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Stimulation of Adrogen Biosynthesis in Rat Fetal Testes in vitro by Gonadotropins

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ABSTRACT

Rat fetal testes in the latter part of gestation (Days 17-21) have enzyme systems for the *de novo* synthesis of androgen. Under the conditions of these *in vitro* experiments the major androgen produced was testosterone, with lesser amounts of androstenedione and 5α-dihydrotestosterone. As little as 10 m IU of hCG added *in vitro* significantly increased androgen synthesis in the fetal testis on Day 19. Bovine, ovine or human LH but not bovine or ovine FSH also stimulated the synthesis of androgens by the rat fetal testis.

The synthesis of testosterone was increased by the addition of precursors: 15-fold by the addition of pregnenolone, 20-fold by progesterone, and 47-fold by dehydroepiandrosterone. However, the increase was marginal in the presence of sulfates of pregnenolone or dehydroepiandrosterone. These results suggest that Δ^5-isomerase-3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase are active in the fetal testes; in contrast, the activity of steroid sulfatase was relatively low.

INTRODUCTION

The extensive studies of Jost and his colleagues have shown that the fetal testis synthesizes masculinizing hormones which stabilize the male accessory reproductive organs during prenatal dvelopment (Jost, 1961, 1966; Jost et al., 1973). The fetal testis contains the enzymes needed to synthesize androgens from steroid substrates and from acetate *de novo*, and androgens can duplicate the effects of masculinizing factors elaborated by the fetal testis (Villee, 1969; Bloch et al., 1973; Price and Ortiz, 1965). It has been suggested that the fetal pituitary influences secretions of the fetal testis (Jost, 1961, 1966; Jost et al., 1973). Measurable quantities of androgens and gonadotropins have been found in the male human fetal circulation (Grumbach and Kaplan, 1974; Reyes et al., 1973, 1974; Clements et al., 1976). Human chorionic gonadotropin (hCG) has been shown to stimulate androgen synthesis *in vitro* in the human and rat fetal testis (Ahluwalia et al., 1974; Eik-Nes, 1974; Warren et al., 1975). The present study was undertaken to explore the possibility that a multifactor control system including pituitary and chorionic gonadotropins as well as steroid substrates may regulate the biosynthesis of androgens in the fetal testis.

MATERIALS AND METHODS

General

Adult female rats (150-200 g) from the Charles River Breeding Laboratories (CD-strain) were maintained under normal laboratory conditions of 14 h of daily illumination, with *ad libitum* Purina Laboratory chow and tap water. Proestrus female rats were mated with experienced breeder males. Rats with sperm in the vaginal smear next morning were considered to be at Day 1 of pregnancy. On Day 17, 19 or 21 of pregnancy they were killed by a blow on the head. Fetal testes were dissected out under a stereobinocular microscope, decapsulated and used in the incubations.

Incubation

The paired decapsulated testes were immersed in 200 μl of Ham's F-10 tissue culture medium (Microbiological Associates, Inc.) containing 1 mM L-glutamine in a 75 × 10 mm culture tube (Kimble Glass Co.) and incubated with gentle shaking in an atmosphere of 5 percent CO_2 and 95 percent O_2 at 37°C according to the method outlined by Dufau et al. (1972). The testes were equilibrated for 30 min in the tubes before

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[2] 17β-hydroxy-5α-androstan-3-one.

the gonadotropin or saline was added.

1) To evaluate the capability of the fetal testis to synthesize androgens *de novo*, testes of pregnancy Day 19 were incubated for periods of 1, 2 or 4 h. These incubations received 5 μl sterile saline and served as controls for experiments in which gonadotropins or steroid substrates were added to the incubation tubes.

