination of steroid 17-hydroxylase

With progesterone as the substrate. The composition of the incubation mixture was as mentioned for the 21-hydroxylase standard assay. After incubation, [1α,2α-$^2H_2$]17OHP was added (50 ng for the low substrate concentrations and 200 ng for the high substrate concentrations) and the reaction was stopped by extraction with dichloromethane. Extraction residues were fractionated on Lipidex-5000 columns (120 × 4 mm) packed in a solvent mixture of *n*-hexane–chloroform (9:1, v/v). The first 2.5 ml of this eluent were discarded, P was eluted in the next 3 ml and DOC thereafter in a 4 ml fraction. The eluent was then changed for a mixture of *n*-hexane–chloroform (8:2, v/v): the first 2 ml were discarded and 17OHP was eluted in the next 4 ml. The MO-TMS derivative of 17OHP was analyzed by GC–MS at ion masses *m/z* 429 and 431.

With pregnenolone as the substrate. The conditions of incubation were similar to those of the 21-hydroxylase assay with a 5P concentration range of 0.5–40 μM. At the end of the incubation time, 100 ng [21,21,21-$^2H_3$]17OH5P were added and the extraction with dichloromethane was immediately performed. The extraction residues were fractionated on Lipidex-5000 columns (120 × 4 mm) packed in a mixture of *n*-hexane–chloroform (8:2, v/v). The first 8.5 ml were discarded and 17OH5P was eluted in the next 8 ml. The EO-TMS derivative was analyzed by GC–MS at ion masses *m/z* 519 and 522.

Steroid determinations in equine follicular fluid

These quantitative determinations were performed by GC–MS with stable isotope dilution, according to procedures described for human FF [15, 22].

RESULTS

Identification and Quantification of 21-Hydroxysteroids Formed After Incubation of Various Progestins with the Enzyme Preparation Isolated from Solubilized Microsomes of Equine Ovarian Follicles

Identification was based on the similarity of retention times of suitable derivatives of authentic and biosynthesized 21-hydroxysteroid on the high resolution GC column and on the presence in the selected ion chromatogram of the molecular ion peak or another prominent ion peak of the native steroid derivative at a slightly higher retention than the one of the deuterium labelled analogue (Fig. 1). Ions selected for quantification by selected ion monitoring are mentioned in Table 1. Deuterium labelled analogues were used in most of the cases, except for 19norDOC and Δ^{16}DOC where deuterated DOC was the internal standard.

Steroids in Equine Follicular Fluid

Pools of FF, constituted by the antral fluid aspirated from ovarian follicles selected for recovery of follicular linings, were analyzed for their principal steroid content (Table 2). The levels of P, E_2 and A indicate that these follicles were at the viable or mature stage, as confirmed by the histological examination of granulosa and theca cell layers (not shown). These concentrations were, however, well below those of mare follicles at the preovulatory stage [23].

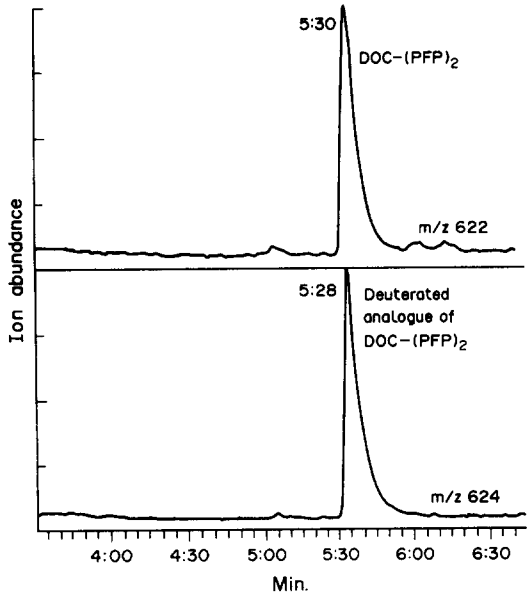

Fig. 1. A representative selected ion chromatogram of a standard assay of steroid 21-hydroxylase activity with progesterone as the substrate (for conditions, see text). Molecular ion abundances of the bis(pentafluoropropionate) derivative of DOC and its deuterated analogue at masses m/z 622 and 624 are plotted vs GC retention time.

