J. steroid Biochem. Vol. 17, pp. 407 to 414, 1982
Printed in Great Britain.

0022-4731/82/100407-08$03.00/0

THE STEROID 17α-HYDROXYLASE ACTIVITY OF THE IMMATURE RAT OVARY

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(*Received* 29 *September* 1981)

SUMMARY

The steroid 17α-hydroxylase activity of the microsomal (105,000 *g*) fraction of ovaries obtained from hypophysectomized immature rats was measured using a tritium exchange assay with 17α[^{3}H]-pregnenolone or 17α[^{3}H]-progesterone as substrates. The tritiated water produced by hydroxylation of progesterone was highly correlated with the amount of 17-hydroxyprogesterone produced and measured by radioimmunoassay. Pregnenolone appeared to be the preferred substrate. Although the substrates competitively inhibited hydroxylation of each other, the K_i for progesterone was greater than that for pregnenolone. The hydroxylation of progesterone was inhibited to a lesser degree than that for pregnenolone when 20α-dihydroprogesterone was present at half the concentration of the substrate. Only hydroxylation of progesterone was inhibited by 20α-dihydropregnenolone. Enzyme activity decreased following hypophysectomy with a half-time of about 33 h. Administration of pregnant mare's serum gonadotropin (PMSG) or human chorionic gonadotropin (hCG) resulted in a dramatic and rapid decrease in activity. However, within 24 h following a single injection of PMSG, or multiple injections of hCG, the specific activity of the enzyme increased. Ovine LH or FSH, but not FSH whose LH activity had been neutralized with antiserum, caused an initial decrease in activity but they did not produce an increase even with repeated administrations at 12 h intervals. Prolactin reduced the stimulatory effects of PMSG as well as greatly reduced the activity which had been increased by PMSG or hCG. The results indicate that the immature rat ovary contains a labile 17-hydroxylase activity which can be quickly altered by gonadotropic action.

INTRODUCTION

In spite of rather extensive investigation, the control of ovarian steroid production is not clearly established. That the rate limiting step is conversion of cholesterol to pregnenolone, and that this step is stimulated by gonadotropin, are not contested (reviewed in [1]). However, several additional steps in the synthesis of progestins, androgens and estrogens are also controlled by gonadotropic action [2]; some of these involve alterations in enzyme activities [3]. Steroid 17α-hydroxylase (EC 1.14.99.9) appears to be a pivotal enzyme for production of androgens and estrogens either via the Δ5 pathway from pregnenolone or the Δ4 pathway from progesterone. However, few studies have focused attention on this enzyme in the ovary. The present study was aimed at determining some of the properties of 17-hydroxylase and changes in its activity in the ovaries of immature hypophysectomized rats.

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The following trivial names and abbreviations are used in the text: pregnenolone, 3β-hydroxy-5-pregnen-20-one; testosterone, 17β-hydroxy-4-androsten-3-one, estradiol, 1,3,5(10)-estratriene-3,17 diol; progesterone, 4-pregnene-3,20 dione; 20α-dihydroprogesterone, 20 DHP, 20α-hydroxy-4-pregnen-3-one; 20α-dihydropregnenolone, 5-pregnene-3β,20-diol; NADP, nicotinamine adenine dinucleotide phosphate.

EXPERIMENTAL

Immature (25 ± 1 day) female rats of the Holtzman strain were maintained in temperature (23 ± 1°C) and light controlled (lights on from 0600 to 2000 h daily) quarters and given free access to laboratory food and tap water. Hypophysectomy was performed using the parapharyngeal approach and ether anesthesia. A 5% solution of glucose was used for drinking water after this operation.

Tissue preparation

(1) Blood was collected from the trunk following decapitation. After clotting at room temperature for 30 min the blood was stored at 4°C overnight prior to centrifugation (1500 *g*); the serum was separated and stored frozen until assayed for steroids. (2) Ovaries were removed from the animals (groups of 6–12) as quickly as possible, weighed on a torsion balance and frozen on dry ice; the tissue was stored at −20°C until processed. The ovaries were thawed, maintained at 4°C, and homogenized (100–400 mg wet wt/ml) in 0.15 M KCl using a Teflon coated pestle in a glass homogenizer. The homogenate was centrifuged at 9000 *g* for 30 min and then at 105,000 *g* for 60 min.

The pellet (microsomal fraction) was resuspended in 0.15 M Na–KPO_4 buffer (pH 7.4); this suspension was used for the assay of hydroxylase.