2) To test the stimulatory effect of hCG (A.P.L., Human Chorionic Gonadotropins, Ayerst Laboratories, Inc., New York, N.Y.) on androgen biosynthesis, 1, 10, 100 or 1,000 mIU of hCG dissolved in 5 μl of sterile saline were added at the beginning of the incubation to culture tubes containing fetal testes (pregnancy Day 19). The testes were then incubated for 4 h. In a second series of experiments, 1000 mIU of hCG dissolved in 5 μl of saline were added to paired fetal testes, and the incubation was terminated after 1, 2 or 4 h.

3) To evaluate the androgen biosynthetic capacity of testes on different days of gestation, testes from fetuses of Days 17, 19 or 21 were incubated for 4 h with or without 1000 MIU hCG added following a 30 min preincubation equilibration period.

4) To evaluate the effect of heterologous pituitary gonadotropins on androgen biosynthesis by the fetal testes, 5 ng each of bovine FSH (B-1), LH (B-9), ovine FSH (S-8), LH (S-18) separately or a combination of FSH and LH were added to the incubation tubes after 30 min equilibration period and the incubation was continued for 4 h. These hormone preparations were obtained from the National Institute of Arthritis, Metabolic and Digestive Diseases, Pituitary Distribution Program. Testes were incubated similarly in tubes containing human FSH (100 or 1000 mIU, units based upon augmentation reaction assay, The National Pituitary Agency, NIAMDD, Lot No. 3), LH (100 or 1000 mIU, units based upon ventral prostate weight assay, The National Pituitary Agency, NIAMDD, Lot No. A3), or a combination of 100 mIU of FSH and LH.

5) To evaluate the effects of exogenous steroid substrates on the synthesis of androgen by the fetal testis, 5 nmol of authentic pregnenolone (Searle Chemicals, Inc.), progesterone (Steraloid Co.), dehydroepiandrosterone (DHA) (Searle Chemicals, Inc.), or the sodium salts of pregnenolone sulfate (Sigma Chemical Co.) of dehydroepiandrosterone sulfate (Ikapharm) dissolved in 5 μl of 50 percent aqueous methanol were added to the F-10 medium containing testes from Day 19 fetuses, equilibrated for 30 min, and the incubations were continued for 4 h. In similar experiments 1000 mIU of hCG were added to testicular incubations after 30 min of equilibration to test effects of gonadotropins on androgen synthesis from steroid substrates. The steroids were free from contaminants, and their purity was verified by thin layer chromatography in several different solvent systems.

Each treatment group included 6 to 12 replicates. Incubations were terminated by the addition of 0.5 ml methylene chloride; the tubes were stored at −60°C until they were utilized for radioimmunoassays.

Chromatography of Steroids and Radioimmunoassay

Each incubation mixture was extracted with 15 ml of freshly opened diethyl ether (Mallinckrodt Chemical Co.), after addition of approximately 600-800 cpm of [1,2,6,7-^{3}H] testosterone (SA 8.5 Ci/m mol), [1,2-^{3}H] 5α-dihydrotestosterone[2] (SA 42.6 Ci/m mol) and [1,2-^{3}H] androstenedione (SA 40.0 Ci/m mol) (New England Nuclear Corporation, Boston, MA). The extracts were dried under N_2 and chromatographed on Celite (Johns-Manville) Microcolumns (0.5 × 5.0 cm) made from 5 ml disposable pipettes (Kimble Glass Co.) (Coyotupa et al., 1972; Barberia et al., 1974; Challis et al., 1975). Each column was packed with 0.5 g Celite from which the impurities had been removed by heating at 600°C overnight and mixed thoroughly with 0.5 volume of ethylene glycol (Stationary phase). The dried extract reconstituted with 1 ml iso-octane (2,2,4-trimethyl pentane, Nanograde quality, Mallinckrodt Chemical Co.) was applied to the column and the steroids were eluted under standardized conditions. Androstenedione was eluted in the first three ml of iso-octane and 5α-dihydrotestosterone in the next 6 ml of the same solvent. Five ml of 30% benzene in iso-octane eluted testosterone (Challis et al., 1975).