Table 2. Principal steroid concentrations (ng/ml) in different pools ($N = 10$) of follicular fluid aspirated from mature ovarian follicles which were subsequently dissected for the isolation of an enzyme with steroid 21-hydroxylase activity

	P	DOC	17OHP	S	E_2	A	5P
Mean	88.7	5.01	127	2.97	1702	93.4	1.43
SEM	27.5	1.77	35	0.50	375	16.5	0.95

Protein concentration. Enzyme activity increases linearly with protein content of the incubation mixture up to a protein concentration of 1 mg/ml (Fig. 4).

Cofactor concentration. The relationship between NADPH concentrations and the initial rate of 21-hydroxylation of P is shown in Fig. 5. Initial velocity reached a plateau (400 pmol/mg protein/h) at 30 μM NADPH. The apparent K_m value was 12.5 μM.

Substrate specificities. Different progestins have been tested for their ability to be 21-hydroxylated by the enzyme preparation of equine ovarian follicles, which appears to have a broad specificity, as indicated by Table 3.

Characteristics of Steroid 21-Hydroxylase in Solubilized Microsome Preparations of Equine Ovarian Follicles

Conversion of progesterone to DOC as a function of substrate concentration

The P dependence of steroid 21-hydroxylase activity is illustrated by Fig. 2, which also indicates that Michaelis–Menten enzyme kinetics are applicable. The apparent K_m of the enzyme for P was 1.1 μM and the sp. act. was between 300 and 400 pmol/mg protein/h, depending on the follicular origin.

Conversion of progesterone to DOC as a function of other parameters

Incubation time. Figure 3 indicates a linear increase of enzyme activity up to an incubation time of 40 min at 37°C.

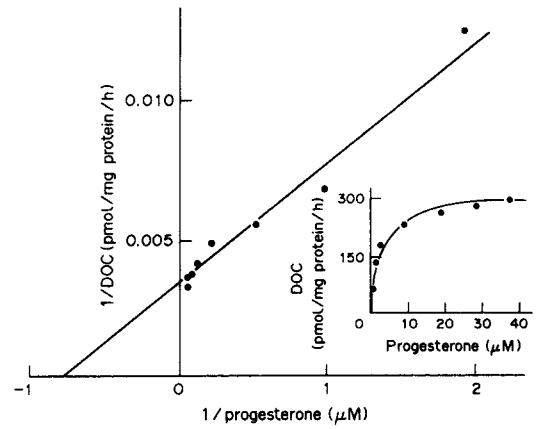

Fig. 2. Conversion rate of progesterone to DOC by the solubilized microsome-enriched fraction of equine ovarian follicles under standard conditions. The apparent K_m value was 1.1 μM and V_{max} was 300 pmol/mg protein/h (insert).

Table 1. Gas chromatographic–mass spectrometric characteristics of the 21-hydroxylated steroids analyzed in this study

			Nominal masses of ions selected for detection and quantitative analysis	
21-Hydroxysteroid	Derivative	RI	Native compound	Internal standard
DOC	bis(PFP)	1.00	622 (M^+)	624 (DOC-d_2)
19norDOC	bis(PFP)	0.93	608 (M^+)	624 (DOC-d_2)
Δ^{16}DOC	bis(PFP)	0.99	620 (M^+)	624 (DOC-d_2)
S	MO-TMS	1.14	517 (M^+-31)	519 (S-d_2)
B	MO-TMS	1.18 1.19*	517 (M^+-31)	519 (B-d_2)
21OH5P	EO-TMS	1.44	519 (M^+)	522 (21OH5P-d_3)

RI = relative retention index on OV-73 stationary phase at 240°C, with a retention index of 2750 for DOC(PFP)$_2$.
*Double peak due to syn/anti isomerism.

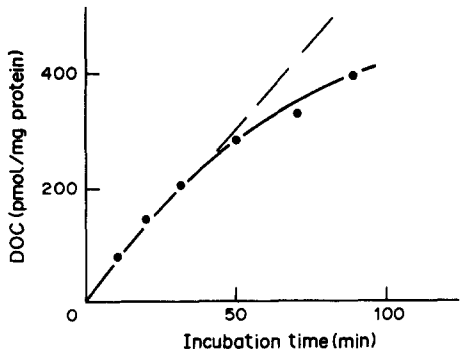

Fig. 3. Conversion of progesterone to DOC by the ovarian steroid 21-hydroxylase preparation as a function of incubation time. Incubation mixture composition was: 30 μM P, 0.3 mM NADPH and 0.5 mg microsomal protein per ml of phosphate buffer (50 mM, pH 7.4).