Enzyme assay

17-Hydroxylase activity was measured by a modification of the tritium exchange assay of Kremers[4]. This assay depends upon the reaction: 17α[^{3}H]-pregnenolone (17α[^{3}H]-progesterone) + NADPH + O_2 → 17OH-pregnenolone (17OH-progesterone) + $NADP^+$ + [^{3}H]OH. The assay was performed in a total volume of 200 μl, using 12 × 75 mm glass culture tubes. In addition to the suspension of ovarian microsomes, the assay medium contained 0.5 mg of Tween 80 to aid in substrate solubilization [4], 0.2 μmol of NADP, 1 μmol of glucose-6-phosphate, 0.2 μ of glucose-6-phosphate dehydrogenase, and 20 nmol of either 17α[^{3}H]-pregnenolone or 17α[^{3}H]-progesterone. The specific activities of the latter compounds, which were prepared and characterized by Dr P. Kremers, University of Liege, were 1.34 and 1.83 μCi/μmol respectively, as determined by radioimmunoassay. Previous studies [4] using $^3H/^{14}C$ ratios of products indicated that 87% of the tritium was in the alpha position on carbon 17 and this value was used for calculation of enzyme activity. The reaction was started by the addition of NADP and the incubation carried out at 37°C in a Dubnoff shaking water bath. The reaction was stopped by the addition of 400 μl of cold (4°C) distilled water followed immediately by a 4 mg pellet of dextran coated charcoal (Reflex Industries Inc., North Hollywood, CA). The tubes were centrifuged (1500 *g* × 15 min) and the supernatant fluid transferred to a 16 × 150 mm glass culture tube. The latter tube was connected, by a 3 mm Teflon tube, to a 10 × 75 mm glass tube which was partially submerged in an acetone + dry ice bath. Negative pressure was applied via a polyethylene tube inserted into the rubber stopper of the latter tube. A 300 μl sample of the distillate was transferred to a vial, 5 ml of scintillation fluid (3a70; Research Products International Corp., Elk Grove, IL) added and the radioactivity determined in a Packard liquid scintillation spectrometer with a 59% efficiency for tritium. Hydroxylase activity was calculated, in triplicate samples, after subtracting blank values (no tissue). Differences between means with a P value of less than 0.05 were considered significant.

In one study the reaction was stopped with 2 ml of diethyl ether after the addition of tritiated 17-hydroxyprogesterone for determination of extraction efficiency. The ether was decanted after freezing the aqueous phase in an acetone + dry ice bath and taken to dryness. The dried residue was redissolved in benzene–methanol (90:10, v/v) and chromatographed on a 6 × 200 mm column of Sephadex-LH 20 using the same solution for elution. The 17-OH-progesterone fraction was collected and measured by radioimmunoassay.

Steroid assay

The concentrations of progesterone, 20α-dihydroprogesterone (20-DHP), 17-OH-progesterone, and testosterone were determined in the blood, or fractions of ovarian homogenates by radioimmunoassay using a specific antiserum for each hormone. The characteristics of the antibodies used have been reported, as well as the details of the procedures [5, 6]. All of the samples in a particular series were assayed simultaneously to avoid interassay variation: intraassay coefficients of variation did not exceed 10%.

Reagents

[1,2,6,7^{3}H(N)]-Testosterone 98.8 Ci/mmol, [1,2,6,7^{3}H(N)]-progesterone 97.9 Ci/mmol, [1,2^{3}H(*N*)]-17-OH-progesterone 40.4 Ci/mmol, [1,2^{3}H(*N*)]-20-DHP 55.7 Ci/mmol, were purchased from New England Nuclear Corp. (Boston, MA) and used without further purification. Unlabelled steroids were obtained from Sigma Chemical Co. (St. Louis, MO) as were NADP, NADPH, NADH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, PMSG and hCG. Ovine FSH (S-8 and S-12), LH (S-18) and prolactin (S-12, 35 IU/mg) were obtained from NIADDK. Anti-ovine LHβ-subunit antiserum was prepared by Dr. Bruce Goldman (Univ. of Conn. Storrs).

RESULTS

The amount of tritiated progesterone utilized in the tritium exchange assay for 17-hydroxylase was compared to the amount of 17-hydroxyprogesterone produced and assayed by immunoassay. Progesterone was used as substrate and in a concentration which would inhibit the action of C 17-20 lyase and prevent the conversion of 17-hydroxyprogesterone to androstenedione [7]. Although ovarian 17-hydroxylase is saturated at 100 μM progesterone, and no further increases in activity occur with higher concentrations [8], our preliminary trials indicated that this concentration was insufficient to completely inhibit the lyase activity of the highest doses of microsomal suspension (data not shown). However, when 1 mM progesterone was used no androstenedione was detected in the reaction products and a linear increase in 17-OH-progesterone was obtained with increasing concentrations of microsomal protein (Fig. 1). For the tritium exchange assay 100 μM progesterone was used, which produced results completely comparable to those obtained with the immunoassay method.