An antiserum raised in the rabbit from antigen testosterone-3-(O-carboxymethyl)-oxime-bovine serum albumin (Dr. Abraham's collection No. S-741 #2; 14) was used for the radioimmunoassay of testosterone and 5α-dihydrotestosterone and an antiserum from androstenedione-3-oxime human albumin complex (S-1557 #2) was used for androstenedione. They were diluted in a gelatin buffer, 1:1400 for testosterone, 1:8000 for dihydrotestosterone and to 1:5000 for androstenedione assays. Quantitation of the steroid in the samples was based upon 10 point standard curves (5-500 pg) of the antisera bound to authentic steroids. Several duplicate aliquots of different volumes of diluted extracts of incubations were assayed for estimation from the sensitive part of the standard curve. A large portion of the diluted extract was utilized for measuring recovery which ranged from 60 to 80 percent. Aliquots of standard curve steroids and samples to be assayed were dried under N_2 and mixed thoroughly with antisera for 1/2 h. To this mixture 8000 cpm of the respective ^{3}H-steroid was added and incubated for 18-20 h at 4°C. The free steroids were separated by dextran-coated charcoal (Sanyal et al., 1974).

The radioimmunoassays are sufficiently specific after separation of the steroids on Celite microcolumn chromatography. The cross-reactivity of the antisera used to a variety of steroids is given in Table 1. Although dehydroepiandrosterone was eluted with 5α-dihydrotestosterone in chromatographic separations, the cross-reactivity of dehydroepiandrosterone with the antisera used for measurements of 5α-dihydrotestosterone was very low. The interassay variability was assessed by calculating the coefficient of variation in the standard curve values of 13-17 different estimations. The average coefficient of variation for testosterone and 5α-dihydrotestosterone was 10 percent, and for androstenedione, 8 percent. In the composite standard graphs 5 pg was significantly different from 10 or 0 pg at the 95 percent confidence limit. The accuracy of the assays was assessed by the addition of authentic steroids in distilled water blanks. One ng testosterone, 5α-dihydrotestosterone and androstenedione read 1.03 ± 0.02, 1.02 ± 0.02 and 1.03 ± 0.01 ngs respectively (n = 25, mean ± S.E.). The assay values were about 10 pg in the controls when no steroids were added to the same volumes of distilled

TABLE 1. Cross reaction of the steroids with the antisera used in the radioimmunoassays*.

Steroids	Testosterone (S741 #2)	5α-dihydrotestosterone (S741 #2)	Androstenedione (S1557 #2)
Cholesterol	< 0.001	< 0.001	< 0.001
Progesterone	< 0.001	< 0.001	< 0.001
17α-OH-progesterone	< 0.001	< 0.001	< 0.001
Pregnenolone	< 0.001	< 0.001	< 0.001
20α-OH-pregn-4-en-one	< 0.001	< 0.001	< 0.001
5α-pregnane-3,20-dione	< 0.001	< 0.001	< 0.001
5β-pregnane-3,20-dione	< 0.001	< 0.001	< 0.001
Pregnanediol	< 0.001	< 0.001	< 0.001
Cortisone	< 0.001	< 0.001	< 0.001
Hydrocortisone	< 0.001	< 0.001	< 0.001
Corticosterone	< 0.001	< 0.001	0.6
Testosterone	100.0	60.0	0.8
Dehydroepiandrosterone	0.04	0.73	20.7
5α-dihydrotestosterone	117.0	100.0	< 0.001
5β-dihydrotestosterone	8.7	3.1	< 0.001
Androstenedione	13.5	2.4	100.0
5β-androstane-3α, 17β-diol	4.2	2.1	0.001
3α-OH-5α-androstane-17-one	9.5	4.4	2.8
3β-OH-5α-androstane-17-one	3.5	2.6	2.9
Estrone	< 0.001	< 0.001	< 0.001
Estradiol-17β	< 0.001	< 0.001	< 0.001
Estriol	< 0.001	< 0.001	< 0.001

*Cross reactivity = [test steroids (pg) required for 50% displacement]/[testosterone, 5α-dihydrotestosterone or androstenedione (pg) required for 50% displacement] X 100.

water or F-10 medium. The assay data for each experiment have been expressed as mean ±S.E. pmol/pair testes, and statistically evaluated by Student's *t* test (Mendenhall, 1967).

