Stimulation of Rat Testis Microsomal 17 α -Hydroxyprogesterone Side-Chain Cleavage Enzyme by Luteinizing Hormone Added *in vitro*

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The addition of purified luteinizing hormone to isolated rat testis microsomes causes an increase in the activity of the enzyme system that converts 17 α -hydroxyprogesterone into C₁₉ steroids in these tissue organelles. The gonadotrophin does not act in this system by altering the Michaelis constants of the enzyme for either steroid or nicotinamide nucleotide, but rather appears specifically to increase the catalytic capability of the protein.

One of the effects of corticotrophin on steroid synthesis is an increase in the activity of the adrenal enzyme that cleaves the side chain from 20,22-dihydroxycholesterol (Stone & Hechter, 1951), and experiments have been reported that implicate adenosine 3',5'-(cyclic)-monophosphate as the immediate effector of this alteration in enzyme activity (Haynes, Sutherland & Rall, 1960). Luteinizing hormone increases the conversion of acetate and cholesterol into testosterone by slices of testis incubated *in vitro* (Brady, 1951; Hall & Eik-Nes, 1962), and here too adenosine 3',5'-(cyclic)-monophosphate appears to serve as an intermediary in the gonadotrophin effect (Marsh, Butcher, Savard & Sutherland, 1966). Since luteinizing hormone has also been reported to affect testicular androgen biosynthesis from pregnenolone (Carstensen, 1961), and since the slowest step in this portion of the pathway is a carbon-carbon bond cleavage reaction, chemically analogous to, but not identical with, the cholesterol side-chain cleavage reaction (Axelrod, 1965; Loutfi & Hagerman, 1967), experiments to examine the effect of the gonadotrophin on partially purified enzyme preparations that catalyse this portion of steroid synthesis were performed. The results show that the gonadotrophin has a direct stimulatory effect on the 17 α -hydroxyprogesterone side-chain cleavage enzyme in rat testis microsomal preparations.

MATERIALS AND METHODS

Materials. Radioactive steroids were purchased from New England Nuclear Corp., Boston, Mass., U.S.A.; unlabelled steroids and nicotinamide nucleotides were from Sigma Chemical Co., St Louis, Mo., U.S.A. The steroids were examined for identity, purity and, with the radioactive compounds, for radiochemical homogeneity, at

frequent intervals in at least two systems by thin-layer chromatography according to the methods of Lisboa (1966). When necessary, repurification of the labelled steroid was done by thin-layer chromatography in Lisboa's (1966) system L [chloroform-ethanol (19:1, v/v)]. Luteinizing hormone was NIH-LH-S8 (0.73 unit/mg.) freshly dissolved in 0.9% NaCl for each experiment. Glucose 6-phosphate dehydrogenase was purchased from Nutritional Biochemicals Inc., Cleveland, Ohio, U.S.A., and solvents were either freshly redistilled or commercial 'spectrophotometer-grade' materials; other chemicals were American Chemical Society Specification reagent grade.

Enzyme assay. The standard assay system contained, in a total volume of 3.0 ml.: 0.1 M-sodium phosphate buffer, pH 7.0; 0.33 mM-MgCl₂; 3.3 mM-glucose 6-phosphate; 3.3 mM-NADPH; 0.05 mM-17 α -hydroxy[4-¹⁴C]progesterone [added to the vessel in 0.1 ml. of propane-1,2-diol-ethanol (1:1, v/v)]; 5 enzyme units of glucose 6-phosphate dehydrogenase; washed rat testis microsomes containing about 10 mg. of protein; washed human placenta microsomes containing about 19 mg. of protein. The testis enzyme preparation was added last to initiate the reaction, which was performed at 37° for 30 min. with gentle mechanical shaking in an atmosphere of air. Identical non-incubated vessels containing the substrate and products were analysed in parallel with every experiment. Variations from the standard assay mixture are indicated in the descriptions of the various experiments.