Enzyme characteristics

Hofstee[9] plots of enzyme activity in ovarian homogenates using pregnenolone or progesterone (Fig. 2) as substrate indicate the apparent K_m for the former was more than double that for the latter. The V_{max} for pregnenolone was 0.29 nmol/mg protein/h while it was 0.43 for progesterone. Because of the

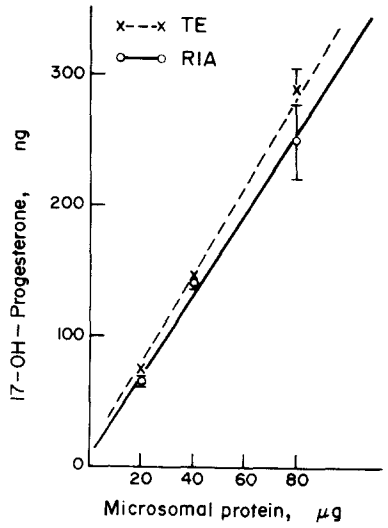

Fig. 1. Comparison of the tritium exchange (TE) and the radioimmunoassay (RIA) methods for the measurement of 17-hydroxylase activity. Ovaries from immature hypophysectomized rats were removed 48 h after an i.v. injection of 20 IU of PMSG. Following homogenization in 0.15 M KCl and centrifugation at 9000 *g* for 30 min, the supernatant fluid was centrifuged for 60 min at 105,000 *g*. The precipitate (microsomal fraction) was resuspended in Na-K phosphate buffer (pH 7.4) and incubated for 60 min with 1 mM progesterone or 100 μM 17α[^{3}H]-progesterone along with an NADPH generating system. The 17-hydroxyprogesterone produced from the former was extracted with ether, purified by chromatography on Sephadex LH-20, and assayed by RIA. The amount of [^{3}H]OH present in the distillate of the incubation medium was used to calculate the amount of labelled progesterone converted to 17-hydroxyprogesterone. In this, and in subsequent figures, the SEM is indicated by a vertical line when it exceeds the area covered by the symbol. There were five samples per dose in the RIA method and three in the TE assay.

limited amount of labelled progesterone available and the greater enzyme activity shown with pregnenolone this substrate was used for most of the subsequent studies.

Pregnenolone competitively inhibited [10] hydroxylation of progesterone with a K_i of 3.01 μM (Fig. 3A) while the reverse reaction yielded a K_i of 10.7 μM for progesterone (Fig. 3B). Because of reports that 20-DHP and 20α-dihydropregnenolone act as inhibitors of 17-hydroxylase, and may be important control factors [11], the effect of these steroids upon ovarian hydroxylase was examined in a study using both pregnenolone and progesterone as substrates. The animals were injected with 20 IU of PMSG (intravenously, i.v.) or with 20 IU of hCG; the hCG was administered in 4 doses of 5 IU each, the first being given i.v. and the others given subcutaneously (s.c.). The ovaries were removed 48 h after the initial injection of gonadotropin and the microsomal fraction of the homogenates prepared. The substrate concentrations were 100 μM while the inhibitor concentrations were 50 μM. The results are shown in Table 1. The activity of the hydroxylase was 2.8 times greater when pregnenolone was the substrate compared to progesterone. 20-DHP severely inhibited 17 hydroxylation of pregnenolone, but was only about half as effective against progesterone. In contrast, 20α-dihydropregnenolone had no effect upon hydroxylation of pregnenolone and significantly inhibited hydroxylation of progesterone only when the enzyme was obtained from ovaries stimulated by PMSG.

Use of NADPH over a range of 10^{-5} M to 10^{-2} M in the reaction mixture indicated an apparent K_m for the cofactor of 3.04×10^{-4} M. A K_m for NADH was not established, but significant 17-hydroxylase activity was obtained with this cofactor. With 10^2 NADH the 17-hydroxylase activity of an ovarian microsome preparation was 19.0 ± 0.45 nmol/h while the activity of the same fraction using 10^{-2} NADPH was 41.2 ± 1.5 nmol/h. Addition of 10^{-2} NADH to 10^{-2} NADPH did not alter the activity of the enzyme found with the latter cofactor alone; other combinations were also without additive effect.