RESULTS

Stimulation of De Novo Biosynthesis of Androgens by hCG

The accumulation of testosterone, androstenedione and 5α-dihydrotestosterone by Day 19 fetal testes as a function of the time of incubation is shown in Fig. 1. Testosterone in the culture medium increased progressively following one half hour of equilibration to a plateau during 2-4 h incubation. The quantity of androstenedione synthesized during incubation remained unaltered. 5α-dihydrotestosterone was produced in small amount and the quantity increased slightly during 4 h incubation period (Fig. 1).

The addition of 1000 mIU hCG after 30 min equilibration markedly stimulated androgen synthesis by the fetal testes compared to the saline controls (Fig. 1). The testosterone accumulated in 1 h of incubation in the presence of hCG was significantly ($P<0.01$) greater than the initial value and the control containing no gonadotropin. The increase was more than 10-fold when compared with the increase which occurred in the controls. The quantity of testosterone in the incubation medium progressively rose as the incubation was continued, reaching a plateau by 2 h ($P<0.01$). The amounts of androstenedione and 5α-dihydrotestosterone were also significantly elevated ($P<0.01$) in an hour of incubation after the addition of hCG. The quantity of androstenedione and 5α-dihydrotestosterone increased further to some extent as the incubation period was prolonged to 4 h (Fig. 1).

The increased synthesis of androgens *in vitro* by the fetal testes was related to the amount of hCG added to the incubation medium (Fig. 2). The accumulation of testosterone was markedly stimulated by the addition of 10 mIU of hCG. The values were 2-3 times with 10 mIU and 6-7 times with 100 mIU of hCG over controls ($P<0.01$). However, testosterone accumulation was not significantly increased further by the addition of a greater amount of hCG. The quantity of 5α-dihydrotestosterone was also