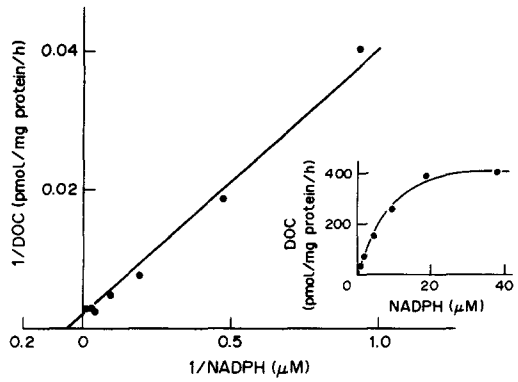

Fig. 5. Double reciprocal plot indicating NADPH dependence of progesterone to DOC conversion by the ovarian steroid 21-hydroxylase enzyme preparation. Incubation mixture composition was similar to the one mentioned on Fig. 3, with a NADPH concentration range up to 40 μM. The apparent K_m value was 12.5 μM and sp. act. reached a plateau at 30 μM NADPH (insert).

Competitive inhibition of the progesterone-to-DOC conversion

Two substrates, 17OHP and 5P, were tested for their inhibitory capacity on the 21-hydroxylation of P. Dixon plots characteristic for competitive inhibition were obtained for 17OHP with an apparent inhibition constant K_i of 18 μM (Fig. 6), and for 5P with K_i at 78 μM (Fig. 7).

Comparison of steroid 17-hydroxylase and 21-hydroxylase activities

As expected, the steroid 17-hydroxylase activities of equine follicular enzyme preparations were high and ranging between 8 and 10 nmol/mg protein/h for the conversion of P to 17OHP. The average sp. act. of 17-hydroxylase,

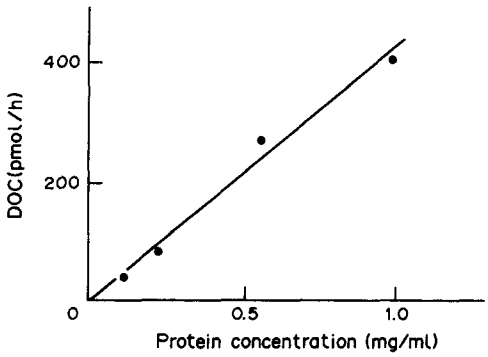

Fig. 4. Conversion of progesterone to DOC by the ovarian steroid 21-hydroxylase enzyme preparation as a function of microsomal protein concentration. Incubation mixture composition was similar to the one mentioned on Fig. 3, with a protein concentration range of 0.1–1 mg/ml.

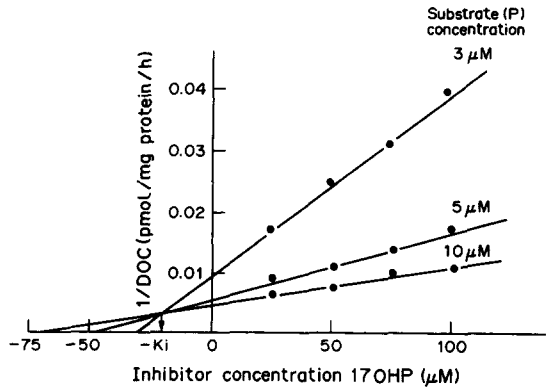

Fig. 6. Dixon analysis of the competitive inhibition of progesterone to DOC conversion by increasing 17OHP concentrations, measured under standard assay conditions. The apparent K_i value was 18 μM.

Table 3. Apparent Michaelis constants (K_m) and maximum specific activities (V_{max}) for the conversion of various substrates to 21-hydroxysteroids by the action of the microsome-enriched preparation with 21-hydroxylase activity, isolated from equine ovarian follicles. Values are means (±SD) of triplicate determinations made with the same enzyme preparation

Substrate	21-Hydroxylated product	K_m (μM)	V_{max} (pmol/mg protein/h)
P	DOC	1.1(±0.1)	308(±18)
17OHP	S	6.4(±0.5)	24(±2)
11βOHP	B	11.7(±1.2)	250(±20)
19norP	19norDOC	43.5(±3.5)	250(±15)
Δ^{16}P	Δ^{16}DOC	1.7(±0.1)	910(±30)
5P	21OH5P	12.7(±1.0)	91(±5)