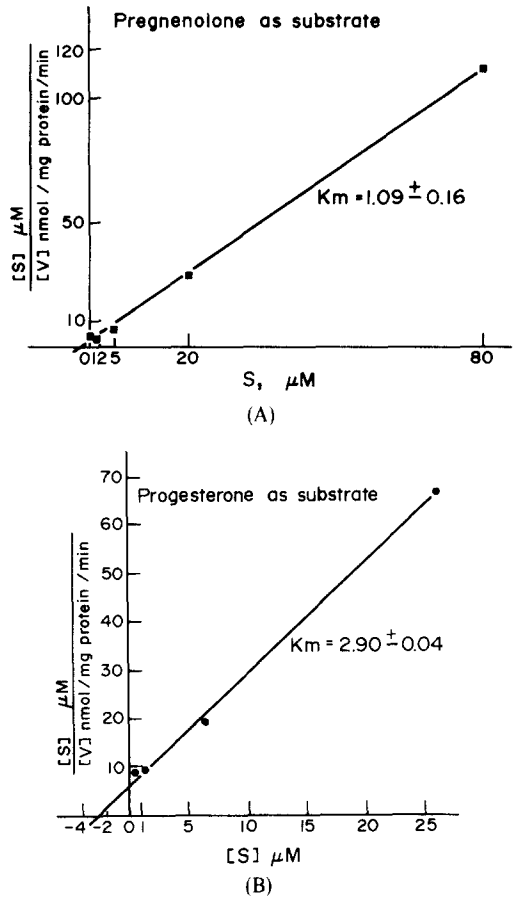

Fig. 2. Calculation of the K_M(μM) from the regression line, determined by the method of least squares, for hydroxylation of pregnenolone (A) and progesterone (B) using the microsomal fraction of ovaries from untreated hypophysectomized immature rats. Triplicate samples at each dose. The calculated V_{max} for the hydroxylation of pregnenolone is 0.29 ± 0.04 nmol/h/mg protein while for the hydroxylation of progesterone it is 0.43 ± 0.01.

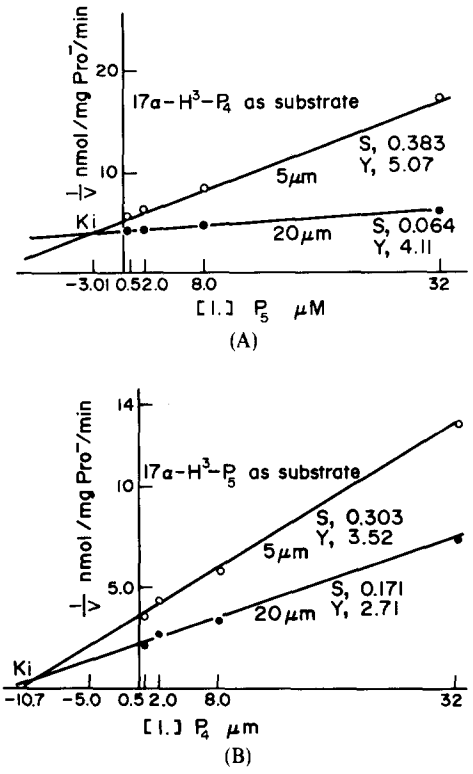

Fig. 3. Inhibition of progesterone (P4) hydroxylation by pregnenolone (P5) (A) or pregnenolone hydroxylation by progesterone (B). The enzyme was prepared as indicated in Fig. 1. and the substrate was used at 5 or 20 μM in an incubation carried out for 1 h. The slope (S) and intercept (Y), as calculated by the method of least squares, are given for each curve. The calculated K_i for pregnenolone was 3.01 μM while that for progesterone was 10.7 μM.

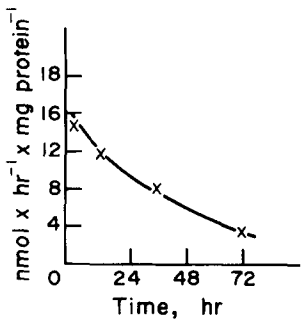

Fig. 4. The disappearance of 17-hydroxylase activity from ovaries of immature rats following hypophysectomy. Groups of 10 animals were killed at the time of pituitary removal or at 12, 36 or 72 h later, the ovaries homogenized and the microsomal fraction prepared as indicated in Fig. 1. The half-time for the enzyme, obtained by a plot of the log of the enzyme activity vs time, is 33.4 h.

Disappearance rate of the enzyme

Three methods were used to assess the half time of ovarian hydroxylase activity: (1) changes at 24, 60 and 96 h after hypophysectomy; (2) changes at 12, 36 and 72 h (Fig. 4) after hypophysectomy in animals injected (s.c.) with 0.1 ml of 0.15 M NaCl every 12 h, and 3) changes at 24 h intervals following an intraperitoneal (i.p.) injection of anti-PMSG antiserum given after the enzyme activity had been increased by an i.v. injection of 20 IU of PMSG. With the first method the slope of the regression line, of the plot of the log of enzyme activity versus time, indicated a half-time of 32.4 h. With the second method, the slope indicated a half-time of 33.4 h. In animals given anti-PMSG the slope of the regression line indicated a half-time of 14.4 h. The latter experiment was repeated (Fig. 5). Animals were given 20 IU of PMSG and 48 h later received either normal rabbit serum or sufficient anti-PMSG antiserum to neutralize the biological activity of 40 IU of PMSG [12]. In controls ovarian weight continued to increase until 96 h after PMSG administration and then declined slightly (non-significant) by 120 h. In animals treated with anti-PMSG the weight decreased within 24 h and continued a more gradual decline thereafter. In control animals ovarian hydroxylase activity increased until 72 h after PMSG and then decreased with a half-time of 28.3 h. Re-