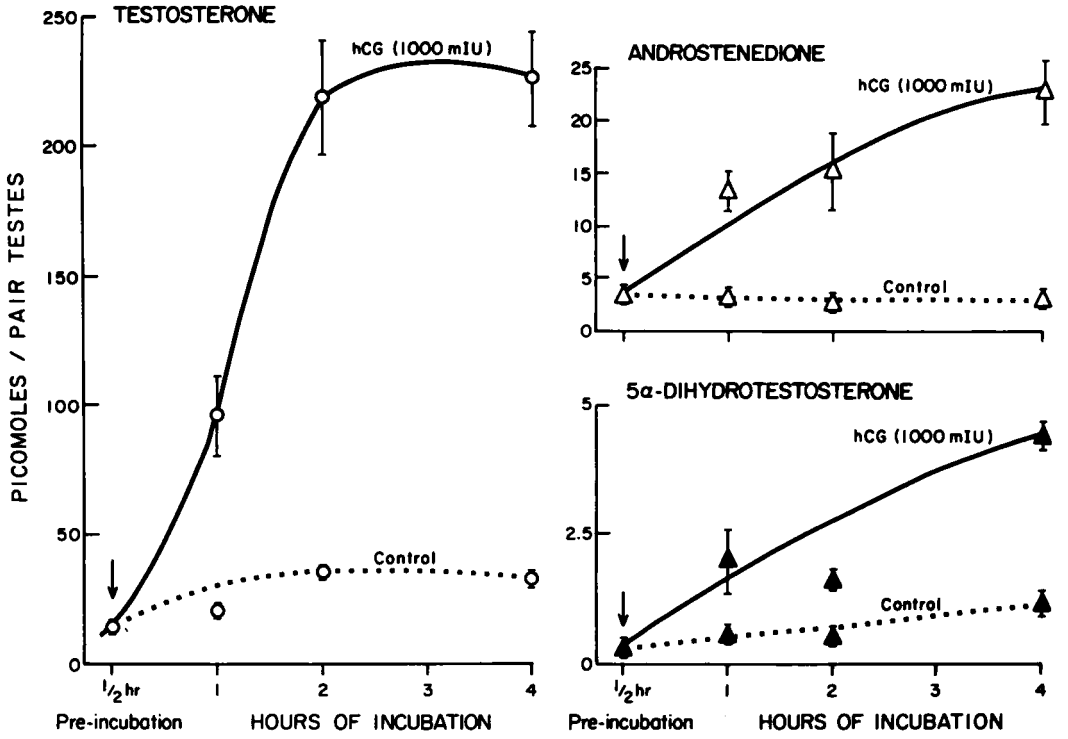

FIG. 1. Production of androgens *in vitro* by fetal testes (Day 19) incubated for different time periods. The arrows indicate the time of addition of 1000 mIU hCG or an equivalent volume of normal saline following 1/2 h preincubation equilibration.

increased with these amounts of hCG. The increments in the amounts of 5α-dihydrotestosterone produced at doses of 10 and 100 mIU hCG were statistically significant ($P<0.01$). The amount of androstenedione produced was also a function of the quantity of gonadotropin added during incubation (Fig. 2). The progressive increase in androstenedione with 10 and 100 mIU hCG was significant ($P<0.01$).

Effect of hCG on Androgen Biosynthesis in Fetal Testes from Different Days of Gestation

The biosynthesis of androgens *in vitro* by fetal testes from the 17th to the 21st day of pregnancy was clearly demonstrable under the conditions adopted in these experiments (Fig. 3). The fetal testes of Days 17, 19 and 21 produced substantial amounts of testosterone, androstenedione and 5α-dihydrotestosterone in 4.5 h of incubation. The increased amounts of testosterone and 5α-dihydrotestosterone synthesized in testes of Days 19 and 21 were statistically significant from those of Day 17 ($P<0.01$).

The addition of 1000 mIU of hCG markedly stimulated biosynthesis of the three androgens by the fetal testes compared to the controls with saline ($P<0.01$), and the quantity synthe-

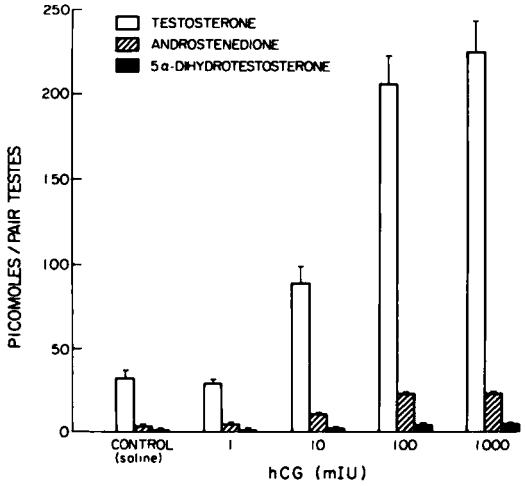

FIG. 2. Accumulation of testosterone, androstenedione and 5α-dihydrotestosterone in 4 h incubations of Day 19 testes as a function of different concentrations of hCG added after 1/2 h equilibration.