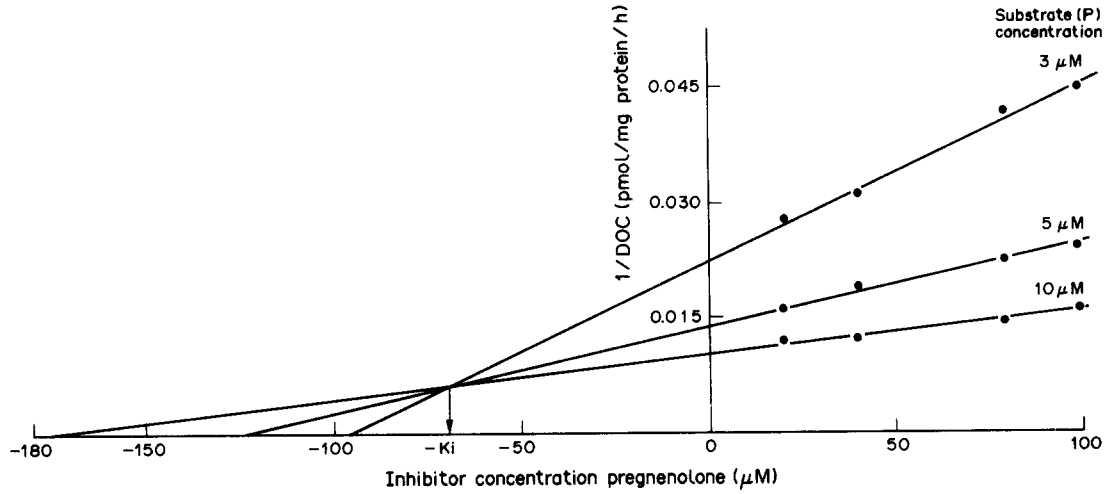

Fig. 7. Dixon analysis of the competitive inhibition of the progesterone-to-DOC conversion by increasing pregenenolone concentrations. Standard assay conditions were used with a NADPH regenerating system. The apparent K_i value was 78 μM.

for the conversion of P to 17OHP, was 30 times higher than the concomitant 21-hydroxylase, as shown by Fig. 8. With 5P as substrate for 17-hydroxylase, V_{max} was 4.1 nmol/mg protein/h and K_m was 13.3 μM. These data, as well as those concerning the conversion of 5P to 21OH5P (Table 3), confirm that in the mare P is a better substrate than 5P for the actions of ovarian 17-hydroxylase and 21-hydroxylase.

DISCUSSION

In search of an animal model that was appropriate for the investigation of steroid 21-hydroxylase activity in ovarian tissue, we have retained the mare ovary, for which it appears that 3 cm in diameter is a major stage in the follicular maturation process [24]. This large diameter also offers the advantage of easy dissection of the follicles relatively free from surrounding tissues.

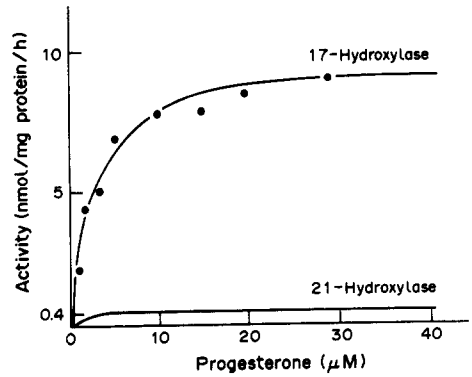

Fig. 8. Comparison of steroid 21-hydroxylase and 17-hydroxylase activities of the follicular enzyme preparation from equine ovaries. The 21-hydroxylase activity represented less than 5% of the 17-hydroxylase activity.

Comparatively to our previous results on the P and E_2 levels in equine FF [23] and to similar findings by Kenney *et al.* [24], the average population of ovarian follicles, recruited for enzyme recovery, was assigned a viable stage close to maturity. Moreover, equine follicular steroid 17-hydroxylase was very active with P as the substrate (Fig. 8), even somewhat more active than the 17-hydroxylase present in human follicular tissue from the late follicular phase (6.4 nmol/mg protein/h) [25].

Mean concentration ratios DOC/P = 5.6% and S/17OHP = 2.3% were found in equine FF; these ratios are considerably higher than those found in human FF from preovulatory follicles of stimulated cycles, which were, respectively, 0.3 and 0.2% [15]. Whether this is due to higher ovarian steroid 21-hydroxylase activity in the mare, or to more extensive biosynthesis of P and 17OHP metabolites other than 21-hydroxysteroids, remains to be established.