Table 1. The effect of 20α-dihydroprogestins on the 17-hydroxylation of pregnenolone and progesterone by the microsomal fraction of ovaries from immature (25 day) hypophysectomized rats treated with PMSG or hCG

Inhibitor 50 μM	Treatment of females	Substrate (100 μM) for hydroxylase			
		Pregnenolone	% Reduction	Progesterone	% Reduction
none	hCG*	49.7 ± 3.1	—	17.4 ± 0.3	—
20-DHP4	hCG*	19.7 ± 0.1	60.4§	12.5 ± 0.14	28.2§
20-DHP5	hCG*	50.2 ± 0.7	0	14.9 ± 0.4	14.4‡
none	PMSG†	92.9 ± 2.8	—	33.2 ± 0.4	—
20-DHP4	PMSG†	31.9 ± 0.4	65.7§	24.3 ± 0.15	26.8§
20-DHP5	PMSG†	94.3 ± 0.6	0	30.0 ± 1.1	9.6

Enzyme activity expressed as nmol per mg of microsomal protein per h ± SEM for at least three samples. *hCG (5 IU) or †PMSG (20 IU) was injected i.v. 24 h after hypophysectomy; 5 IU of hCG was also injected (s.c.) to the former animals every 12 h for three doses. The animals were killed 48 h after the initial injection. 20-DHP4 is 20α-dihydroprogesterone and 20-DPH5 is 20α-dihydropregnenolone. †Indicates a P value of less than 0.05 when compared to controls with no inhibitor; § indicates a P value of less than 0.01.

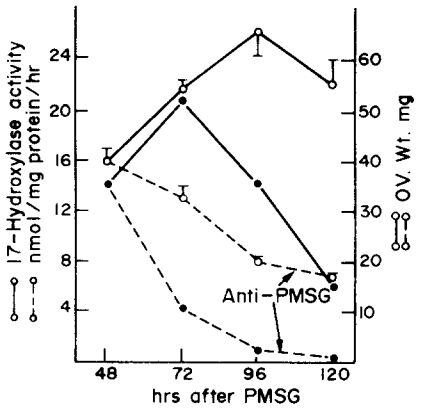

Fig. 5. The effect of anti-PMSG antiserum given (i.p.) 48 h after administration of 20 IU of PMSG to immature hypophysectomized rats. The ovaries (at least six pairs) were removed at 24 h intervals and the microsomal fraction prepared as indicated in Fig. 1. Control animals received normal rabbit serum. The calculated half-time for the loss of enzyme activity in the controls, after 72 h, was 28.3 h but for the animals given antiserum the half-time was 14.8 h calculated from the time of the injection.

moval of the PMSG activity with antiserum produced a more rapid decrease in enzyme activity yielding a half-time of 14.8 h.

Effects of gonadotropin

Injection of 20 IU of PMSG resulted in a prompt increase in 17-OH progesterone concentrations in the cytosolic and microsomal fractions of ovarian homogenates; increases in the serum levels were less pronounced (Fig. 6). The 17-hydroxylase activity however, decreased by 85% during the first 12 h, returned to the original value by another 12 h, but showed a dramatic increase only between 24 and 48 h after PMSG, i.e. at the time when ovarian levels of 17-OH-progesterone were dropping. A closer examination of the initial response indicated that the enzyme activity of the microsomal fraction decreased by 40% within 2 h and by 81% within 4 h after injection of PMSG. Changes in the ovarian and serum levels of other steroids are shown in Fig. 7 A, B, C and D. All except testosterone reached peak levels by 12 h after PMSG; the latter steroid increased only after 12 h.

The changes induced by administration of 5 IU of hCG (i.v.) 24 h after hypophysectomy, and 5 IU(s.c.) every 12 h thereafter, are shown in Fig. 8. As with PMSG there was an initial 85% decrease in enzyme activity with a concomitant increase in serum 17-OH-progesterone; the data are not shown but the serum levels of the latter steroid were the same as those obtained with PMSG (Fig. 7). Recovery of enzyme activity was somewhat slower with hCG than with PMSG but the pattern of changes that occurred between 24 and 72 h were similar.

Prolactin given at a dose of 250 μg (s.c.) every 12 h had no effect upon ovarian hydroxylase activity in hypophysectomized animals. However, this hormone altered the enzyme activity which had been increased either by PMSG or hCG. Injection of 250 μg of prolactin 48 h after giving 20 IU of PMSG reduced the hydroxylase activity by 70.6 ± 0.4% within 12 h. When given at the same time as PMSG, and every 12 h thereafter, prolactin prevented much of the increase in hydroxylase activity produced by PMSG (Fig. 9). Prolactin had a similar effect upon hydroxylase stimulated by hCG. When given 36 h after the first injection, and at the same time as the 4th dose of hCG, 250 μg of prolactin reduced hydroxylase activity by 80.8 ± 2.1% within 12 h. Repeated injections at 12 h intervals, beginning 36 h after the first dose of hCG, resulted in a 95.7 ± 1.7% reduction in enzyme activity compared with that obtained with hCG alone.