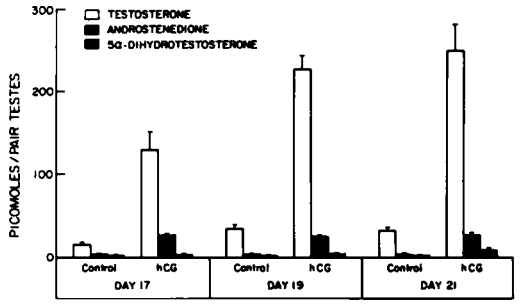

FIG. 3. The effects of 1000 mIU hCG on androgen biosynthesis by testes from fetuses of 17, 19 and 21 days of gestation. One half hour preincubation, 4 h incubation.

sized was related to the gestational age of the fetus (Fig. 3). Testosterone and 5α-dihydrotesterone values for Days 19 and 21 were significantly greater than that of Day 17 ($P<0.01$). However, the amounts of androstenedione produced were identical in incubations of testes of all three age groups.

Influence of Heterologous Pituitary Gonadotropins on Androgen Biosynthesis in Fetal Testis

Five ng ovine or bovine LH augmented androgen biosynthesis considerably relative to the controls containing saline ($P<0.01$) in 4 h (Fig. 4). However, when 5 ng ovine or bovine FSH were added to the incubation mixture the testes produced testosterone, androstenedione and 5α-dihydrotestosterone during the same period in amounts similar to those of saline controls. There was no synergistic effect on androgen production of LH and FSH added together.

Human FSH, 100 or 1000 mIU added to the incubation mixture following a 30 min equilibration, stimulated testes to produce significantly ($P<0.01$) greater amounts of testosterone, androstenedione and 5α-dihydrotestosterone than the controls (Fig. 4). Human LH, 100 or 1000 mIU added to the incubation mixture, led to the accumulation of even greater amounts of testosterone, androstenedione and 5α-dihydrotestosterone ($P<0.01$). One hundred mIU of human LH plus 100 mIU of FSH added together resulted in the synthesis of more of these steroids than either added separately. However, the values were not statistically significant from those incubations to which 100 mIU LH alone were added.

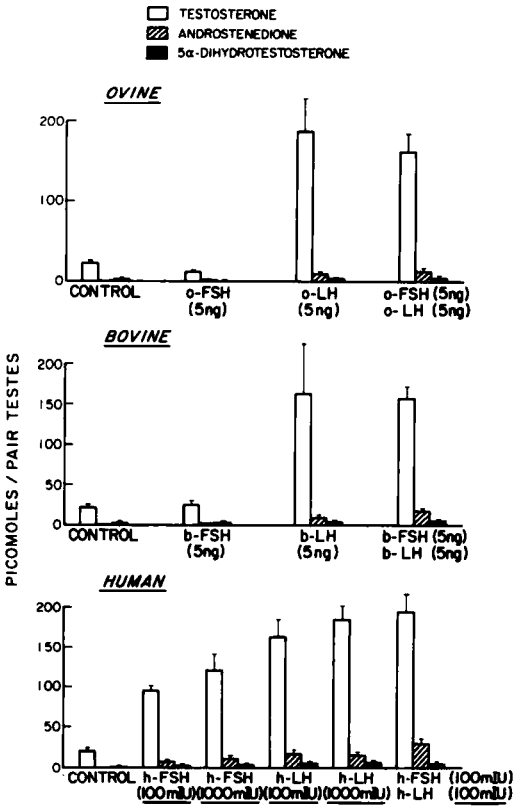

FIG. 4. Effects of heterologous gonadotropins on androgen synthesis by testes from rat fetuses on Day 19 of gestation. Time of incubation 4 h with 1/2 h preincubation equilibration.

Androgen Biosynthesis by Fetal Testes from Exogenous Steroid Precursors

The quantities of testosterone, androstenedione and 5α-dihydrotestosterone produced in incubations containing 5 nmol of pregneneolone, progesterone or dehydroepiandrosterone were greatly increased ($P<0.01$) over the amounts produced in the controls to which no steroid was added (Fig. 5). Androgen biosynthesis by the fetal testes was most markedly increased in the presence of dehydroepiandrosterone.