Owing to the low quantitative importance of ovarian steroidogenesis along the 5-ene pathway in the mare, the FF concentrations of 5P were low and 21OH5P remained undetectable (below 0.1 ng/ml), although the enzyme activity required for the conversion of 5P to 21OH5P is present in the equine ovarian follicle (Table 3).

An analytical technique of high specificity, based on GC–MS associated with stable isotope dilution, has been applied to detect and quantify the 21-hydroxylated progestins. This was a requisite since the turnover rate of P to DOC exerted by the equine ovarian enzyme

preparation, under standard assay conditions with saturating substrate concentrations, was low (ranging from 0.3 to 0.5%). Under similar conditions, the turnover rate of P to 17OHP was much higher (12.5%). This corroborates earlier results reported by Mahajan and Samuels on the steroid 17-hydroxylase activity in follicular walls from mare ovaries [26]. Other enzyme activities (20α- and 20β-hydroxysteroid dehydrogenase, lyase, e.g.), which may be in competition with steroid 21-hydroxylase for the common substrate (P), make that only apparent kinetic parameters can be measured with the crude enzyme preparation isolated from equine ovarian follicles.

The enzyme kinetics of the P-to-DOC conversion by steroid 21-hydroxylase activity present in microsome-enriched preparations of equine ovarian follicles have been determined graphically in Figs 2–5. As expected, the specific activity of DOC formation by the equine ovarian 21-hydroxylase was considerably higher (30 fold) than the corresponding specific activity in the human fetal ovary (10 pmol/mg protein/h), which is the only ovarian data available for comparison [3].

No increase of microsomal steroid 21-hydroxylase activity could be observed upon addition of an aliquot of the 105,000 *g* cytosol to the incubation mixture. Such an activation has been evidenced with bovine adrenal steroid 21-hydroxylase [27].

Concerning substrate specificity, the data mentioned in Table 3 indicate that P is a better substrate than 17OHP for equine ovarian 21-hydroxylase action. The strength of the enzyme–substrate complex is similar for both P and Δ^{16}P, but the specific enzyme activity with the latter substrate is nearly three times higher than with P. This seems to be related to the Δ^{16} unsaturation which renders 17-hydroxylation impossible and probably decreases the rate of 20α-reduction. Remarkably, 11βOHP was converted significantly to B by the action of equine ovarian 21-hydroxylase, thus indicating that, at least *in vitro*, unusual 21-hydroxylation of 11β-hydroxylated metabolites can be achieved by this microsomal enzyme preparation.

Competition for binding to the same active site of the equine ovarian 21-hydroxylase was evidenced by the results shown in Figs 6 and 7. As expected from the data on substrate specificity, the strengths of the enzyme–inhibitor complexes for 17OHP ($K_i = 18\ \mu$M) and 5P ($K_i = 78\ \mu$M) were much lower than the corresponding strength of the enzyme–substrate complex for P ($K_m = 1.1\ \mu$M). Thus, high inhibitor concentrations were needed to reduce significantly the P-to-DOC conversion.

In conclusion, the mature equine ovarian follicle is capable of 21-hydroxylation of various progestins *in vitro*, and most probably *in vivo*, since some 21-hydroxysteroids are present in FF at levels higher than in peripheral plasma. Whether these conversions are mediated by cytochrome *P*450 specific for 21-hydroxylation of both gluco- and mineralocorticoids, is unknown. According to Miller [28], other cytochrome *P*450 enzymes may have gratuitous 21-hydroxylase activity. This may be the case in the ovarian follicle, as no mRNA specific for cytochrome *P*450c21 (21-hydroxylase) was detectable, neither in the bovine ovary [29], nor in the human fetal ovary [30]. Nevertheless, the enzymatic conversion of P to DOC seems to be a normal metabolic process in the mammalian ovarian follicle. The physiological role of ovarian DOC in mammals remains to be established, but a possible action on the maturation and the ovulation of oocytes should be considered, in the light of what has been suggested for some fish ovaries [7].

Acknowledgements—We are very grateful to Messrs Jacques Dubois and Pierre Salvador for technical assistance and to Mr Pierre Ducrot (Abattoir Industriel Persoons & Bertola, 95460 Ezanville, France) who kindly agreed to collect numerous mare ovaries.

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