Injection (i.v.) of 50 μg of ovine LH 24 h after hypophysectomy, and the same dose subcutaneously every 12 h thereafter, caused a prompt decrease in hydroxylase activity (Fig. 10). The decrease in activity seen with FSH was somewhat less than that for LH. In neither case was the activity returned to that of the starting level even though hormone was being administered at 12 h intervals. If the FSH was treated with anti-ovine LHβ subunit, then the initial decrease did not occur, but rather the enzyme activity was significantly increased 12 and 24 h after injection. However, activity decreased with time and at 72 h it was below that found in ovaries from rats treated with saline. Progesterone, 20 DHP, and 17-OH-progesterone were undetectable in the sera of animals treated with FSH + anti-LH, as well as in animals injected with saline.

DISCUSSION

The close agreement between results obtained by immunoassay of 17-OH-progesterone produced and the tritiated progesterone consumed supports the validity of the tritium exchange assay that was estab-

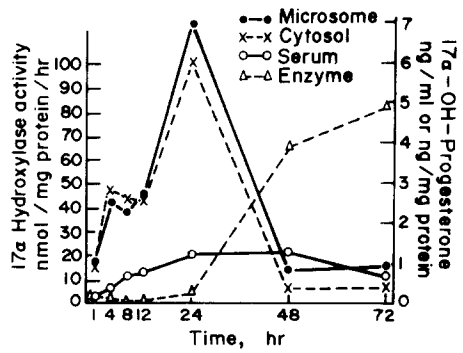

Fig. 6. Changes in ovarian and serum concentrations of 17-hydroxyprogesterone, assayed by RIA, and the microsomal 17-hydroxylase activity in immature hypophysectomized rats treated with 20 IU of PMSG at time 0. Note the reciprocal changes in the *in vivo* and *in vitro* enzyme activity.

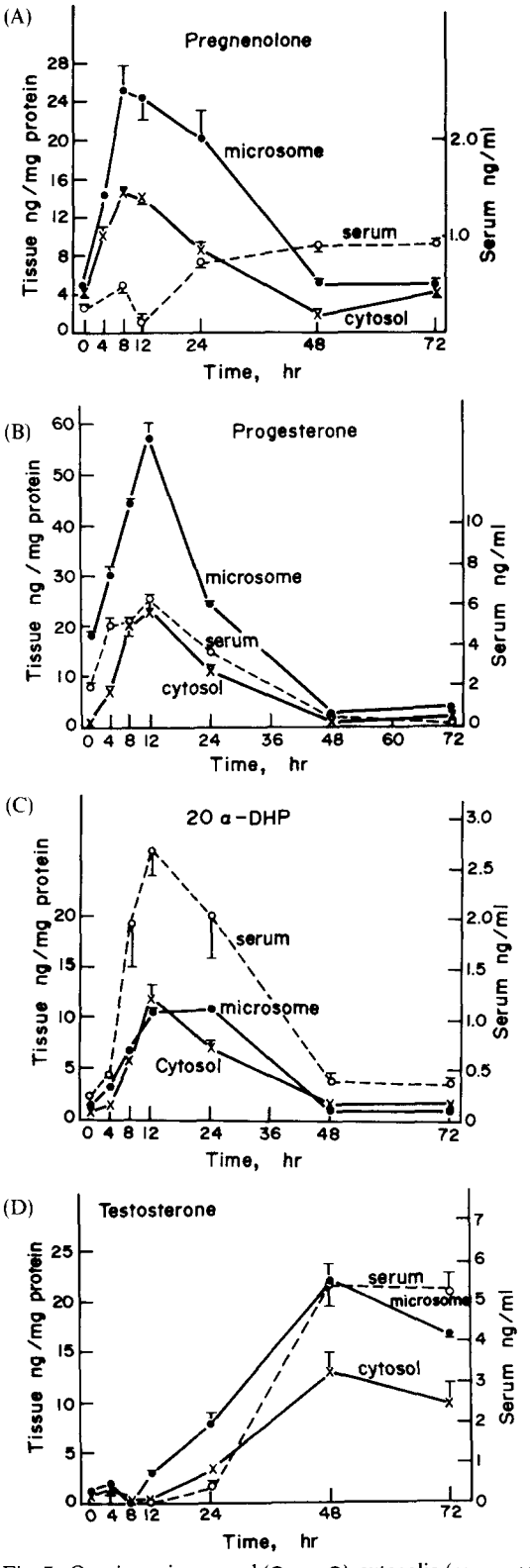

Fig. 7. Ovarian microsomal (●——●), cytosolic (×——×) and serum (○----○) changes in pregnenolone (A), progesterone (B), 20α-dihydroprogesterone (C) and testosterone (D) concentrations in immature hypophysectomized rats (six/group) injected with 20 IU of PMSG at time 0. The pattern of changes for the three C-21 steroids was the same as that for hydroxyprogesterone but that for testosterone followed the changes in 17-hydroxylase (Fig. 6).