Extraction and separation of reaction products from substrates. At the end of the incubation (or immediately after addition of the testis microsomes for the non-incubated vessels) 50 μ g. each of 17 α -hydroxyprogesterone and [7-³H]oestrone in 0.1 ml. of ethanol were rapidly added to the flasks and mixed. The contents of the flasks were then immediately transferred to separatory funnels containing 30 ml. of water-saturated chloroform. The vessels were rinsed into the funnels once with 3 ml. of N-H₂SO₄ and twice with 10 ml. of chloroform. After 100 vigorous shakes of the funnels they were allowed to stand for a few minutes and then the clear organic layer was drawn off into a round-bottomed flask. Extraction with 50 ml. of chloroform was repeated once and the combined extracts were evaporated

on a rotary still operated at about 30 mm. Hg pressure and at 40°. Remaining traces of water were removed by adding a few millilitres of benzene-ethanol (7:2, v/v) and re-evaporating. (The dry extracts could be stored at 4° overnight without loss.) The extracts were dissolved in 20 ml. of light petroleum (b.p. 30–60°)–benzene (1:1, v/v) and transferred quantitatively to a separatory funnel. Then 60 ml. of 0.4 N-NaOH was added and the two phases were mixed by vigorous shaking. After separation of the phases, the aqueous layer was drawn off into another funnel containing 30 ml. of ethyl acetate and vigorously re-extracted. The aqueous phase was then discarded. The light petroleum–benzene (neutral) and the ethyl acetate (phenolic) extracts were dried under vacuum, dissolved in 1 ml. of ethanol and quantitatively transferred to vials for scintillation counting.

Determination of radioactivity and calculations. A 15 ml. volume of scintillation fluid [containing 0.4% 2,5-diphenyl-oxazole and 0.05% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene] was added to each of the vials and mixed. They were counted at 2° in a Nuclear–Chicago model 726 counter (Nuclear–Chicago Corp., Des Plaines, Ill., U.S.A.) fitted with external standardization, and a sufficient number of counts were always collected to reduce the standard error of the counting process to less than $\pm 1\%$ of the observed counting rate. The efficiency of the counting process was determined with the aid of the external standard and the disintegrations/min. of ^3H and ^{14}C in each vial were calculated. Cross-contamination of the extracts (phenolic steroid radioactivity in the neutral fractions and neutral steroid radioactivity in the phenolic fractions) and recoveries of radioactivity were calculated directly from the analyses of the non-incubated vessels. The resulting values for cross-contamination and recovery were used to form a pair of simultaneous equations whose solution gave directly the corrected amount of phenolic products made and neutral substrates remaining in each vessel at the end of the incubation. The sums of these two values for both ^3H and ^{14}C should, of course, be equal to the amount of radioactivity originally added to the incubation vessel, and whenever this result was not obtained within $\pm 5\%$ the analysis was discarded.

Protein analyses were done by the biuret method with crystalline bovine serum albumin as standard and, from the specific activity of the substrate, the results were expressed in μmoles of phenolic steroid made/min./mg. of testis microsomal protein. One unit of enzyme activity was defined as 1 μmole produced/min. under the specified conditions.

Preparation of microsomes. Mature male rats (90–180 days old) of the local inbred strain were killed by a blow on the head and the testes were rapidly removed and placed in ice-cold 0.15 M-NaCl. The capsules of the glands were removed by sharp and blunt dissection and the parenchymal tissue was homogenized in 0.25 M-sucrose–1 mM-EDTA–0.01 M-nicotinamide, pH 7.0, with 5 ml. of homogenizing medium/g. of tissue. Homogenization was for 30 sec. at 60 v in a Lourdes Multi-Mix homogenizer (Lourdes Instrument Corp., Brooklyn, N.Y., U.S.A.). The resulting suspension was centrifuged for 20 min. at 8000g (rotor no. 856, model HR-1; International Equipment Corp., Boston, Mass., U.S.A.). The resulting supernatant fluid was recentrifuged for 60 min. at 78500g (rotor no. 30, model L-2; Spinco Division, Beckman Instruments Inc., Palo