Human chorionic gonadotropin (1000 mIU) influenced androgen production from progesterone and pregnenolone in 4 h. In incubations performed with hCG and pregnenolone more testosterone ($P<0.01$) was produced than in the saline controls. However, with progesterone and hCG the increase in testosterone amount was not statistically significant. Human chorionic gonadotropin did not significantly influence

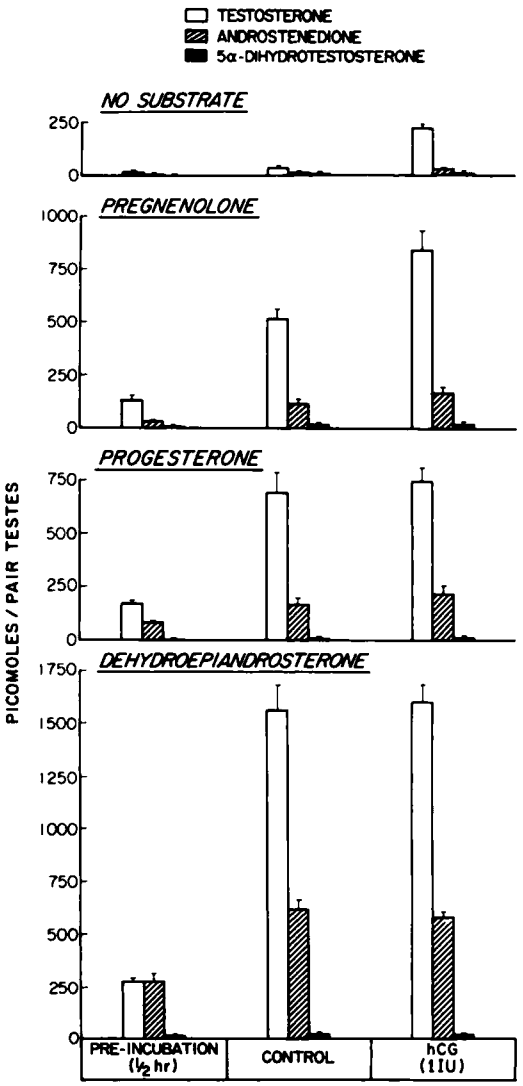

FIG. 5. Production of androgens by testes of Day 19 fetuses incubated with steroid substrates in the presence or absence of hCG (1000 mIU). Period of incubation 4 h and 1/2 h equilibration.

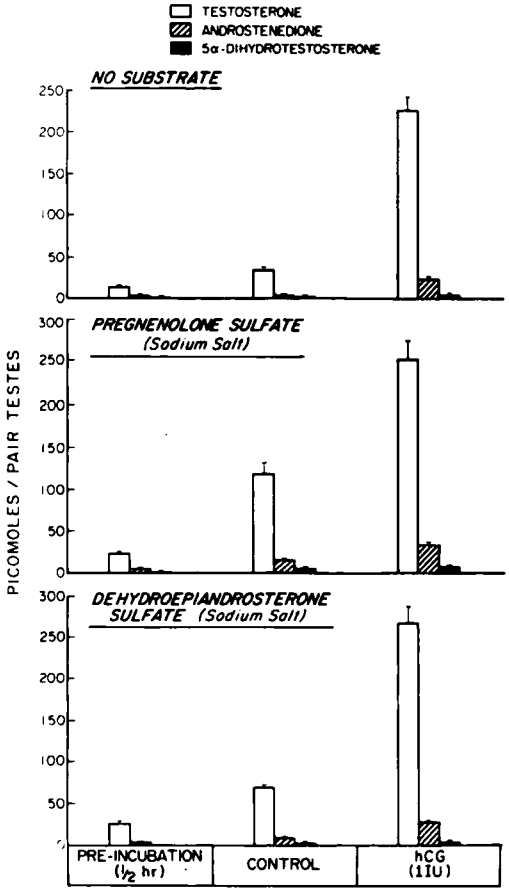

FIG. 6. Production of androgens by testes of Day 19 fetuses incubated with steroid sulfate substrates in the presence or absence of hCG (1000 mIU). Four h incubation and 1/2 h preincubation.

androgen biosynthesis from dehydroepiandrosterone under the conditions of incubation utilized in these experiments.