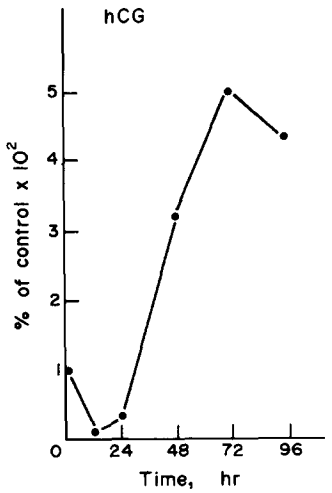

Fig. 8. Changes in 17-hydroxylase activity in the microsomal fraction of ovaries removed from immature hypophysectomized rats injected (i.v.) at time 0 with 5 IU of hCG; the animals received 5 IU s.c. at 12 h intervals. At least eight animals were used for each point and the assay run in triplicate.

lished, and discussed in detail, by Kremers[4]. While the immunoassay method is more sensitive than the tritium exchange method, particularly with 17α-[^{3}H]-progesterone of relatively low specific activity, the latter method is more rapid and more easily accomplished.

Although the affinity for pregenolone by rat ovarian 17-hydroxylase appears to exceed that for progesterone a predominance of the Δ5 pathway for androgen, and estrogen, production is certainly not established, even though several indications seem to favor such a view. In previous studies [13] serum concentrations of 17-OH-pregnenolone were considerably higher than those of 17-OH-progesterone in intact or hypophysectomized immature rats given

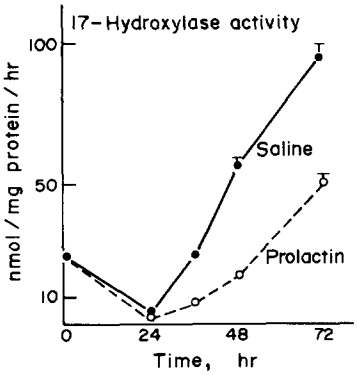

Fig. 9. The inhibition of 17-hydroxylase activity by the injection (s.c.) of 250 μg (8.75 IU) of ovine prolactin at time 0 and every 12 h into immature hypophysectomized rats given (i.v.) 20 IU of PMSG at time 0. Control received saline plus PMSG. Prolactin had no effect upon 17-hydroxylase activity and the loss of enzyme did not differ from that shown in Fig. 4.

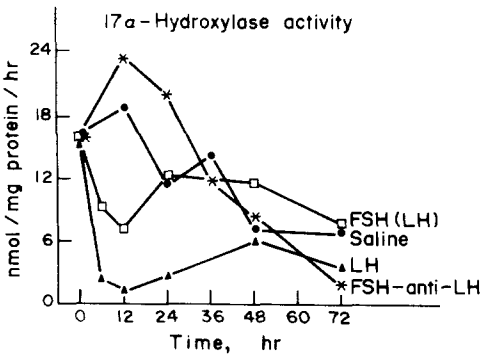

Fig. 10. Changes in ovarian microsomal 17-hydroxylase activity in immature hypophysectomized rats injected (i.v.) at time 0 with 0.1 ml of saline, saline containing 250 μg of FSH(NIH-S-8), 50 μg of LH(NIH-S18) or 25 μg of FSH(NIH-S-12) diluted (1:1) with a 1:100 dilution of anti LHβ-subunit antiserum. The same dose of hormones was given (s.c.) at 12 h intervals. The effect of the antiserum was not different from that of saline. While gonadotropins containing LH activity caused an immediate decrease and FSH, whose LH component was neutralized, caused an increase in activity, none of the preparations prevented the decline seen without gonadotropin (Fig. 4) and contrast the results obtained with PMSG or hCG.

PMSG, suggesting an active Δ5 pathway *in vivo*. The competitive inhibition between the two substrates also appears to favor use of the Δ5 pathway. The K_i for progesterone inhibition of pregnenolone hydroxylation was 3.5 times the K_i for pregnenolone inhibition of progesterone hydroxylation. Similar differences in K_i values for progesterone and pregnenolone using adrenal or testicular hydroxylase have been reported [14].