Alto, Calif., U.S.A.). The supernatant fluid from the second centrifugation was discarded and the residual lipid on the walls of the tube was wiped out. The pellet was resuspended in a volume of the homogenizing medium equivalent to that of the second supernatant fluid and the particles were dispersed by gentle homogenization in a motor-driven smooth-glass homogenizer with a Teflon pestle. The resulting suspension was centrifuged at 10000g for 20 min. in the no. 30 Spinco rotor and the resulting supernatant fluid was again centrifuged at 78500g for 60 min. The supernatant fluid was discarded and the washed microsomal pellets were stored at -90° . No loss in enzyme activity was observed under these conditions for at least 6 months. Microsomes from spontaneously delivered term human placentas were prepared in exactly the same fashion except that only 2 ml. of medium/g. of tissue was used.

RESULTS

The placental microsomal preparation containing a steroid-aromatizing system (Ryan, 1958) was added to the assay mixture to simplify the separation of the products of the cleavage enzyme of the testis from the original substrate. It was therefore necessary to demonstrate that the amount of placental enzyme preparation used and that the conditions of the assay permitted the aromatization of all the C_{19} steroid (androst-4-ene-3,17-dione, 17β -hydroxyandrost-4-en-3-one, androst-5-ene- 3β , 17β -diol or 3β -hydroxyandrost-5-en-17-one) that could be produced by the testis enzyme preparation under the conditions used. This was achieved by showing that, under the conditions prescribed except for omission of the testis enzyme preparation, each of the C_{19} steroids mentioned as substrates was converted into phenolic steroid at a rate at least twice that found for the cleavage enzyme in any of the measurements of its activity reported here. It is customary to use somewhat greater excesses of the ancillary enzymes than this when performing enzyme assays in a coupled system, but in the present instance the impossibility of obtaining the aromatizing enzyme complex at a higher specific activity precluded its use at a higher concentration. The addition of luteinizing hormone to the aromatizing system had no effect on the rate of phenolic steroid production from any of these substrates. In other spectrophotometric experiments it was shown that the specified amounts of glucose 6-phosphate and its dehydrogenase were sufficient to maintain the amount of NADP used in the completely reduced form even in the presence of both the testis and placental microsome preparations.

The experiments described by Sobrevilla, Hagerman & Vilee (1964) imply that the aromatizing system used in the present assay would not contribute any side-chain cleavage activity. Likewise, Little, Shaw & Purdy (1963) found side-chain

Fig. 1. Time-course of side-chain cleavage reaction. Experimental details are given in the text. The points represent individual assays.

Fig. 2. Proportionality between rate of side-chain cleavage and amount of testis microsomal protein incubated under the standard conditions. Experimental details are given in the text. The points represent individual assays.

cleavage activity only in the soluble fraction of the placenta, and Warren & Cheatum (1964) observed side-chain cleavage only in whole homogenates of placenta, not in isolated microsomes. (The fact that both of these groups used progesterone as the substrate for their incubations is immaterial in the present context, since the testis preparation contains an active steroid 17-hydroxylase.) Control experiments were done to confirm these observations under the conditions of my assay. Incubations were performed in the usual way except for the omission of the testis preparation and with luteinizing hormone either absent or present. In no case was any more radioactivity detected in the phenolic fraction than in non-incubated controls. Thus the side-chain cleavage that occurs in the complete

system is due to the testis microsomes and any observed effect of luteinizing hormone is on the testis preparation.

In a consecutive series of 17 sets of enzyme assays the mean recoveries (expressed as percentages of the original amount of steroid added to the vessels $\pm$ S.E.M. of these percentages) were 89.8 ± 1.5 in the neutral fraction and 64.2 ± 2.3 in the phenolic fraction. In the same series the mean phenolic cross-contamination of the neutral fraction was 8.2 ± 1.1 and neutral steroid contamination of the phenolic fraction was 2.8 ± 0.4 . (These results are the means of the individual values determined and used in each experiment to calculate the true distribution of radioactivity and steroid between substrate and product as explained above.)