The addition of pregnenolone sulfate or dehydroepiandrosterone sulfate to the incubation medium led to only a slight increase in the synthesis of androgens (Fig. 6). The increased androgen synthesis in response to hCG was similar in incubations containing pregnenolone sulfate, dehydroepiandrosterone sulfate or no substrate.

DISCUSSION

The present study indicates that the fetal testis in the latter part of pregnancy can synthesize androgens, testosterone, androstenedione, and 5α-dihydrotestosterone either from steroid substrates or *de novo* corroborating previous findings (Lipsett and Tullner, 1965; Noumura et al., 1966; Bloch 1967; Warren et al., 1972, 1973). Under the experimental conditions used in this study the testis of a 17-day fetus produced appreciable quantities of testosterone, androstenedione, and 5α-dihydrotestosterone *de novo* (Fig. 3). The synthetic ability of the testes increased with the growth of the fetus, presumably reflecting the increased number of interstitial cells present (Lording and Dekrester, 1972). The amount of testosterone accumulated by fetal testes was about 5 times

greater than androstenedione and 50 times greater than 5α-dihydrotestosterone in incubations of Day 17. These ratios change to 10 and 30 respectively in incubations of testes from Days 19 and 21. In testicular incubations of all three states of development only small quantities of 5α-dihydrotestosterone were produced; the major androgen was testosterone.

The relative ability of the fetal testis to synthesize androgens from exogenous steroids under identical *in vitro* conditions was assessed (Fig. 5). Large quantities of testosterone, androstenedione and 5α-dihydrotestosterone were produced in incubations with pregnenolone, progesterone and dehydroepiandrosterone indicating that they were effective substrates for androgen synthesis. These data, therefore, suggest that the fetal testis has very active Δ^5-isomerase-3β-hydroxysteroid dehydrogenase, 17α-hydroxysteroid dehydrogenase, 17α-hydroxylase and C_{17-20} lyase enzymes. Inhibitors of these enzyme systems when administered to pregnant rats caused failure of normal masculine development due to diminished production of testosterone by the fetal testis (Goldman et al., 1966; Goldman, 1971, 1971/1972; Bloch, 1971; Jost, 1971/1972).

Steroid sulfates have been shown to be present in high concentrations in the circulating blood of both male and female human fetuses (Huhtaniemi et al., 1970). Some investigators have considered them to be precursors of the active steroid hormones (Kawano et al., 1973; Payne et al., 1975). In the present experiments only a limited sulfatase activity was demonstrable in the fetal testis of the rat (Fig. 6). The synthesis of testosterone and other androgens from free steroids was far greater than that from steroid sulfates added as substrates.

The stimulatory effects of gonadotropins on *de novo* androgen synthesis *in vitro* by the fetal testis were similar to that shown for the adult testis. Neither ovine nor bovine FSH stimulated androgen synthesis in the fetal testis (Fig. 4). However, LH from both sources and hCG markedly increased androgen production. Human FSH as well as LH caused stimulation of androgen synthesis; the increase observed with human FSH may possibly be due to contamination of the FSH with LH (Dufau et al., 1972). In the present studies no significant stimulation of androgen production from progesterone or dehydroepiandrosterone by hCG added *in vitro* was observed (Fig. 5). However, a very large amount, 1000 mIU of hCG, increased androgen accumulation from pregnenolone as measured by radioimmunoassay. It is not clear in this study whether gonadotropins may be acting in the rat fetal testis at one or more sites that are different from that of the adult testis since the gonadotropin stimulation in the adult testis occurs before pregnenolone formation in the steroid biosynthetic pathway (Hall and Eik-Nes, 1964; Menon et al., 1965; Dorfman, 1972).

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