Fan *et al.*[11] proposed an important role for 20-ketone reduced steroids in regulating testicular androgen synthesis by the human testis. Whether such a role exists in the ovary is uncertain. The results of the present study indicated that 20-DHP was more inhibitory for the hydroxylation of pregnenolone than for progesterone. On the other hand 20-dihydropregnenolone had no effect upon pregnenolone hydroxylation but did inhibit hydroxylation of progesterone. Interestingly the hydroxylase activity of rat testicular microsomes showed the same pattern of inhibition as that for the ovary but the activity in porcine testicular microsomes was greatly inhibited by both 20-DHP and 20αdihydropregnenolone (unpublished data). Although the production of 20-dihydropregnenolone by the ovary has been demonstrated [15] its importance as a substrate for progesterone synthesis and/or as a control factor for steroidogenic enzyme remains in doubt.

The disappearance rate of ovarian hydroxylase activity appears to be quite different from that in testis. Purvis *et al.*[16] reported a half-life for hydroxylase from rat testis, following hypophysectomy, of 2.3 days; Kremers *et al.*[17] reported a half-life of rat testicular hydroxylase stimulated by hCG of 2.5 days. With no treatment, or with saline injections, the ovarian enzyme activity of hypophysectomized rats decreased with a half-time of only about 33 h. A similar value was obtained for loss of activity when the PMSG levels fell after 72 h. However, if the PMSG was removed by antibody the half-time was reduced by about 50% (Fig. 5). The mechanisms of this rapid loss, as well as the much more rapid loss seen after injection of gonadotropin, remain to be determined.

The effect of gonadotropin upon ovarian 17-hydroxylase was biphasic. The initial effect, seen within 2 h, was a loss of activity. There is a question whether this is real or only an apparent loss of activity due to dilution of substrate in the assay with endogenous steroid. With a typical assay, using 50 μg of microsomal protein, a maximum of about 0.016 nmol of progesterone plus pregnenolone (Fig. 7; 12 h) would be added to the 20 nmol of labelled pregnenolone. Obviously such a small amount would not significantly decrease the specific activity of the label or alter the calculation of enzyme activity.

The gonadotropin responsible for the loss in activity appears to be LH. All of the gonadotropic preparations, except the FSH whose LH component was neutralized by antiserum, caused a rapid reduction in enzyme activity. Considering that granulosa cells require several hours of exposure to FSH before they gain receptors for LH we assume that the initial change in enzyme activity occurred in theca/interstitial cells.

The second phase of gonadotropic action on the enzyme occured after about 24 h. PMSG was more effective at raising the specific activity of the 17-hydroxylase in the ovary than was hCG (Table 1). One reason for this could be that PMSG disappears from circulation more slowly than does hCG. Repeated injections of hCG at 12 h intervals were used for hCG in an attempt to overcome this difference. A second more important factor is that PMSG, which has both FSH and LH actions, stimulates more than one ovarian compartment. That is, the granulosa cells initially have receptors only for FSH, but after they attain receptors for LH they can also show increases in 17-hydroxylase activity [8]. Without the exposure to FSH the granulosa cells do not gain LH receptors and therefore they do not respond to hCG; i.e. the only ovarian compartment with 17-hydroxylase activity in animals given hCG is the theca/interstitium. The immediate rise in enzyme activity seen with FSH, which had been treated with antiserum, is difficult to understand. Presumably it involves the granulosa cells because they alone have receptors for this hormone [17].

The failure to obtain an increase in enzyme activity with purified FSH or LH probably relates to the disappearance rate of the hormones and the frequency of administration. Previous studies [19] with the estrogen synthesizing system indicated that frequent administration of large doses were necessary to sustain the process of enzyme induction.

The profound effect of prolactin on ovarian hydroxylase is unexplained. The enzyme activity increased by the action of hCG is quickly lost when prolactin is administered even though the hCG treatment continues. Since only theca and interstitium are stimulated by the hCG we assume that the prolactin is altering the enzyme activity in the cells in these compartments. Prolactin suppressed the increase in enzyme activity produced by PMSG and also drastically reduced the activity that had been increased by this gonadotropin. However, in the latter case administration of hCG to animals that have received PMSG causes a greater reduction in enzyme activity, along with ovulation and luteinization, than does prolactin [20].

In conclusion, the immature rat ovary contains an active 17-hydroxylase which has some of the same properties as the enzyme activity in testis. However, the ovarian enzyme also appears to have several properties which significantly differ from those of the testis. In addition to those discussed above, the difference in utilization of cofactor can be added. Recently Yoshida *et al.*[21] reported an NADH-linked hydroxylation of progesterone by human testicular microsomes. This was in addition to NADPH-linked hydroxylation which indicated to these authors that testicular hydroxylase has two active sites for hydroxylation. While NADH can be used as cofactor by the ovarian enzyme the activity is not increased by addition of NADPH. The significance of these findings remains to be elucidated.

Acknowledgements—The excellent technical assistance of Mrs Murriel Wagoner is gratefully acknowledged. Also thanks are given to Dr P. Kremers of the University of Liege for preparing the labeled pregnenolone and progesterone, and to Dr Bruce Goldman for supplying the anti-LHβ subunit antiserum. This research was supported in part by a grant from the National Institute of Aging.

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