The curve shown in Fig. 1 shows that the activity of the cleavage enzyme measured by the standard assay system with 17α -hydroxyprogesterone as substrate is proportional to the time of the incubation for at least 1 hr. with 23.5 mg. of testis microsomal protein/incubation vessel. On the basis of this result a standard incubation time of 30 min. was selected. Fig. 2 shows the proportionality between the amount of testis enzyme preparation added and quantity of phenolic steroid produced under the standard assay conditions. These results demonstrate the validity of the assay system.

I have confirmed (unpublished work) the reports of earlier investigators that the side-chain cleavage enzyme is confined to the microsomal fraction of rat testis homogenates (Shikita & Tamaoki, 1965), and in the experiment shown in Table 1, I attempted a further subfractionation of this tissue element by using the procedure of Lundkvist & Perlmann (1966). All the activity present in the original microsomal preparation was recovered in the three fractions and there was a somewhat higher specific activity of the enzyme in one of the 'smooth'-membrane fractions, as might have been expected from the work of Jones & Armstrong (1965). The procedure was not, however, of a substantial preparative value and was therefore not investigated further.

Many attempts were made to solubilize the enzyme by means of ultrasonic oscillation, treatment with organic solvents, partial digestion with proteases or lipases, repeated freezing and thawing, treatment with deoxycholate or detergents etc. No procedure was found that released any appreciable amount of the enzyme system into true solution. This failure was disappointing, for it made it impossible to examine by straightforward preparative means the question whether a single enzyme complex catalyses both 17α -hydroxylation and side-chain cleavage, as postulated by Dorfman & Ungar (1965), or whether different protein molecules are responsible for these two reactions.

Table 1. *Side-chain cleavage activity of subfractionated microsomes*

Rat testis microsomes prepared as described in the text were fractionated as described in the Materials and Methods section, and the fractions were assayed in duplicate by the standard procedure.

Microsomal subfraction	Activity (% of total)	Specific activity (units/mg. of protein)
'Rough' membranes with attached ribosomes	36	108
'Smooth' membranes that sediment in Mg^{2+} but not in Ca^{2+}	47	163
'Smooth' membranes that do not sediment in either Mg^{2+} or Ca^{2+}	17	46

Fig. 3. Effect of pH on side-chain cleavage reaction. Experimental details are given in the text. Each point represents the mean of three to seven determinations with standard errors less than 10% of the mean values.

The optimum pH for the reaction was determined to be near neutrality in both phosphate and tris buffers (Fig. 3). It was shown in separate experiments that a sufficient excess of the coupling enzymes was present at all pH values studied to ensure that the cleavage enzyme was rate-limiting. In other similar experiments it was shown that the addition of luteinizing hormone to the incubation mixture did not alter the pH optimum.

The effects of addition of luteinizing hormone to the standard assay system defined by the above results was explored at two concentrations of NADPH. As would have been predicted, increasing the NADPH concentration (in the absence of added luteinizing hormone) from $6\mu M$ to $3.3mM$ increased the apparent activity of the side-chain cleavage enzyme from 26.7 ± 1.2 to 88.0 ± 2.8 (units/mg. of testis preparation protein $\pm$ S.E.M. for four and three determinations respectively). When luteinizing

hormone was present at a concentration of $0.5\mu g./ml.$ the corresponding values were 25.0 ± 1.2 and 95.4 ± 8.0 (four determinations each). The enzyme is more active at the higher cofactor concentration, and a suggestion of a stimulatory effect of the gonadotrophin was observed at the higher nucleotide concentration. The fact that no effect is seen at the lower cofactor concentration indicates that the trophin is not acting via an inhibition of alternative NADPH-oxidizing systems (cf. Hagerman, Smith & Day, 1966).

Table 2 summarizes the principal results of the investigation. Activity of the side-chain cleavage enzyme was measured at various concentrations of the two substrates, steroid and nucleotide, in the presence and absence of luteinizing hormone. When NADPH was the variable substrate (with the steroid at a saturating concentration) the generating system ensured a constant concentration of the variable substrate throughout the incubation, and the kinetic constants could therefore be calculated in the usual way from the Michaelis-Menten equation. A least-squares computer programme was used for these calculations (Cleland, 1963). When the steroid was the variable substrate (with the nucleotide at a saturating concentration) it was impossible to maintain the substrate concentration constant throughout the incubation and still obtain sufficient product for accurate analyses. The integrated form of the equation was therefore used and the data were fitted by the general least-squares method (Dixon & Webb, 1964; Sweat, Samuels & Lumry, 1950; Scarborough, 1958). Since in every case the residual variance after curve fitting was not significantly different between the comparable experiments without and with added luteinizing hormone the values of the kinetic constants were compared by simple unpaired *t* tests. The Michaelis constants for both steroid and nucleotide were unchanged by addition of the trophic hormone *in vitro*, which strongly implies that any observed effect was not due to an alteration in the binding of the substrates to the enzyme. In contrast, addition of luteinizing hormone caused a statistically significant increase in the maximal velocity of the reaction with either the steroid or the nucleotide as the variable substrate.

DISCUSSION

Slow steps in a reaction sequence are recognized as likely sites for control of the sequence by a variety of mechanisms including general cell environment (e.g. pH), feed-back (final end product) inhibition or stimulation, substrate activation, product inhibition, repression-depression mechanisms, hormonal effects etc. In the best studied case, phosphofructokinase, it is now

Table 2. *Effect of luteinizing hormone on the kinetics of the side-chain cleavage enzyme*

The values for $V_{\max}$ are in μmoles of product/mg. of protein/min.; K_m is given in moles/l.; n is the number of determinations and the values are means $\pm$ S.E.M. calculated as described in the text; * denotes a statistically significant effect of luteinizing hormone ($P < 0.04$).

Substrate varied ...	Steroid		Nucleotide	
Luteinizing hormone ...	—	+	—	+
n	5	5	11	10
$V_{\max}$	87.1 ± 1.8	$95.5 \pm 2.1^*$	75.5 ± 4.9	$95.4 \pm 8.3^*$
$10^6 \times K_m$	3.4 ± 0.6	2.9 ± 0.8	12.7 ± 2.6	13.1 ± 3.0

apparent that multiple factors can be involved in the control by both genetic and immediate mechanisms (Lowry & Passonneau, 1966). The same appears to be true for the enzyme investigated here. I and others have previously shown that the measured activity (which is an approximate estimate of the amount) of the cleavage enzyme in testis varies with the endocrine status of the animal (Brady, 1951; Hall & Eik-Nes, 1963), and my present results are compatible with the stimulatory effects *in vitro* observed by other investigators in tissue-slice preparations. The effect observed here is small, since the experiments were performed with tissue preparations from intact rather than hypophysectomized animals. Removal of the source of endogenous gonadotrophin was not done, to examine the gonadotrophin effect on enzyme from as nearly normal tissue as possible. All of the effects *in vitro* suggest an immediate action of the pituitary hormone on the enzyme complex. My results imply that this action is to increase, by a chemically unspecified mechanism, the catalytic activity of the active site of the enzyme. Further details of the chemical mechanism itself will probably not be susceptible to examination until the enzyme has been brought into solution and more fully characterized. However, there would appear to be two mechanisms for pituitary control of steroid synthesis in testis at this point in the biosynthetic scheme, one acting rapidly by a direct action on the enzyme and one acting rather more slowly by way of the genetic apparatus and/or protein-synthesizing machinery, the latter being demonstrable by enzyme measurements on tissues of animals previously treated *in vivo* with the gonadotrophin.

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